COMPARATIVE BIOACTIVITIES OF *IN VIVO* AND *IN VITRO* MATERIALS OF *PIPER LONGUM* L. AND *PARIS POLYPHYLLA* SM.

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF BOTANY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN BOTANY

BY

CHANDRA BAHADUR THAPA

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Institute of Science and Technology

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Kirtipur, Kathmandu, Nepal

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DECLARATION

Thesis entitled **"Comparative Bioactivities of *in Vivo* and *in Vitro* Materials of *Piper longum* L. and *Paris polyphylla* Sm."** is being submitted to the Central Department of Botany, Institute of Science and Technology (IoST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Bijaya Pant of Central Department of Botany, Tribhuvan University and co-supervised by Prof. Dr. Hari Datta Bhattarai and Associate Prof. Dr. Krishna Kumar Pant of Central Department of Botany, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

CB Thapa
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Chandra Bahadur Thapa

RECOMMENDATION

This is to recommend that **Mr. Chandra Bahadur Thapa** has carried out research entitled **"Comparative Bioactivities of *in Vivo* and *in Vitro* Materials of *Piper longum* L. and *Paris polyphylla* Sm."** for the award of Doctor of Philosophy (Ph.D.) in **Botany** under our supervision. To our knowledge, this work has not been submitted for any other degree.

He has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of a Ph.D. degree.

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LETTER OF APPROVAL

19/11/2024

On the recommendation of **Prof. Dr. Bijaya Pant, Prof. Dr. Hari Datta Bhattarai, and Associate Prof. Krishna Kumar Pant**, this Ph.D. thesis submitted by Mr. Chandra Bahadur Thapa, entitled **"Comparative Bioactivities of *in Vivo* and *in Vitro* Materials of *Piper longum* L. and *Paris polyphylla* Sm."** is forwarded by Central Department Research Committee (CDRC) to the Dean, IoST, T.U.

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Chandra Bahadur Thapa

November, 2024

शोधसार

Piper longum L. (पिपला) एक बहुमुल्य उष्णप्रदेशीय औषधीय गुण भएको बिरुवा हो, जुन विभिन्न प्रकारका रोगहरु निको पार्न प्रयोग गरिन्छ । त्यस्तै, *Paris polyphylla* Sm. (सतुवा) एक समशीतोष्ण वा हिमाली भागमा पाईने जोखिमयुक्त (Vulnerable) औषधीय गुण भएको बिरुवा हो, जुन विभिन्न प्रकारका रोगहरुको उपचार गर्न प्रयोग गरिन्छ । तथापि, दिगो उपयोगिताको अभाव, अवैध संकलन र निर्यात, बासस्थानको ह्रास र जलवायु परिवर्तनले प्राकृतिक बासस्थानमा यिनीहरुको अस्तित्वलाई खतरामा पारेको छ । फलस्वरुप, प्राकृतिक बासस्थानमा यिनीहरुको संख्या घट्दै गइरहेको छ । अर्कोतर्फ, अधिकांश अनुसन्धान गतिविधिहरु यी विरुवाहरुका प्राकृतिक परिवेशमा (*in vivo*) उत्पादन भएका विभिन्न भागहरुका bioactivity र रसायनिक विश्लेषणमा केन्द्रित छन् । तर तन्तु प्रविधिबाट (tissue culture) उत्पादित callus को bioactivity र रसायनिक विश्लेषणको अनुसन्धान अत्यन्त सीमित छ । तसर्थ, यस अनुसन्धानमा पिपला र सतुवाको प्राकृतिक परिवेशमा (*in vivo*) उत्पादन भएका विभिन्न भागहरु र कृत्रिम परिवेशमा (*in vitro*) उत्पादित callus को तुलनात्मकरुपमा bioactivity र रसायनिक विश्लेषणको अध्ययन गरिएको छ।

Tissue Culture प्रविधि प्रयोग गरि कृत्रिम पोषक माध्यम (MS media) मा पिपलाको काण्डको आँख्ला (node) र पात (leaf) बाट स-साना बिरुवाहरु उत्पादन गरियो, अनि सतुवाको पातबाट पनि यसका बिरुवाहरु उत्पादन गरियो । पिपलाको आँख्लाबाट बिरुवाहरु उत्पादन गर्दा प्रत्यक्ष organogenesis विधिवाट र यसको पातबाट र सतुवाको पातबाट बिरुवाहरु उत्पादन गर्दा अप्रत्यक्ष organogenesis र somatic embryogenesis उपयुक्त देखियो । त्यसैगरी, पिपलाको आँख्ला र callus बाट धेरै संख्यामा टुसाहरु (Shoots) उत्पादन गर्नको लागी र सतुवाको callus बाट धेरै संख्यामा टुसाहरु (Mini-rhizome) उत्पादन गर्नको लागी MS पोषक माध्यममा TDZ र KN हर्मोनहरु उपयुक्त देखियो । साथै टुसाबाट जराहरु उतपादन गर्नको लागी MS पोषक माध्यममा IBA हर्मोन उपयुक्त देखियो ।

पिपलाको जराको Dichloromethane (DCM) extract ले अरु extract ले भन्दा बढी antioxidant activity, total phenolic content (TPC) र total flavonoid content (TFC) देखायो । यस extract ले ५ प्रकारका ब्याक्टेरियाहरु (*Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumannii, Staphylococcus aureus* र *Bacillus subtilis*) को बृद्धिलाई रोकेको देखायो । त्यसैगरी, यसले Brine shrimp झिंगाका लार्भाहरुलाई सबैभन्दा कम मात्राको प्रयोगमा मारेको पाईयो । तर पिपलाको फलको crude extract ले अन्य extract ले भन्दा हड्डि र पाठेघर क्यान्सर कोषहरु प्रति उच्च क्यान्सर प्रतिरोध देखायो । जराको DCM extract ले मधुमेह रोगसंग सम्बन्धित α-amylase र α-glucosidase ईन्जाईम प्रति

उच्च प्रतिरोध प्रदर्शन गर्यो । यसबाहेक, पिपलाको callus लाई MS तरल पोषक माध्यममा salicylic acid प्रयोग गरी culture गर्दा यसको तौल (biomass) अरुको तुलनामा बढाएको पाईयो, जबकि 100 mg/L phenylalanine तरल माध्यममा प्रयोग गर्दा सबैभन्दा बढी antioxidant activity, TPC, र TFC को मात्रा पाईयो । तसर्थ, tissue culture माध्यमबाट उत्पादित callus ले bioactive compound हरु उत्पादन गर्यो, जसले गर्दा anti-oxidant, antibacterial, anticancer र antidiabetic प्रतिक्रिया देखायो ।

त्यसैगरी, सतुवाको राईजोमको DCM fraction ले सबैभन्दा बढी antioxidant activity, TPC र TFC को मात्रा देखायो । यसले परिक्षण गरिएका सबै ५ प्रकारका ब्याक्टेरियाहरुको १००% बृद्धि प्रति अवरोध देखाएको छ । यसले Brine shrimp झिंगाका लार्भाहरुलाई सबैभन्दा कम मात्राको प्रयोगमा मारेको पाईयो । साथै यस extract ले अन्य extract ले भन्दा छातीका क्यान्सर कोषहरु प्रति उच्च प्रतिरोध देखायो । त्यसैगरी, राईजोमको crude extract ले मधुमेह रोगसंग सम्बन्धित α-amylase र α-glucosidase ईन्जाईम प्रति उच्च प्रतिरोध प्रदर्शन गर्यो । यसबाहेक, सतुवाको callus लाई MS तरल पोषक माध्यममा salicylic acid प्रयोग गरी culture गर्दा यसको तौल (biomass) अरुको तुलनामा बढेको पाईयो, जबकि MS media मा NAA र BAP हर्मोन प्रयोग गरी तरल माध्यममा culture गर्दा सबैभन्दा बढी antioxidant activity, TPC, र TFC को मात्रा पाईयो । तसर्थ, salicyclic acid र phenylalanine लाई callus suspension culture मा प्रयोग गर्दा secondary metabolite बढेको पाईयो ।

LC-HRMS प्रविधी प्रयोग गरी पिपलाको DCM fraction र callus को crude extract को रसायनिक परिक्षण गर्दा कुल २७ वटा रसायनिक यौगिकहरु पाईयो, तर सतुवाको राईजोमको DCM fraction र callus को crude extract को रसायनिक परिक्षण गर्दा कुल ३१ वटा रसायनिक यौगिकहरु पाईयो । केहि रसायनिक यौगिकहरु दुवै callus र विरुवाका in vivo भागहरु (जस्तै: जरा, राइजोम) मा पाइएको हुनाले यस अध्यनमा callus ले रसायनिक यौगिकहरु उत्पादन गरेको पाइयो ।

खोजशब्द: अल्फा–ग्लूकोसिडेज_भ्रुण_ एक्सट्र्याक्ट_आईसी ५०_एलसि–एचआरयमयस_तरल कल्चर

ABSTRACT

Piper longum L. (Piperaceae) is a valuable tropical medicinal plant used to cure a variety of diseases. Similarly, *Paris polyphylla* Sm. (Melanthiaceae) is a temperate or subalpine vulnerable medicinal plant used to treat a variety of diseases. However, unsustainable utilization, illegal collection and export, habitat destruction, and climate change are threatening its survival in its natural habitats. As a result, their population in natural habitats is declining. On the other hand, most research activities focus on bioactivity and chemical analysis of *in vivo* parts; however, research on bioactivity and chemical analysis of *in vitro*-cultured calli is extremely limited. In the present study, the bioactivity of *in vitro* and *in vivo* cultured parts of *P. longum* and *P. polyphylla* were compared.

The most effective medium for direct organogenesis by regeneration of multiple shoots (5.33±1.15) in *P. longum* from nodal explants was MS medium supplemented with 1.0 mg/L TDZ. The maximum number of roots (7.0±1.0) was regenerated in MS + 1.0 mg/L IBA from *in vitro* shoots. Plants were also regenerated by indirect organogenesis from leaf and node explants, and by somatic embryogenesis. Callus was induced from leaf explants in MS medium enriched with 2,4-D or 2.4-D + KN, while it was induced from nodal explants in MS media enriched with NAA only. Multiple shoots were obtained in MS + 0.25 mg/L TDZ (25.33±1.52) for leaf callus and 2.0 mg/L NAA (18.33±1.50) for nodal callus. Subsequently, the maximum number of roots was regenerated from *in vitro* shoots by using 0.5 mg/L IBA from the leaf callus and 2.0 mg/L NAA from the nodal callus. In addition, the maximum embryogenic/nodular calli with somatic embryos (SEs) were found in MS + 1.5 mg/L 2,4-D + 1.0 mg/L KN (28.33±3.05 SEs) for leaf callus, and 1.0 mg/L NAA (12.66±2.51 SEs) for nodal callus. Nodular calli with SEs were differentiated into plantlets/seedlings in MS + 0.5-2.5 mg/L TDZ and full-strength MS media without PGRs. Similarly, *P. polyphylla* was also propagated *in vitro* using leaf explants, in which the best media for callus formation and mini-rhizomes with root differentiation were MS + 0.25 mg/L 2,4-D + 0.5 mg/L KN and MS + 2.5 mg/L KN respectively.

The dichloromethane (DCM) fraction of *P. longum* root showed the highest antioxidant activity (IC_{50}: 134.81±1.16 μg/mL), the highest total phenolic content (TPC), and the highest total flavonoid content (TFC) compared to other extracts of *in vivo* parts and *in*

vitro callus. It showed 100% growth inhibition against five bacterial strains, such as *Pseudomonas aeruginosa, Escherichia coli*, *Acinetobacter baumannii*, *Staphylococcus aureus,* and *Bacillus subtilis*, with the lowest minimum inhibitory concentration (MIC) of 5.0 mg/mL, and the minimum bactericidal concentration (MBC) of 8.35 mg/mL for *S. aureus*. It was more cytotoxic to brine shrimp nauplii (LC_{50}: 156.78±5.49 µg/mL), while the crude extract of the fruit showed higher anticancer activity (IC_{50}: 146.55±2.31 µg/mL) on U-2 OS and HeLa cell lines (LC_{50}: 273.31±17.89 µg/mL). Similarly, the DCM fraction of the root showed higher inhibition of α-amylase (IC_{50}: 365.21±31.021 µg/mL) and α-glucosidase (IC_{50}: 489.07±27.966 µg/mL) compared to other extracts of *in vivo* parts and *in vitro* callus. In addition, callus suspension culture in MS medium showed that salicylic acid increased callus biomass compared to other treatments, while 100 mg/L phenylalanine resulted in the maximum antioxidant activity (IC_{50}: 22.44±1.96 µg/mL), TPC, and TFC.

Similarly, the DCM fraction of *P. polyphylla* showed the highest antioxidant activity (IC_{50}: 197.06±0.635 µg/mL), TPC, and TFC compared to other extracts of rhizome and callus. It also showed 100% growth inhibition against the five bacterial strains tested, with the lowest MIC at 5.0 mg/mL and MBC at 7.5 mg/mL for *E. coli*. It was more cytotoxic on brine shrimp nauplii (LC_{50}: 201.78±70.97 µg/mL). It also showed higher anticancer activity (IC_{50}: 235.94±0.72 µg/mL) to HeLa cell lines, but the methanol fraction was more cytotoxic to MCF-7 cell lines (IC_{50}: 211.36±0.57 µg/mL). However, the crude extract of the rhizome inhibited α-amylase (IC_{50}: 95.45±8.71 µg/mL) and α-glucosidase (IC_{50}: 51.40±8.62 µg/mL) more effectively than other extracts of rhizome and callus. In addition, suspension culture in MS medium revealed that salicylic acid increased callus biomass than other treatments, but liquid MS media supplemented with BAP+NAA resulted in the highest antioxidant activity, TPC, and TFC than phenylalanine or salicylic acid.

LC-HRMS analysis of the DCM fraction of the root and crude extract of the callus of *P. longum* revealed a total of 27 compounds. Similarly, the DCM fraction of the rhizome and the crude extract of the callus of *P. polyphylla* exhibited a total of 31 compounds.

Keywords: *Alpha-glucosidase_embryogenesis_extracts_*IC_{50}*_LC-HRMS_suspension culture*.

LIST OF ACRONYMS AND ABBREVIATIONS

°C	:	Degree Centigrade
μL	:	Microliter
μM	:	Micromolar
2,4-D	:	2,4- Dichlorophenoxy Acetic Acid
ANOVA	:	Analysis of Variance
BAP	:	6-Benzylaminopurine
Cm	:	Centimeter
$CNPG_3$	:	2-Chloro-4-nitrophenyl-α-D-maltotrioside
CTAB	:	Cetyl Trimethyl Ammonium Bromide
CW	:	Coconut Water
DCM	:	Dichloromethane
DPPH	:	2,2diphenyl-1-picrylhydrazyl
DPR	:	Department of Plant Resources
DW	:	Dry Weight
EDTA	:	Ethylene Diamino Tetra Acetate
eg	:	As an Example,
et al.	:	And Others
etc	:	Etcetera
FCR	:	Folin–Ciocâlteu Reagent
Fig.	:	Figure
FW	:	Fresh weight
g	:	Gram

GA	:	Gallic Acid
GNPS	:	Global Natural Products Social Molecular Networking
$HgCl_2$	:	Mercuric Chloride
HPLC	:	High Pressure Liquid Chromatography
IAA	:	Indole-3-acetic Acid
IBA	:	Indole-3-butyric Acid
KN	:	Kinetin
L	:	Liter
LC-HRMS	:	Liquid Chromatography-High Resolution Mass Spectrometry
M	:	Molar
MBC	:	Minimum Bactericidal Concentration
mg	:	Milligram
MIC	:	Minimum Inhibitory Concentration
mL	:	Milliliter
mM	:	Millimolar
Mm	:	Millimeter
MS	:	Murashige and Skoog
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NAA	:	α-Naphthalene Acetic Acid
PCR	:	Polymerase Chain Reaction
PGRs	:	Plant Growth Regulators
PHE	:	Phenylalanine
pNPG	:	4-Nitrophenyl α-D-glucopyranoside

ppm	:	Parts Per Million
Psi	:	Pound Per Square Inch
Q	:	Quercetin
Rpm		Round Per Minute
SA	:	Salicylic Acid
TDZ	:	Thidiazuron
TFC	:	Total Flavonoid Content
TPC	:	Total Phenolic Content
T.U.	:	Tribhuvan University
UV	:	Ultraviolet
Wt.	:	Weight
ZoI	:	Zone of Inhibition

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CHAPTER 1

1. INTRODUCTION

1.1 Medicinal plants

A large part of the Earth's flora is made up of medicinal plants. Approximately 70,000 plant species, ranging from lichens to flowering plants, have reportedly been used medicinally at some point (Kuruppu *et al.*, 2019). Nepal is home to about 6,973 different species of flowering plants (Shrestha *et al.*, 2018) and about 1700 to 2331 species of them are medicinal and aromatic plants (MAPs) (Kuwar *et al.*, 2022). The majority of them are found in their natural state, however, some are exotic, naturalized, or have been long-cultivated. More than 701 species are used medicinally, 30 species are targeted for research and development, and 12 species are prioritized for agrotechnology development in Nepal (DPR, 2012; 2017).

Since ancient times, medicinal plants have been utilized to cure a variety of ailments and disorders worldwide in ethnomedicine, Ayurveda, Homeopathy, Traditional Chinese Medicine (TCM), and other forms of medication. Medicinal plants offer a significant potential for developing new drug molecules that can cure different diseases (Chauhan *et al*., 2011). Furthermore, researchers and herbalists are paying much more attention to natural products of plant origin because they are safer than modern western medicines with fewer adverse effects. In developing nations, eighty percent of people depend solely on herbal remedies for their primary healthcare (Pant, 2014), whereas in developed nations, more than 25% of prescribed medications are made from wild plant species (Hamilton, 2004). However, the sustainability of medicinal plants in the natural environment is under threat due to heavy exploitation, habitat loss, illegal collection and trade, and global climate change. Therefore, *in-situ* and *ex-situ* management tactics are used to propagate rare, threatened, and valuable medicinal plants to conserve them for the benefit of mankind.

1.2 *Piper longum* L.; Family-Piperaceae; Local name-Pipla

It is a unisexual perennial aromatic climber. Leaves are simple, exstipulate, alternate, petiolate, broadly ovate or oblong-oval; adventitious roots clasping at swollen nodes. The inflorescence is a spike, male spikes narrow, female circular, female and male flowers develop on different plants; fleshy berry fruits embedded in the spikes, one

seeded with three-layered pericarp, ovoid, attains yellowish orange color when ripens and on drying becomes black colored; flowering and fruiting in June-August (Anonymous, 2007).

Figure 1: (A) *Piper longum* L. (Male plant), (B) *P. longum* L. (Female plant), (C) Male catkin (D) Female Fruits, and (E) Seeds.

P. longum grows naturally in the tropical and subtropical woodlands of India, Bhutan, Srilanka, Malaysia, Indonesia, Nepal, Singapore, Myanmar, America, etc. In Nepal, it occurs in the east-west Terai, and Churia hills from 100 to 1000 m above sea level (Press *et al*., 2000).

1.2.1 Traditional uses and bioactive constituents of *P. longum*

Fruits, roots, leaves, and thick roots with the segment of the stem (Piplamul) are used in several ailments in traditional medicine using various modes of herbal formulation preparation. Fruits or spikes are used for cold, cough, asthma, and bronchitis (Ghimire and Bastakoti, 2009; Acharya and Acharya, 2009; Sigdel and Rokaya, 2011; Kumar and Bharati, 2014); leaves and stems for cough (Dangol and Gurung, 1991); and roots for cough, headache, dyspepsia, as anti-dote in snake biting and scorpion bite, and jaundice (Chopra *et al.*, 1956; Kumar *et al*., 2011; Singh, 2017). It is also used for stomach disorders, purgative, vermifuge, flatus-relieving, and also in bronchiolitis, pyrexia, cold, bronchial asthma, urinary discharge, cancer, piles, sleeplessness, hepatitis, leprosy, arthritis, and rheumatoid-arthritis (IUCN, 2004). Sivarajan and Balachandran (1994) reported the use of roots, stems, and fruits for the treatment of respiratory tract infections like bronchitis and asthma. It is a powerful stimulant of the

respiratory system and shows a rejuvenating effect on the lungs (Sharon and Maurya, 2004).

Chemical Constituents: The alkaloid piperine is the primary and therapeutic ingredient which is 3-5% (on a dry weight basis) in *Piper longum*. Other constituents are methyl piperine, piperettine, piperundecalidine, piperlongumine, piperlonguminine, pregumidiene, piperderidine, longamide and tetrahydropiperine, terahydro piperlongumine, dehydropipernonaline piperidine, piperine, and piperlongumine (Zaveri *et al.,* 2010).

1.3 *Paris polyphylla* Sm.; Family- Melanthiaceae; Local name-Satuwa

It is a 30-45 cm tall, erect perennial herb with a creeping rootstock. There are 4–9 leaves per whorl. Flowers are terminal, solitary, short-stalked, long-pointed segments of the perianth that resemble long, green leaves, and they have an inner whorl of thread-like segments that are either as long as or shorter than the outer whorl. Fruits are globose capsules with many red seeds and a yellowish-brown exterior (Anonymous, 2007).

Figure 2: *Paris polyphylla* Sm. (A) Habit of the plant, (B) Rhizome, (C) Fruit, (D) Seeds.

Chemical Constituents: The rhizome of *P. polyphylla* contains polyphyllin D, polyphyllin I, polyphyllin VII, diosgenin, Paris saponin I, II, VI, VII, and H, pariphyllin A, pariphyllin B, parsterone, and trillin (Lee *et al.,* 2005; Zhang *et al*., 2014; 2019; Thapa *et al*., 2020).

1.3.1 Traditional uses and bioactive constituents of *P. polyphylla*

Since ancient times, people in Nepal and other Asian countries including China and China have used the rhizome of *P. polyphylla* as a drug to treat a range of ailments and diseases in the form of infusions, juices, powders, and pastes. Researchers have found many uses of rhizome in humans and cattle from Nepal, including treating cuts, wounds, blisters, burns, sprains, headaches, fever, anthelmintic, vermifuge,

expectorant, antispasmodic, digestive, gastritis, diarrhea, dysentery, menstruation pain, tonic, an antidote for poisoning (aconite poisoning, snake and insect poisoning), jaundice, vasoconstriction in the kidney, vasodilation in spleen and limbs (Rajbhandary, 2001; Manandhar, 2002; IUCN, 2004; Kuwar *et al.*, 2006; Bhattarai *et al*., 2006; KC *et al*., 2010; Lamichhane *et al*., 2014). *P. polyphylla* is widely used in China as a component of topical medicaments in traditional Chinese medicine (TCM) to cure boils, venomous snake bites, carbuncles, sore throat, and traumatic pain (Chinese Pharmacopoeia Commission, 2015). Rhizomes are the primary source of raw material for the '*Yunnan Baiyao*' and Gong Xue Ning (GXN) capsules. The '*Yunnan Baiyao*' is used for various diseases and injuries like back pain, bleeding, fractured bones, wound healing, pain reliever, fungal diseases, poisonous snakes or insect bites, skin allergies, tumors, and a variety of cancers (Long *et al*., 2003). Gong Xue Ning (GXN) capsule has been developed from the saponin extract of *P. polyphylla* var. *yunnanensis* in China to cure abnormal uterine hemorrhage (AUB) (Zhao and Shi, 2005; Guo *et al*., 2008). It is also a source for Chinese patent drugs "Jidesheng Sheyaopian". Now, it has become the main source of anticancer drug preparation.

1.4 *In vitro* culture of plant materials and types of culture techniques

In vitro micropropagation, referred to as micropropagation, is the method of cutting small sections of plants in aseptic conditions and cultivating them on an appropriate nutrient media (Bhatia, 2015). Explants are small fragments of plant tissue that are cultured in a nutrient media under sterile conditions. Plants can be made to grow quickly by using a suitable growing environment for every kind of plant material (explant) due to the totipotent nature of plant cells (Sharon and Maurya, 2004). These plantlets can be multiplied as well, typically during the shooting stage, to develop a significant number of additional plantlets. Then, they can also develop new adventitious roots when the appropriate plant growth regulators (PGRs) are added. The newly developed plants can then be planted in soil and cultivated normally.

In vitro culture methods have recently gained significant commercial significance in the fields of plant improvement, disease eradication, secondary metabolite production, and plant propagation. Under controlled conditions, an individual explant may be replicated into thousands of plants in a comparatively short period and with less space all year round (Akin-Idowu *et al.*, 2009). Due to the high multiplicative efficiency and

minimal demands on the starting plant population and growing environment, micropropagation has been used to successfully grow and safeguard rare, vulnerable, and endangered species. The separation of important mutants in well-suited, high-producing genomes with enhanced resistance to disease and tolerance to stress capacity is another huge potential benefit of the micropropagation technology (Brown and Thorpe, 1995). Furthermore, because somaclonal variability can exist, some callus cultures produce clones with inheritable traits that are distinct from those of the parent plants (George, 1993), which results in the production of commercially significant good varieties. Moreover, the micropropagation approach may be a trustworthy way for the *ex-situ* management of important medicinal plants. Because they act as an "insurance policy" against extinction, *ex-situ* management strategies can be utilized in conjunction with *in situ* conservation techniques (Pant, 2013; 2020).

Micropropagation or *in vitro* propagation of plants has major three stages, i.e., stage I, II, and III (Murashige, 1974). However, Debergh and Maene (1981) described the treatment with disinfectants for surface sterilization of stock plants as stage 0. In modern times, a fourth stage (IV), during which plants are introduced to the outside environment, is also widely identified. The 0-IV stages of micropropagation are as follows:

Stage 0: Choosing and preparing mother plants

At this stage, stock plants devoid of disease symptoms are chosen, and explants are surface sterilized using a variety of disinfectants to rid them of environmental microorganisms.

Stage I: Establishment of culture

At this stage, sterile explants are inoculated in suitable nutrient media to produce an aseptic culture with fresh shoot tips or calli. A group of explants are typically introduced to culture at the same time.

Stage II: Multiplication of propagules

At this stage, newly regenerated axillary buds, adventitious buds, or somatic embryos are multiplied by repeated subculturing in suitable nutrient media. Media are generally not modified between stage I and stage II.

Stage III: Rooting of *in vitro*-grown propagules

At this stage, *in vitro-grown* shoots are encouraged to grow longer and start adventitious roots that will eventually develop into full-fledged plants. Often the media composition is changed at this stage.

Stage IV: Acclimatization and transplantation of plants

At this stage, the *in vitro*-grown plants are transplanted to the suitable soil after acclimatizing in greenhouse conditions. *In vitro* plants are grown under humid conditions with minimal light intensity in the lab, so acclimatization of *in vitro*-grown plants is necessary to adjust to the harsh environment.

Based on the explant used in culture, *in vitro* plant tissue cultures can be divided into several different types, including meristem-tip, shoot-tip, callus, root, cell suspension, protoplast, anther, ovary, embryo, and seed cultures. Some are described below.

1.4.1 Meristem-tip and shoot-tip culture

Meristem-tip culture involves removing the organized shoot apex (less than 1 mm) using a sterile blade under the microscope from a desired plant for further tissue culture. The meristem-tip consists of the apical dome and a few juvenile leaf primordia, with no distinct pro-vascular or vascular structures (Grout, 1990).

Shoot-tip (3-4 mm to 2 cm in length) culture comprises the culture of the growing point that comprises the apical meristem and relatively a large number of immature leaf primordia. Shoot-tip culture is a frequent method of micropropagation, especially when researchers look to generate several plants from a single shoot tip (Nehra *et al.*, 1994).

1.4.2 Callus culture

A callus is a loosely arranged homogenous mass of thin-walled cells that is either regenerated from an injured part of a plant in nature or generated *in vitro* by the culture of explants in nutrient media. It has the biological potential to produce shoots, roots, and embryos by changing the auxin to cytokinin proportion in the media, eventually producing a plantlet.

1.4.3 Cell or suspension culture

It is the culture of individual cells or cell aggregates in a liquid medium by agitation and bubbling to avoid cell settling. Individual cells can be isolated from the callus or other plant tissues (explants), especially leaves, using both mechanical and enzymatic methods (pectinase solutions) for the preparation of cell suspension.

1.4.4 Somatic embryogenesis

It is the technique of generating somatic embryos instead of fusing the gametes *in vitro* from explant somatic cells in solid or liquid nutrient media to regenerate whole plantlets. Somatic embryos develop directly through the explant (direct somatic embryogenesis) or indirectly by producing callus in the media. It has significant applications in the fields of genetic engineering, clonal propagation, and the utilization of doubled haploids in conventional plant breeding.

1.5 Importance of plant growth regulators (PGRs) in *in vitro* culture

Plant growth regulators are chemical substances, either synthetic or natural, which can control the physiological, metabolic, and developmental processes of higher plants at very low concentrations (Hu and Wang, 1984). For various stages of *in vitro* culture, auxins and cytokinins have been widely utilized, either separately or in combination. Gibberellin, jasmonic acid, salicylic acid, strigolactones, and brassinosteroids are also used in tissue culture. These PGRs are paving the way for innovative plant regeneration techniques including suspension cultures (Sehgal and Joshi, 2022).

1.5.1 Auxins

Auxins are mainly responsible for cell elongation. Indole acetic acid (IAA) is the naturally occurring auxin in plants. Synthetic auxins like 2,4-dichlorophenoxy acetic acid (2,4-D) are utilized in callus induction and suspension culture, while an α-naphthaleneacetic acid (NAA) is also frequently used when organogenesis is essential in plant tissue culture. Auxins frequently work best when paired with cytokinins to produce the desired results. According to Bandurski *et al.* (1995) auxins have a significant impact on processes like cell elongation, cell wall acidification, cell division, and formation of meristems that give rise to either callus or defined organs (often roots) and support vascular development. Other auxins like picloram (pyridinemonocarboxylic acid) and dicambra (3,6-dichloro-o-anisic acid) are

frequently useful in causing the production of nodular/embryogenic tissue or in sustaining suspension cultures (Hagen *et al*., 1991; Bandurski *et al.*, 1995)

1.5.2 Cytokinins

Cytokinins are mainly responsible for cell division and cytokinesis, and they are useful in a variety of different processes, including wound healing, plant abscission, cell expansion, feminizing impact, and promoting shoot growth over root growth. There are two major categories of cytokinins utilized in *in vitro* culture: natural (such as zeatin, isopentenyl adinine, and cis-zeatin) and synthetic (such as kinetin, 6-benzylaminopurine, diphenylurea, and thidiazuron). The "6-amino purine moiety" is the most fundamental structural prerequisite for a molecule to be a cytokinin.

One of the important functions of cytokinins is to control cell proliferation, primarily in shoots. According to Chang *et al.* (2013) cytokinins have an impact on the production of lateral roots and hinder the development of root architecture. They restrict root growth by preventing primary roots from growing longer, interfering with how roots work, and promoting cell differentiation activity in the apical meristem of roots (Werner *et al.*, 2001). Moreover, indirect organogenesis through callus or direct organogenesis are both possible when cytokinin is combined with various auxin concentrations (Sehgal and Joshi, 2022).

1.5.3 Gibberellins

Gibberellins, which are naturally occurring plant hormones, are utilized to control plant growth by promoting cell proliferation and internode prolongation. A small number of gibberellic acids, such as GA_1, GA_3, GA_4, and GA_7, are bioactive whereas others are inactivated or precursor gibberellins (Igielski and Kpczyska, 2017). Numerous growth and development processes are governed by GA, including seed germination, stem lengthening, flowering, fruiting, and control gene activity in the layer of cereal aleurone. Only GA_3 and GA_{4+7} are frequently utilized in plant tissue culture out of the more than 130 gibberellins that exist in plants. Gibberellins frequently reduce or stop the development of roots, shoots, or somatic embryos when supplemented with plant tissue culture media (Gaspar *et al.*, 1996). According to Lance *et al.* (1976) some endogenous GAs may be required for proper callus development.

1.6 Metabolite production in suspension culture using elicitor and precursor

Suspension culture is a kind of cell culture whereby single cells or tiny clusters of cells proliferate in an agitating liquid media. As a result, the cells can divide and grow without sticking to a surface. Small fragments of loosely friable callus are cultured in liquid media to produce cell suspension cultures. Suspension culture helps produce bioactive compounds like phenolics, alkaloids, enzymes, recombinant proteins, and antibodies as well as for researching the development and proliferation of cells (Stewart 2008; Murthy *et al*., 2014). Over twenty pharmacological substances, including antibodies, erythropoietin, interleukins, human macrophage-granulocyte colony-stimulating factor (hGM-CSF), and hepatitis B antigen, have already been developed in cell suspension cultures (Shadwick and Doran, 2005).

Furthermore, by inducing stress reactions, many tissue culture techniques like suspension cultures are utilized to increase the quality and quantity of secondary metabolites. These strategies include employing elicitors, precursors, biotransformation, changes in environmental conditions, changes in medium ingredients, etc. to activate stress responses (Fazili *et al*., 2022). Plant tissue grown *in vitro* is one of the best sources for the synthesis of bioactive compounds, and elicitation is one of the key techniques to enhance the synthesis of these chemicals utilizing both abiotic and biotic elicitors (Rezaeieh *et al.*, 2012). Elicitors are organic or inorganic substances used to encourage metabolic changes in plants that manifest as increased amounts of bioactive chemicals from the perspective of a plant (Zhao *et al*., 2005). Elicitors can be classified as either abiotic or biotic depending on their type, and they can also be classified as exogenous or endogenous depending on where they come from. Elicitors have boosted the production of coumarins, isoflavonoid phytoalexins, sesquiterpenoids phytoalexins, and terpenoid indole alkaloids in a plant cell culture. Since they have a significant impact on indole alkaloid production, both biotic and abiotic elicitors are frequently used (Siddiqui *et al*., 2013).

Similarly, plants use molecules known as precursors for producing and enhancing the synthesis of bioactive metabolites. The plant can make higher secondary metabolites than it typically would by introducing precursors into the growth media (Murthy *et al*., 2014; Fazili *et al.*, 2022). The components and amount of the generated secondary metabolites may additionally be changed by the inclusion of precursors. For instance,

phenylalanine increased the formation of vinblastine, a compound having anti-cancer effects, in a suspension of *Catharanthus roseus* cells (Fazili *et al*., 2022).

1.7 Phytochemical production in plants

According to Liu (2013) phytochemicals are non-nutrient bioactive molecules found in a variety of plant parts that have been associated with a decrease in the threat of diseases. They are widely categorized into six groups based on their chemical characteristics and structures: carbohydrates, lipids, phenolics, terpenoids, alkaloids, and other nitrogen-containing compounds. Different subgroups originated within each category by additional categorization based on biosynthetic origin. Phytochemicals produced in plants include both primary and secondary metabolites, with secondary metabolites performing specific activities beyond fundamental plant functions.

1.7.1 Secondary metabolites

Several secondary metabolites are produced at the stationary phase of growth by the plant cell through secondary metabolic pathways, which are end products derived from the primary metabolic pathways (Bourgaud *et al.*, 2001). These are not necessary for primary metabolic activities. However, they might be significant in other ways besides the ecological function, including defense mechanisms. Recently, there has been a trend toward the study of naturally occurring secondary plant metabolites for their cost-effectiveness and fewer side effects (Newman and Cragg, 2016).

1.7.2 Types of bioactive secondary metabolites and their role in various bioactivities

A substance that affects a living thing, such as a cell, tissue, or organism, is called a bioactive compound (Guaadaoui *et al.*, 2014). Secondary metabolites can be categorized into main three types based on their chemical structures and functional groups, such as terpene or terpenoids, phenolics, and nitrogen-containing compounds. Some of the important bioactive secondary metabolites with their bioactivities are described below.

1.7.2.1 Terpenes

The terpenes [general formula: $(C_5H_8)_n$ for $n \geq 2$] are the organization of isoprene units, which are naturally occurring molecules, and are primarily found in plants, however,

larger groups of terpenes like sterols and squalene can also be found in animals. A basic unit of terpene is isoprene, which is a gaseous hydrocarbon with the chemical formula C_5H_8. Terpenes that contain oxygen are called terpenoids. They are in charge of giving plants their flavor, aroma, and color (Cox-Georgian *et al*., 2019). Terpenes are divided into hemiterpene, monoterpene, sesquiterpene, diterpene, triterpene, and tetraterpene based on the number of isoprene units.

Monoterpenes isolated from *Quercus ilex* are used as fragrances and repellents (Loreto *et al*., 2002); Sesquiterpenes isolated from *Helianthus annuus* are used to treat malaria, bacterial infections, and migraines (Chadwick *et al*., 2013); diterpenes isolated from *Salvia miltiorrhiza* are used as anti-inflammatory, and in cardiovascular diseases (Zhang *et al.*, 2012; Vasas and Hohmann, 2014); and triterpene isolated from *Centella asiatica* is used in wound healing, and increases circulation (James and Dubery, 2009). Moreover, limonene (monoterpene) has potent anti-cancer properties both *in vivo* and *in vitro* (Bishayee and Rabi, 2009). Curcumin obtained from *Curcuma longa* (Nabavi *et al.,* 2015), and andrographolide, a diterpenoid lactone obtained from *Andrographis paniculata* are useful in treating diabetes (Brahmachari, 2017).

1.7.2.2 Phenols

Phenolic compounds have a hydroxyl (OH) functional group on an aromatic ring, and polyphenols represent compounds with two or more phenol rings attached. The majority of secondary metabolites fall into the phenolic compound category, which is extensively dispersed throughout the plant kingdom (Giada, 2013). Plant species have more than 8000 phenolic compounds that have been linked to the flavor and color of fruits and vegetables (Cartea *et al*., 2010). Polyphenolic compounds are categorized into several subgroups such as phenolic acids, flavonoids, tannins, coumarins, stilbenes, xanthones, and lignans. Among them, flavonoids are large groups of polyphenols that are again sub-divided into flavans, flavan-3-ols, flavanones, flavanonols, flavones, flavonols, anthocyanidins, isoflavones, and chalcones.

Polyphenols are bioactive compounds that show various biological activities such as antifungal and antibacterial activities (Rao *et al.*, 2013), antioxidative stress (Tsao, 2010), prevention of cancer, heart disease, diabetes, osteoporosis, neurodegenerative disorders, hypertension, and asthma (Pandey and Rizvi, 2009). Depending on the

structural variations and glycosylation patterns, flavonoids exhibit a variety of biological properties, such as antioxidant activity (Pandey and Rizvi, 2009).

1.7.2.3 Alkaloids

According to Wink (2016), all nitrogen-containing natural compounds, basic that are not non-protein amino acids, peptides, cofactors, amines, cyanogenic glycosides, phytohormones, glucosinolates, or primary metabolites (such as pyrimidine and purine bases) are considered to be alkaloids. They are categorized into three groups based on their origin: true alkaloids, which are derived from amino acids and have an N atom in a heterocyclic ring; proto alkaloids, which are derived from amino acids but don't have an N atom in a heterocyclic ring; and pseudo alkaloids, which come from sources other than amino acids but have an N atom in a heterocyclic ring. Similarly, alkaloids can be classified as either heterocyclic alkaloids, also known as typical alkaloids, which have an N atom in the heterocyclic ring (indole alkaloids), or non-heterocyclic alkaloids, which do not have an N atom in the heterocyclic ring.

Alkaloids exhibit a wide range of biological actions both *in vivo* and *in vitro*. Ephedrine is a central nervous system (CNS) stimulant; caffeine, theophylline, and theobromine are stimulants; morphine is a hallucinogen and analgesic; mescaline causes hallucinations; cocaine is a stimulant and analgesic, and quinine is antimalarial (Wink, 2016). Although morphine is a potent opioid used to treat pain, its utility is constrained by its propensity for addiction (Chisholm, 2015). Numerous physiological effects of alkaloids include antimicrobial, antimitotic, anti-inflammation, analgesic, local anesthetic, hypnoses, psychedelic, and anticancer action (Kurek, 2019).

1.7.2.4 Glycosides

Glycosides are naturally occurring complex organic substances in which sugar (carbohydrate) is bonded by hydroxyl groups on non-sugar molecules. Non-sugar part is called aglycone and can be terpenoids, steroids, flavonoids, and other phenols, while the sugar part is called glycone and may be glucose, L-fructose, L-arabinose, galactose, mannose, L-rhamnose, and D-xylose. Water, mineral acids, and enzymes like α-glucosidase can easily hydrolyze the unstable glycosidic linkages to produce the glycone and aglycone components.

Glycosides can be categorized into phenolic glycosides, coumarin and chromone glycosides, flavonoid glycosides, anthraquinone glycosides, saponin glycosides, cardiac glycosides, cyanogenic-glycosides, and thioglycosides based on the aglycon structure (Bartnik and Facey, 2017). These glycosides show various biological activities, for example salicin anti-inflammation and antirheumatic, esculetin anti-haemorrhoidal, anti-phlogistic, anti-rheumatic, hyperoside diuretic and anti-inflammatory, alizarin and rhein antiviral, diosgenin antimicrobial, hypoglycemic, and anti-atheromatic, antihypertension, anti-diabetes, and anti-cancer activities (Barnard *et al.*, 1992; Bartnik and Facey, 2017).

1.8 *In vitro* bioactivities of plant extracts or secondary metabolites

According to Karas *et al.* (2017) bioactivity refers to the particular impacts, such as cell absorption, metabolism, or biological reactions of a substance/extract. Bioactivity can be evaluated using many methods, including *in vivo* (animal model or human studies), *ex vivo* (laboratory organs), and *in vitro* (artificial membranes, cell cultures, chambers). *In vivo* experiments are necessary to accurately assess the bioactivity of plant extracts/compounds (Carbonell-Capella *et al.* 2014). Some bioactivities of plant extracts tested in this study are discussed below.

1.8.1 Antioxidant activity

Antioxidants are chemical compounds, either exogenous or endogenous, that help to reduce oxidative or nitrosative stress and its repercussions (Kurutas, 2015). Sindhi *et al.* (2013) define an antioxidant as a chemical that slows down the oxidation of carbohydrates, lipids, proteins, and DNA at low concentrations. Similarly, free radicals are characterized as independent existing chemical entities with one or more electrons that are unpaired. Reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) include reactive molecules and are free radicals related to the oxygen atom or equivalents. The excessive production of ROS and RNS in plants and animals may harm proteins, lipids, carbohydrates, and DNA, resulting in oxidative and nitrosative stresses. Oxidative and nitrosative stresses damage tissues and lead to many different diseases and illnesses. However, antioxidants assist in avoiding diseases by neutralizing the effects of ROS and RNS.

Endogenous antioxidant mechanisms comprise enzymes such as catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase, as well as non-enzymatic

substances like vitamin C, vitamin E, a-tocopherol, thiol antioxidants (glutathione, thioredoxin), melatonin, carotenoids, and flavonoids (Wojcik *et al.*, 2010; Kurutas, 2015). Several medicinal and aromatic plants have been proven to have antioxidant properties. Numerous antioxidant compounds, including phenolics (phenol and polyphenols), flavonoids, carotenoids, steroids, and thiol compounds, are present in natural antioxidants and can aid in preventing damage to cells brought on by oxidative stress (Lotito and Frei, 2006).

1.8.2 Antibacterial activity

Antibacterial activity describes the capacity of substances/agents to kill or hinder the growth of bacteria. Antibacterial agents may be bacteriostatic or bactericidal based on their effect on bacterial cells. Bactericidal antibacterial agents kill bacteria by destroying their cell walls or membranes, whereas bacteriostatic antibacterial agents slow, hinder, or stop the growth of bacteria (Ullah and Ali, 2017). Antibacterial properties are important in many industries, including medicine, agriculture, and food preservation, because they help regulate and avoid infections caused by bacteria and contamination.

Antibacterial agents prevent the growth of bacterial cells or kill the bacterial cells in several ways, including prevention of cell wall formation, protein formation, DNA synthesis, metabolic pathway inhibition, and cell membrane disruption (Yan *et al.*, 2021). Researchers conduct antibacterial tests on plant extracts against various types of bacteria for a variety of reasons, including the discovery of new antibiotics and novel mechanisms of action, the validation of traditional medicine, the identification of bioactive compounds, and pest disease control in the agricultural field.

1.8.3 Anticancer activity

Cancer is a condition marked by the human body's aberrant cells growing out of control. Anticancer activity of plants refers to the ability of plant-originated compounds or agents to prevent, inhibit, or destroy cancer cells or tumor cells. Anticancer agents can suppress cancer cell proliferation, induce apoptosis, halt the cell cycle, prevent metastasis (stop cancer cells from spreading throughout the body), and reduce angiogenesis (inhibit the development of new blood vessels). Apoptosis is a type of programmed cell death that aids in the elimination of cancerous cells (Renehan *et al.*, 2001).

Calman *et al.* (1980) described three mechanisms of anticancer activity exhibited by anticancer agents: (i) DNA damage and repair modulation: certain plant compounds can cause damage to DNA in cancer cells or hinder their repair systems, resulting in cell death, (ii) Signal transduction pathway modulation: plant extracts may affect cell proliferation, differentiation, and apoptosis pathways (for example, MAPK, PI3K/AKT, and p53), and (iii) Immune system modulation: many plant extracts can help boost the body's immunological response against cancer cells.

1.8.4 Antidiabetic activity

Diabetes mellitus, also referred to as diabetes, is a health problem marked by high amounts of glucose (a form of sugar) in the blood. It develops if the pancreas does not produce sufficient insulin or if the human body fails to adequately utilize the insulin it releases. Three kinds of diabetes are identified, such as type 1, type 2, and gestational diabetes. Among them, type 1 diabetes is mainly a genetic disorder characterized by insulin breakdown and elevated glucose levels. Type 2 diabetes is the most frequent type and is caused by resistance to insulin. Gestational diabetes develops while pregnant when a woman has elevated blood sugar levels.

Several natural substances discovered in plants have shown potential effects in treating diabetes. Antidiabetic agents lower blood sugar levels in a variety of ways, each addressing a particular element of glucose metabolism and insulin regulation. According to Chaudhury *et al.* (2017) the mechanisms by which various types of antidiabetic agents reduce blood glucose levels are explained below.

- Antidiabetic agents act as insulin and insulin analogs and mimic endogenous insulin. They promote glucose absorption into cells, particularly muscle and adipose tissue.
- Antidiabetic agents act as insulin secretagogues. They increase insulin secretion from pancreatic β-cells.
- Antidiabetic agents act as α-glucosidase inhibitors. They suppress intestinal enzymes that degrade carbs, delaying the breakdown of carbohydrates and glucose absorption, resulting in a slower increase in postprandial blood glucose levels.

- Antidiabetic agents act as amylin analogs. They imitate the activity of amylin, a hormone released with insulin that slows stomach emptying, suppresses postprandial glucagon production, and increases satiety.

1.9 Metabolomics of natural resources

Metabolomics is a life science study topic that uses high-throughput technology such as liquid chromatography-high resolution mass spectrometry (LC-HRMS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) spectroscopy, etc. to identify and analyze all metabolites produced in a cell, tissue, or organism or derived from external sources, including nutrition, microorganisms, and xenobiotics (Nicholson *et al.*, 1999; Fiehn, 2002). Metabolites (molecular wt. <1500 Da) are tiny compounds that serve as intermediates or end products in the metabolic processes of cells (Fiehn, 2002). Metabolomic research is classified into two categories: targeted or untargeted, depending on the scope of the metabolite study (Patti *et al.*, 2012). Targeted metabolomics emphasizes studying a particular category of metabolites where as non-targeted studies aim to cover the maximum number of metabolites in a biological system.

A common metabolomics process involves collecting samples from plants, fungi, or microbes and preparing methanol, ethanol, or ethyl acetate extracts to address research issues (Fig. 3). These extracts contain various metabolites that can reveal key cellular processes. Then, metabolites in the biological sources are measured using high-throughput analytical techniques such as NMR spectroscopy, GC-MS, and LC-HRMS (Bhattarai *et al.*, 2022).

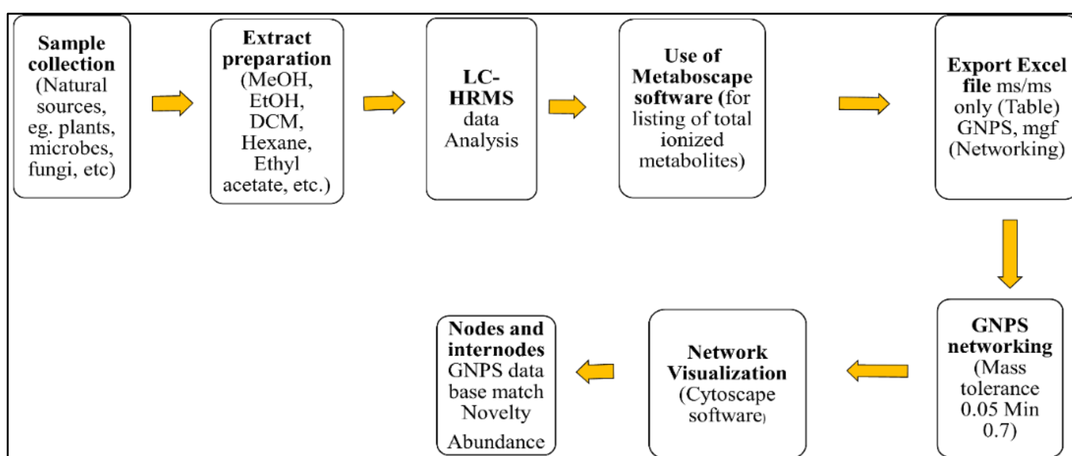


Figure 3: Workflow for metabolomics

Some of the important uses of metabolomics are listed below:

- It is useful for the metabolite profiling of plant species. By examining metabolite profiles, researchers gain knowledge about numerous chemicals, including amino acids, carbohydrates, phenols, polyamines, terpenes, and others (Salam *et al.*, 2023). Therefore, this profile may aid in identifying distinctions among plant species, ecotypes, and how they respond to biotic and abiotic factors.
- Metabolomics helps understand stress tolerance to both abiotic and biotic stresses in plants. Researchers can identify metabolic alterations caused by stresses like dryness, infections, or severe temperatures. Knowing these reactions informs methods for improving plant resistance and adaptation (Salam *et al.,* 2023).
- Metabolomics helps with the discovery of secondary metabolites or natural products that contain bioactive compounds with possible uses in healthcare, farming, and industries (Yan *et al.*, 2022).

1.10 Rationale of the study

P. polyphylla is a vulnerable medicinal plant under the IUCN threat category (Chauhan, 2020). Threats to *P. polyphylla* include habitat degradation, harmful commercial harvest, and occasionally even a small geographical distribution (Cunninghama *et al.*, 2018). The main hazards to plant renewal are animal trampling, historical excessive harvesting, unethical rhizome collection, harvesting of the plant before seed maturation, decreased quantity of healthy seed production, lengthy dormancy of seeds, or extremely poor germination of seeds (KC *et al.*, 2012). The rhizomes of this plant are utilized in ethnomedicine, Traditional Chinese Medicine (TCM), and Ayurveda to cure various disorders, such as cuts, wounds, stomach disorders, antitoxins, anthelmintic, anticancer, etc. Similarly, *P. longum* is a medicinal plant threatened by over-collection for the export trade in Nepal (www.floraofnepal.org; accessed on 20/8/2022). Various parts, especially, fruits/spikes and roots with small segments of stem (Piplamul) are used in various ailments and diseases, such as cough, cold, bronchitis, asthma, diabetes, antidote in snake and scorpion bites, etc. The vegetative propagation of *P. longum* in soil through nodes can result in the production of a single plant from each node. However, the *in vitro* culture approach can be used for *ex-situ*

conservation to regenerate several shoots from each node. Medicinal plants that are selected in the present investigation are in the threatened category so there is a critical need for their sustainability conservation. Tissue culture is the best supportive technology that can preserve the germplasm, as well as mass propagation is possible without making a direct impact on its natural habitat.

Furthermore, *P. polyphylla* and *P. longum* are prioritized medicinal plants for research and development as well as for agrotechnology development by the Department of Plant Resources (2017), Government of Nepal. Therefore, there should be a high-priority to conserve these plant species in their natural habitat as far as practicable and also cultivate them on a commercial plantation basis by the production of disease-free, cost-effective, and genetically homogenous plants through *in-vitro* culture followed by their effective field trial. It may help to strengthen the Government's policy regarding the conservation of these plant species.

P. polyphylla contains valuable bioactive metabolites, such as steroidal saponins, triterpenoid saponins, flavonoid glycosides, sterols, and polysaccharides (Xiao *et al*., 2009; Wu *et al.,* 2012a; Wu *et al.,* 2012b). The above compounds, especially, steroidal saponins have antioxidant, antimicrobial, anticancer, antiviral, antileishmanial, anthelmintic, and antityrosinase enzyme activities (Thapa *et al.*, 2022). Similarly, the fruits of *P. longum* contain various bioactive compounds, such as alkaloids and amides (piperine, piperlongumine, piperetine, piperlonguminine, piperderidine, pipercide), lignans, esters, and volatile oils (Zaveri *et al*., 2010). Various parts of *P. longum* have antioxidant, insecticidal and acaricidal, antifungal, antiamoebic, antimicrobial, antiasthmatic, antidiabetic, anti-inflammatory, anti-tumor, anti-depressant, and hepatoprotective activities (Zaveri *et al.,* 2010). However, there has been insufficient research into the bioactivities of wild-grown different parts of *P. longum* and *P. polyphylla* for the comparison to *in vitro*-raised callus, as well as for the identification of bioactive compounds to utilize in natural drugs.

Callus, on the other hand, has biological potential for whole-plant regeneration and bioactive compound production. Extraction, screening, identification, and profiling of bioactive metabolites, and bio-prospecting of these plants and their *in vitro*-raised callus are very important to prove their medicinal properties, which will be in great demand in pharmaceutical industries in the future for drug discovery. Similarly, the

callus suspension culture using elicitor (salicylic acid) and precursor (phenylalanine) in *P. longum* and *P. polyphylla* for the synthesis, increase, or enhancement of secondary metabolites may be an alternative source of natural products that may help for the management and conservation of these medicinal plants in the wild habitat by utilizing callus instead of wild plants. However, a thorough investigation into the bioactivity, metabolite production, and enhancement of metabolite production through suspension culture using elicitors and precursor of *in vitro*-raised callus of *P. longum* and *P. polyphylla* to identify useful metabolites is inadequate.

1.11 Hypotheses

The present work was based on the following research hypotheses.

- Micropropagation of *P. longum* and *P. polyphylla* is possible from various explants due to the totipotent nature of cells.
- *In vitro*-produced callus and its suspension cultures of *P. longum* and *P. polyphylla* can synthesize bioactive compounds, which can exhibit various bioactivities in *in vitro* conditions.
- Enhancement of the bioactive compounds is possible in plant cell suspension culture by using an abiotic elicitor (salicylic acid) and precursor (phenylalanine).

1.12 Objectives

1.12.1 General objective

This research aims to develop an effective protocol for micropropagation and other culture types as well as to compare the bioactivities and bioactive compounds of *in vitro*-raised callus and *in vivo* parts of *P. longum* and *P. polyphylla*.

1.12.2 Specific objectives

Some specific objectives are:

- To find out the best explants and culture type for *in vitro* propagation of *Piper longum* and *Paris polyphylla.*
- To assess *in vitro* antioxidant, antibacterial, anticancer, and antidiabetic properties of extracts from *in vitro*-raised callus and *in vivo* various parts of *P. longum* and *P. polyphylla*.

- To develop a metabolite enhancement strategy of phenolic and flavonoid contents production in suspension culture by using precursor (phenylalanine) and elicitor (salicylic acid) in *P. longum* and *P. polyphylla*.
- To screen phytoconstituents and dereplicate the possible plant metabolites in *in vitro*-raised callus and *in vivo* parts of *P. longum* and *P. polyphylla* by LC-HRMS analysis.

CHAPTER 2

2. LITERATURE REVIEW

2.1 In vitro culture of *Piper longum* and *Paris polyphylla*

Micropropagation of plants can be achieved through three approaches, viz. multiplication of axillary shoots (axillary budding), initiation of adventitious shoots (adventitious budding), and somatic embryo development (somatic embryogenesis). The axillary budding utilizes the culture of the nodal explants on a medium enriched with cytokinin and auxin; the adventitious budding utilizes the culture of the petiole, root segments, or intermodal segment on a medium; and the somatic embryogenesis utilizes the culture of leaf segment, callus, micropylar tissue of seeds, immature zygotic embryo, etc. on a suitable media. The axillary budding is the most reliable method of micropropagation producing about 90% of the current production of the micro-propagated plants with genetic stability.

2.1.1 *In vitro* propagation of *P. longum*

The literature demonstrates that the two types of approaches were used for the *in vitro* propagation of *P. longum.* In other words, they used direct organogenesis using nodal explants, and indirect organogenesis utilizing leaf explants (callus) to regenerate plantlets. However, micropropagation through somatic embryogenesis techniques was not reported in *P. longum*.

2.1.1.1 Plant regeneration through direct organogenesis

Earlier researchers used a variety of explants, including nodal segments, internodal segments, petioles, shoot-tips, and fruit/spikes for direct organogenesis in MS media. Soniya and Das (2001) regenerated plants from the shoot-tips in MS medium in *P. longum*. They successfully induced the greatest number of shoots from shoot tips in 8.9 µM BA and 4.64 µM KN. Additionally, they directly regenerated the greatest number of adventitious shoots from leaf segments using 17.76 µM BA and 8.28 µM picloram (P). The MS enriched with 2.46 µM IBA was used to develop roots from *in vitro*-developed shoots. Padhan (2015) was able to regenerate plantlets in *P. longum* from nodal segments through the induction of several shoots in MS + 1.0 mg/L KN + 1.5 mg/L BAP. The roots were induced in 0.5 mg/L IAA from tissue culture-generated shoots. Parida and Dhal (2011) developed several shoots from each nodal explant in

MS medium enriched with 1.0 mg/L BA as well as 1.0 mg/L IAA. From *in vitro*-grown shoots, they produced numerous roots in 1.0 mg/L BA and 0.2 mg/L IAA. Bhat *et al.* (1992) induced many shoots from *in vitro*-grown shoots in MS media enriched with 0.5 mg/L IAA as well as 1.5 mg/L BA. They used 0.1 mg/L IAA and 0.2 mg/L BA for shoot elongation and made rooted in half-strength MS media supplemented with 0.1 mg/L IAA. Tiwari (2016) generated shoots (95%) from the nodal segment in MS media supplied with 5.0 mg/L BAP, 0.5 mg/L NAA, and 15 mg Kanamycin, and roots (70%) in MS media enriched with 0.5-1.0 mg/L IBA.

Fonseka and Wickramaarachchi (2018) produced multiple shoots in *P. longum* using nodal segments in MS medium supplied with 0.5 mg/L KN, 3.0 mg/L BAP, and 0.2 mg/L NAA. In 1/2 MS media enriched with 100 mg/L ascorbic acid, 15 g/L glucose, 0.8% agar, and 1.0 mg/L NAA, the *in vitro* shoots regenerated adventitious roots. Sharon and Maurya (2004) produced 6 root-like structures per explant using the axillary bud in MS media added with 0.01-0.5 mg/L NAA; multiple shoots from *in vitro* root-like structures in 0.5-3.0 mg/L BAP, and roots from *in vitro* shoots in 0.1-3.1 mg/L IBA. Similarly, Sarasan and Nair (1991) regenerated direct multiple shoots (289) using shoot tips in MS media supplemented with 1.0 mg/L BA and 0.1 mg/L GA_3; multiple shoots from leaf discs in 3.0 mg/L BA and 10% CW; direct multiple shoots from internode in 3.0 mg/L BA and 10% CW.

Moreover, Rani and Dantu (2012) regenerated axillary buds directly using nodal segments in MS medium added with 0.5 mg/L BA and 40 mg/L ascorbic acids. They also developed multiple shoots from petiole and internode, and multiple roots from *in vitro* shoots in MS medium enriched with 0.25 mg/L IBA. Ravindran *et al.* (2016) regenerated multiple shoots directly from nodal segments in MS medium added with 1.0 mg/L BAP, and multiple roots from *in vitro* shoots containing 300 mg/L IBA in greenhouse conditions. Similarly, Prajapati (2019) regenerated multiple shoots from fruit spikes in MS media combined with 0.25 mg/L TDZ; and multiple roots from *in vitro* shoots in 1.0 mg/L IBA.

2.1.1.2 Plant regeneration through indirect organogenesis

Earlier researchers used leaf segments and internodal segments for indirect organogenesis in MS media. Sathelly *et al.* (2016) induced callus using leaf discs in MS media enriched with 1.0 mg/L IAA and 1.0 mg/L BAP; multiple shoots from callus

in 2.0 mg/L BAP + 1.0 mg/L KN; and multiple adventitious roots were produced from *in vitro* shoots in 1/2 MS media enriched with 1.0 mg/L NAA. Prajapati *et al.* (2019) produced callus using a leaf segment in MS media supplied with 0.5 mg/L TDZ, multiple shoot buds from the callus in in 0.25 mg/L TDZ, and roots in 1.0 mg/L IBA. Sarasan and Nair (1991) regenerated callus and multiple shoots from internode in MS medium containing 2.0 mg/L 2,4-D + 1.0 mg/L BA, but could not regenerate roots.

According to Malthi *et al*. (2016) callus was formed using leaf segments of *P. longum* in MS medium containing 1.5 mg/L BAP + 1.0 mg/L 2,4-D; multiple shoot buds from *in vitro*-raised callus in MS +n1.0 mg/L KN + 1.0 mg/L BAP + 0.5 mg/L IAA; and many roots from *in vitro*-raised shoots in MS + 1.0 mg/L NAA.

2.1.2 *In vitro* propagation of *P. polyphylla*

There are a few studies on micropropagation of *P. polyphylla*. Earlier researchers utilized different kinds of explants, such as rhizomes, leaf segments, immature zygotic embryos, shoot apex, and transverse thin cell layer (tTCL) of shoot buds for the regeneration of *P. polyphylla* in MS media.

2.1.2.1 Plant regeneration through direct organogenesis

Devi *et al.* (2017) regenerated *P. polyphylla* using rhizome as explants in half-strength MS media enriched with two cytokinins with 6% sucrose concentrations. They induced mini-rhizomes and plantlets in ½ MS + 0.5 mg/L BAP and ½ MS + 1.0 mg/L 2iP with the best response having 1.27±0.02 g and 1.36±0.10 g fresh weight of mini rhizome respectively. Devi *et al.* (2018) regenerated shoots and roots from the rhizome in MS medium containing 2.0 mg/L BAP + 2.0 mg/L NAA + 2.5 mg/L charcoal + 2% sucrose + 0.8% agar. Danu (2016) found that 50 g/L sucrose was the most efficient in giving maximum shoot bud breakage in MS medium supplied with 4.44 µM BA+2.85 µM IAA from the rhizome. The 1/4 MS medium added with 2.45 µM IBA was the most suitable for *in vitro* root formation from *in vitro*-grown shoots.

Similarly, Raomai *et al.* (2014b) used the transverse thin cell layer (tTCL) of terminal young shoot buds to regenerate plantlets in *P. polyphylla* by growing mini-rhizomes (MRs) in MS media. They found that basal sections of shoot bud grown on half MS media enriched with 0.5 mg/L BAP produced the highest response of MRs with the greatest fresh weight (1.05±0.08 g). These MRs developed into plantlets in the absence of PGRs. They successfully acclimatized plantlets in a greenhouse environment with a

lifespan rate of greater than 95%. Teerawatsakul *et al.* (2014) utilized the shoot-tip of the rhizome of *P. polyphylla* var. *chinensis* for micropropagation in MS media without the addition of hormones. For 45 days, shoot-tips were grown in a gradient incubator with temperatures ranging from 5 to 18ºC. They found that temperature was a key component in regulating *in vitro* culture and that temperatures between 14 and 16°C were preferable to other temperature ranges for regulating endophytic bacteria and plant growth.

2.1.2.2 Regeneration of plant via indirect organogenesis

Danu (1016) induced callus from leaf explants in MS medium enriched with 4.44 µM BAP + 4.53 µM 2,4-D for the regeneration of plants.

2.1.2.3 Plant regeneration through direct somatic embryogenesis

Raomei *et al.* (2014a) induced somatic embryos directly and regenerated plantlets in *P. polyphylla* using immature zygotic embryos (IZEs) in MS media. They found the maximum frequency of somatic embryogenesis and mean no. of somatic embryos (SEs) for each explant in ½ MS medium directly without an intermediary callus phase. On a 1/2 MS medium in the absence of PGRs, they generated secondary somatic embryos (SSEs) on the primary somatic embryos (SEs) in a persistent manner. The medium that had been added with 0.05 mg/L BAP and 0.1 mg/L NAA had the greatest percentage of seedlings to plantlet conversions.

2.2 Callus suspension culture and enhancement of secondary metabolites in *P. longum* and *P. polyphylla*

The culture of plant cells involving cell growth in a liquid media is known as suspension culture. It is a practical *in vitro* technique for the elicitation of secondary metabolites and their enhancement in plants. Several investigations on elicitation and enhancement of bioactive compounds using abiotic and biotic elicitors, and precursors in cell suspension culture have been carried out. However, no reports have been found regarding the suspension culture of *P. longum.* To date, one study has been found on the suspension culture and enhancement of bioactive compounds using elicitors in *P. polyphylla*.

Delgado-Paredes *et al.* (2013) established a cell suspension culture from the callus culture of *Piper aduncum, P. cernuum, P. crassinervium*, and *P. regnellii*, and investigated their growth and metabolite accumulation. Similarly, Rodrguez-Sánchez

et al. (2020) found that the kind of elicitor (Methyl Jasmonic Acid or Salicylic Acid), the amount of elicitor (10 mM and 100 mM), and the period of exposure (3,12,24 h) on *Piper cumanense* cell suspension all affected the differential synthesis of bioactive compounds. The addition of 100 mM salicylic Acid in cell suspension was successful in increasing the formation of certain metabolites, such as (Z)-9 octadecenamide (8.8%), phenol, and 5-hydroxymethylfurfural (6.3%).

Rawat *et al.* (2023) assessed the production of callus biomass, steroidal saponin (dioscin and diosgenin), antioxidant activity, total phenolic, total flavonoids, total tannin contents in callus suspension culture of *Paris polyphylla* rhizome using Thidiazuron (TDZ), Methyl Jasmonate (MeJA), and Yeast Extract (YE). According to them, the MS medium supplemented with 50 mM MeJA had a high phenolic and tannin content, but no other treatment showed a noticeably higher level of overall tannin synthesis. Similarly, the medium with 1.0 mM TDZ and 50 mM MeJA had the best antioxidant activity. In contrast to the wild sample, which had lower levels of dioscin and diosgenin, the HPLC analysis revealed an elevated level of these compounds.

2.3 Bioactivity of various extracts of *P. longum*

Several researchers have examined the bioactivities of different crude extracts, their fractions, and pure compounds isolated from different parts including fruits, roots, as well as leaves of *P. longum* both in *in vitro* and *in vivo*. The bioactivity test of *in vitro*-grown callus has received relatively little attention in *P. longum*.

2.3.1 Antioxidant Activity

The antioxidant properties of different parts including roots, seeds, fruits, and leaves of *P. longum* have been investigated using a variety of extracts, including methanol, chloroform, aqueous, ethanol, ethyl acetate, and hexane. Most researchers used DPPH or free radical scavenging assay to determine the *in vitro* antioxidant activities.

Jagdale *et al.* (2009) assessed the free radical scavenging activity of piperine isolated from *P. longum* roots and petroleum ether extract from roots. They induced myocardial ischemia in rats, which is a significant cardiac condition, and antioxidants help in treating it. They reported that petroleum ether extract and piperine demonstrated 74.12% and 72.13% free radical inhibition at 50 mg/mL concentration, respectively, and benefits in rat myocardial ischemia. Barua *et al.* (2014) demonstrated the greatest

in vitro free radical scavenging properties of the chloroform extract of seeds of *P. longum* compared to all other extracts, such as aqueous, hydro-ethanol, ethanol, ethyl acetate, and hexane extracts utilizing DPPH assay.

Archana *et al.* (2015) determined the antioxidant properties of methanol (MeOH) extracts of *P. longum* fruit. The MeOH extract had the maximum free radical scavenging activity at a concentration of 40 µg/mL, which was close to the ascorbic acid standard. Parida and Dhal (2011) investigated the free radical scavenging activity of water and EtOH extracts of *P. longum* leaf by using the DPPH method. When contrasted with water extract at the 160 µg/mL concentration, the ethanol fraction had the maximum free radical scavenging activity at the same concentration. Similarly, by using the DPPH test, Banerjee *et al.* (2017) compared the antioxidant activity of leaves of *P. longum* both *in vivo* as well as *in vitro*. They reported that *in vitro*-grown callus in hot extracts displayed greater antioxidant capacity than *in vivo*-grown leaves. However, the total phenolic content was 43.75 percent for *in vivo* leaves and 44.7 percent for *in vitro*-grown callus extracts.

Krishna *et al.* (2014) assessed the free radical scavenging properties of *P. longum* fruit using the DPPH test, hydroxyl radical scavenging test, total phenolic content (TPC), total flavonoid content (TFC), and total reducing power (TRP). The ethyl acetate (EtOAc) extract demonstrated better free radical scavenging activity in the DPPH test and the hydroxyl radical scavenging test. Similarly, Sultana *et al.* (2019) found higher antioxidant activity (IC_{50} of 149.42 µg/mL) of the methanol extract of *P. longum* leaf by DPPH assay.

2.3.2 Antibacterial activity

Antibacterial test on plant extracts acts for several crucial purposes, including determining the potential medicinal properties of plant extracts, screening a variety of medicinal plants for antibacterial properties, recognizing the action of plant extracts on bacterial pathogens, assessing plant extract safety and toxic effects, and making a contribution to antibacterial drug development.

Various parts such as root, stem, leaf, and fruits of *P. longum* extracts have demonstrated *in vitro* antibacterial activity in previous research. Sawhney *et al.* (2011) assessed the antibacterial activity of ethyl acetate, methanol, and aqueous extracts of *P. longum* fruits on some bacterial strains by agar well diffusion method. According to

them, *Streptococcus mutans* proved to be the most resilient bacterium since none of the extracts could stop the bacterial growth. *S. aureus* was shown to be strongly inhibited by methanol extract, but *S. pyogenes, S. pneumoniae,* and *Klebsiella pneumoniae* were very slightly affected. Banerjee *et al.* (2017) reported that hot extracts of *in vitro*-grown callus of *P. longum* showed better antibacterial activity in some bacterial strains, such as *Escherichia coli, Bacillus subtilis,* and *S. aureus* than *in vivo* leaf extracts. Lokhande *et al.* (2007) showed noteworthy antibacterial activities of various *Piper* species on a variety of bacterial strains, such as *Salmonella typhi, Staphylococcus albus, Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus megaterium,* as well as one fungus, *Aspergillus niger*. They also found that the aqueous extract failed to demonstrate any antimicrobial activity toward the pathogens that were investigated.

Ali *et al.* (2007) showed that the majority of the tested microorganisms (5-gram-positive and 8-gram-negative) were only mildly to moderately affected by EtOAc, chloroform, and MeOH root extracts of *P. longum*. For the stem, EtOAc, chloroform, and MeOH extracts showed modest to moderate effectiveness on the majority of the bacteria examined, but the petroleum ether extract was effective on *S. aureus* and *Shigella boydii*. Similarly, regarding leaves, all crude extracts (apart from petroleum ether) showed mild to moderate efficacy towards the majority of bacterial strains, whereas petroleum ether extract demonstrated effectiveness only toward *S. aureus*. It demonstrates that the extracts from the various parts of *P. longum* do not contain the same bioactive antibacterial compounds. Sultana *et al.* (2019) exhibited that, at various doses, the methanol extract of *P. longum* leaf exhibited excellent activity towards *S. typhi* and *S. aureus* but decreased effectiveness towards *S. paratyphi*, *B. subtilis*, and *S. dysenteriae*, as well as weak action toward *E. coli*. Barua *et al.* (2014) found that MICs (minimum inhibitory concentrations) of various extracts of *P. longum* seeds on *Mycobacterium smegmatis* (non-tuberculosis) bacteria in the ascending order of chloroform >ethanol>hexane>ethyl-acetate respectively. Similarly, MBCs (minimum bactericidal concentrations) were reported as 20.23 mg/mL in chloroform, 33.43 mg/mL in ethanol, 36.23 mg/mL hexane, and 64.09 mg/mL ethyl acetate extracts respectively. Khan and Siddhiqui (2007) also compared the antibacterial activity of seven extracts, such as carbon tetrachloride, benzene, chloroform, EtOAc, acetone, EtOH, and distilled water of *P. longum* fruits on five bacterial strains including *E. coli, S. albus, S. typhi, P. aeruginosa,* and *B. megaterium.* Among all the extracts, benzene

extract was reported to be the most susceptible to all of the examined bacterial strains. Moreover, Saraf and Saraf (2014) compared the antimicrobial activity of alcohol, chloroform, and aqueous extracts of *P. longum* fruits by both disc diffusion and agar well diffusion methods. They claim that while all of the extracts were sensitive to the tested bacterial strains, including *S. aureus, S. pyogenes*, and *K. pneumoniae*, the alcoholic extract had the largest zone of inhibition.

Multiple drug resistance (MDR) microbial strains have recently emerged, posing a serious risk to the well-being of humans and posing one of the major obstacles to international drug development projects (Shriram *et al*., 2010). The aqueous and MeOH extracts of *P. longum* fruits have been evaluated by Kumar *et al.* (2013) on clinically relevant isolates of MDR strains of bacteria such as *S. typhi, Shigella sonnei, S. aureus*, along with reference strains of *B. subtilis* (pUB110) and *E. coli* (RP4). They claimed that the crude methanol extract had a 42% cure rate for clinically isolated strains of *S. sonnei* and showed substantial antibacterial efficacy with a MIC of 400 µg/mL versus *B. subtilis*. Moreover, Singh *et al.* (2011) claimed that MDR bacteria like *M. smegmatis* (3000 µg/mL) and *M. tuberculosis* (39 µg/mL) were more sensitive to an ethyl acetate fraction than petroleum ether, hexane, chloroform, and MeOH extracts of *P. longum* fruits.

2.3.3 Antidiabetic activity

Diabetes develops as a consequence of poor glucose utilization, resistance to insulin, and excessive production of glucose (Ginsberg and Huang, 2000). *P. longum* can be utilized as a substitute medication for managing diabetic problems brought on by oxidative damage and diabetes. Various parts and extracts of *P. longum* have been examined for their *in vivo* antidiabetic activities by previous researchers. However, there have been a few research on *in vitro* antidiabetic activity of *P. longum*.

To study antidiabetic activity *in vivo*, Nabi *et al.* (2012 and 2013) administered streptozotocin/STZ (50 milligrams per kg body wt) intraperitoneally to male Wister albino rats, causing them to develop diabetes. In comparison to placebo diabetic rats, they found that giving root aqueous extract to diabetic rats at a dosage of 200 milligrams per kg body wt every day for 30 days caused a substantial decrease in fasting blood glucose (FBG) levels and repair of diabetic dyslipidemia.

In streptozotocin-induced diabetic rats and other groups of rats, Kumar *et al.* (2013) administered aromatic oils (100 and 200 mg/kg), piperine (25 and 50 mg/kg), and glibenclamide (0.6 milligram per kg) isolated from *P. longum* fruits for 28 days. This decreased the blood sugar levels in diabetic rats induced by STZ. They also determined the IC_{50} value of essential oil for α-glucosidase, aldose reductase, and pancreatic lipase *in vitro*, and was found to be 150±2.5, 120±1.2, and 175±1.2 µg/mL, respectively. These values were equivalent to those of the standard drug acarbose (90±2.3 µg/ml), quercetin (80±2.3 µg/ml), and orlistat (25±0.5 µg/ml), respectively.

Kumar *et al.* (2011) administered EtOH, hexane, and EtOAc extracts of *P. longum* fruits in type 2 diabetic rats induced by a single intra-abdominal inoculation injection, or IV of 60 milligrams per kg STZ and 120 milligrams per kg body wt of nicotinamide. The Oral glucose tolerance test (OGTT) results revealed a considerable blood sugar-reducing impact of the residual ethanolic fraction. On the OGTT test, both of the other two fractions, hexane extract, and ethyl acetate extract, failed to produce any notable results. Similarly, the amount of blood sugar in diabetic rats was restored after 45 days of oral administration of the ethanolic extract, indicating that the extract boosted the liver's normal homeostasis-maintaining processes (Puneshwar and Pradeep, 2016).

2.3.4 Anticancer activity

The second-most common cause of mortality worldwide is cancer, but because of advancements in detection, therapy, and preventative measures, rates of survival are rising for numerous kinds of cancer. A few studies have shown the anticancer properties of crude extracts as well as fractions of various parts of *P. longum* on various kinds of human carcinogenic cell lines *in vitro* as well as *in vivo.*

Sawhney *et al.* (2011) assessed the cytotoxic properties of the EtOAc, MeOH, and water extracts of *P. longum* fruits using an *in vitro* MTT assay on human lung epithelial adenocarcinoma cell line (HCC-827). They reported that the methanol extract was more potent to HCC-827 cell lines (1.57×10^5 cells in control but 0.5×10^5 cells in treated after 24 hrs; 2.17×10^5cells in control but 0.46×10^5 cells in treated after 48 hrs) than EtOAc and water extracts. Benerjee *et al.* (2017) found that the hot extracts of *in vivo*-grown leaves of *P. longum* showed better cytotoxic activity in the leukemic cell line (K562) than *in vitro*-grown callus extracts.

According to Sunila and Kuttan (2004) malignant cells like Ehrlich ascites carcinoma (EAC) cells and Dalton's lymphoma ascites (DLA) cells were destroyed by the alcoholic extract of *P. longum* fruits at concentrations of 500 and 250 μg/mL respectively. They also reported that at a concentration of 250 μg/ml, piperine that was extracted from fruit extracts was found to be toxic to DLA and EAC cells. The mouse fibroblast-like cell line (L929) was obtained to be cytotoxic to alcohol extract and piperine at concentrations of 100 and 50 μg/ml, respectively.

According to Ovadje *et al.* (2014) oral feeding of EtOH extracts of *P. longum* fruits inhibited the growth of colon cancer tumors *in vivo* in immunodeficient mice without causing any linked toxicity. They utilized CD-1 nu/nu immunodeficient mice (efficacy) and Balb/C mice (toxicity). Additionally, they observed that crude ethanol extract had a dose- and time-dependent effect on lowering the growth of cancer cells *in vitro*, comprising pancreatic (BxPC-3), colon (HCT116), melanoma cells, and ovarian cancer (OVCAR-3).

Sharma *et al.* (2014) studied the anticancer properties of chloroform, acetone, hexane, benzene, ethyl alcohol, and aqueous extracts of *P. longum* fruit against human cancer cell lines, such as prostrate (DU-145), lung (A549), leukemia (THP-1), ovary (IGR-OVI-1), and breast (MCF-7) *in vitro*. For the majority of the tested cell lines, hexane extracts showed 90–92% toxicity to cells, whereas benzene extract showed 84–87% cellular toxicity. They also stated that in the Sulforhodamine B dye (SRB) assay, benzene, hexane, and acetone extracts showed substantial toxicity (91–95%) to the lung cancer cell line (A549).

2.4 Bioactivity of various extracts of *P. polyphylla*

The rhizome is the main part of *P. polyphylla* that is used to treat different types of ailments and diseases in traditional medicine. Therefore, crude extracts and fractions of rhizomes have been examined for various biological activities both *in vitro* and *in vivo* by several researchers. There is, however, little research reported on the biological activities of aerial parts (leaf and stem). Similarly, the bioactivity test of *in vitro*-grown callus has received relatively little attention in *P. polyphylla*.

2.4.1 Antioxidant activity

Ethanol, petroleum ether, methanol, aqueous extracts, and pure compounds (steroidal saponins) extracted from the rhizome of *P. polyphylla* demonstrated antioxidant properties. By using the DPPH assay and Folin's Ciocalteu reagent, Devi *et al.* (2018) assessed the free radical scavenging activity of EtOH and petroleum ether extracts of *P. polyphylla* rhizome as well as the total phenol content (TPC). The TPC of the petroleum ether extract was 0.47 mg/g catechol, whereas the total phenol content (TPC) of the EtOH extract was 0.68 mg/g catechol. The ethanol extract exhibited a significant free radical scavenging activity, which was 68 µg/mL as inhibitory concentration. According to Mayirnao and Bhat (2017) the methanolic extract of *P. polyphylla* rhizome had a higher antioxidant activity (IC_{50} of 1.09 mg/mL) than the aqueous extract. They also assessed the total phenolic content and total flavonoid contents (TFC) of methanolic extract, which was 43.01±0.17 mg gallic acid equivalent (GAE)/g DW and 28±0.12 mg quercetin equivalent/g DW, respectively. Moreover, the free radical scavenging activity of a methanolic extract of *P. polyphylla* rhizome from two different locations was examined by Lepcha *et al.* (2019). They claimed that rhizomes harvested from higher altitude Tholung (PPT) have higher TPC, TFC, and free radical scavenging activity than rhizomes collected from lower altitude Uttaray (PPU). Thus, it showed that the antioxidant activity of PPT was 2.01±0.16 µg/mL and that of PPU was 2.55±0.004 µg/mL.

2.4.2 Antimicrobial activity

P. polyphylla leaves, rhizome, and aerial parts in the form of MeOH, EtOH, and aqueous extracts demonstrated antibacterial (Mayirnao and Bhat, 2017; Qin *et al*., 2018; and Joshi *et al*., 2020) and antifungal (Deng *et al*., 2008; Qin *et al*., 2018; and Joshi *et al*., 2020) activity in studies. With >31 mm diameter zones of inhibition at 5 mg/mL on *E. coli* and 30 mm diameter zones of inhibition at 5 mg/mL against *Staphylococcus aureus*, Mayirnao and Bhat (2017) demonstrated the antimicrobial activity of ethanol extract of the rhizome. They noticed that the percentage inhibition grows (95-97%) as the sample concentration increases (up to 5 mg/mL) in the case of *E. coli* and *S. aureus*, indicating a dose-dependent inhibition.

Quin *et al.* (2018) determined the antimicrobial activity of the total steroidal saponins isolated from the rhizome (TSSRs) as well as total steroidal saponins isolated from the

above-ground parts (TSSAPs) of *P. polyphylla* var. *yunnanensis*. They found that MIC of TSSRs and TSSAPs on *Candida albicans* 5314 and *C. albicans* Y0109 were 5.15 and 10.3 µg/mL, respectively. Moreover, the MIC of TSSRs and TSSAPs on *E. coli* was the same that is 156 µg/mL, but *Staphylococcus aureus* and *Pseudomonas aeruginosa* were resistant.

Joshi *et al.* (2020) assessed the antimicrobial activity of MeOH extracts of *P. polyphylla* leaves. The methanol extract proved specific in its activity (percentage inhibition) against six different species of bacteria, including *Pseudomonas aeruginosa* (100%), *Staphylococcus aureus* (80%), *Salmonella enteric* (67%), *Listeria innocua* (65%), *Escherichia coli* (57%), and *Shigella sonnei* (47%), and one species of fungus *Candida albicans* (99%).

2.4.3 Antidiabetic activity

There are a few research studies available on the anti-diabetic properties of *Paris polyphylla.* Kshetrimayum *et al.* (2023) assessed the anti-diabetic properties of *P. polyphylla* rhizome *in vitro* using α-amylase and α-glucosidase inhibition, as well as *in vivo* in streptozotocin-induced diabetic rats. This study demonstrated that diosgenin-enriched extract inhibited α-amylase (IC_{50}=275.7±0.59 µg/mL) and α-glucosidase (IC_{50}=121.9±1.21 µg/mL). Similarly, they demonstrated that a daily oral dose of extract for 28 days results in a substantial reduction in fasting blood sugar levels and weight restoration in comparison to the diabetic control group.

2.4.4 Anticancer activity

Anticancer activity is the most intensively investigated bioactivity of *P. polyphylla* by several researchers. They studied crude extracts, fractions, and pure compounds isolated from *P. polyphylla* rhizome or above-ground parts for their *in vivo* and *in vitro* anticancer activity. Crude extracts and fractions such as methanol, ethanol, petroleum ether, aqueous, hexane, chloroform, ethyl acetate, butanol, and dichloromethane (DCM) from various parts of *P. polyphylla* like a rhizome, and above-ground parts showed activity against lung, oesophagus, cervical, bone, stomach, prostate, breast, liver, colon, and blood cancer.

According to Li *et al*. (2013) methanol extract of *P. polyphylla* rhizome at 2.5, 5.0, and 7.5 mg/kg prevented cancer cell growth in Lewis bearing-C57BL/6 mice, however, *in*

vitro, steroidal saponins (0.25, 0.50, and 0.75 mg/mL) caused apoptosis in human lung adenocarcinoma A549 cell lines. Lepcha *et al*. (2019) studied *in vitro* cytotoxic activity of MeOH extracts of *P. polyphylla* collected from higher altitude Tholung (PPT) and lower altitude Uttray (PPU) on three kinds of cancer cell lines. In a dose-related way, methanol extracts slowed the growth of HeLa cells within 72 hours, reaching >90% inhibition at 100 μg/mL concentrations. HepG2 cells (non-tumorigenic hepatic cells) were somewhat affected by PPT and PPU up to a dose of 30 μg/mL, however, PPT reduced growth by 73.47% at a concentration of 100 μg/mL. At a concentration of 100 μg/mL, both of the extracts suppressed PC3 (a prostate cancer cell line) cells. Similarly, Ruamrungsri *et al*. (2016) compared the cytotoxicity of methanol and dichloromethane (DCM) extract of *P. polyphylla* rhizome on bone cancer cell lines or chondrosarcoma cells (SW 1353). They noticed that the DCM extract increased apoptosis in SW 1353 cells, however, normal canine primary chondrocyte cells were less affected by the percentage of survival and apoptosis. The lowest IC_{50} (less than 10 μg/mL) was demonstrated by methanol extract in both primary chondrocytes and SW 1353 chondrosarcoma cells.

Ethanol extracts were more frequently used for the evaluation of anticancer activity than methanol due to their non-toxic effect on human health. Li *et al*. (2012) studied the anticancer activity of EtOH extracts of *P. polyphylla* roots on human oesophageal cancer cells (ECA109). By inducing cell apoptosis, the EtOH extract decreased the development and expansion of ECA109 cells *in vitro* at concentrations of 25 mg/mL, 50 mg/mL, 100 mg/mL, and 200 mg/mL. However, it increased the activity of the cancer suppressor gene (connexin26) at the mRNA and protein levels, which had anti-tumor effects on ECA109 cells. Sun *et al*. (2007) compared the cytotoxic activity of ethanol and aqueous extracts of *P. polyphylla* stem on six digestive carcinoma cell lines and two liver cancer cell lines. The IC_{50} values for the six human digestive carcinoma cell lines (BGC-823, SMMC-7721, LoVo, HepG-2, SW-116, and CaEs-17) varied from 10 to 30 μg/mL, demonstrating anticancer activity. The lowest IC_{50} values for the two liver tumor cell lines, SMMC-7721 and HepG-2, were 12 and 10 μg/mL, respectively. In contrast, the aqueous extracts demonstrated low cytotoxicity, having a cytotoxicity rate of below fifty percent. Moreover, Hu *et al*. (2017) assessed the anticancer activity of ethanol extracts of *P. polyphylla* leaves on lung cancer cell lines. By producing a cell cycle halt during the G0/G1 phase in 24 and 48 hours and

preventing the passage from G1 to S phase, the ethanol extract at a concentration of 40 μg/mL reduced the total number of human lung cancer cells (A549) in the G1, S, and G2/M phases. Zhang *et al*. (2018) assessed the *in vitro* and *in vivo* anticancer activity of ethanol extract of whole parts of *P. polyphylla* on human prostate cancer cell lines. Prostate tumor cells (PC3 and DU145) experienced concentration-related apoptosis when exposed to ethanol extract *in vitro*. After 48 hours, the extract had an IC_{50} of 3.98 μg/mL on PC3 cells, causing cell arrest in the G0/G1 and G2/M phases, however had an IC_{50} of 8 μg/mL on DU145 cells, causing cell arrest in the G0/G1 phase. In PC3 xenograft development in BALB/c nude mice, the ethanol extract generated an anti-tumor effect in a dose-dependent way *in vivo*, with the greatest dose showing an effect comparable to 5-FU (positive control).

According to Guo *et al.* (2018) ethanol extracts of the whole plant had an IC_{50} of 1.2 μg/mL on bladder cancer cells with mutant p53 (such as HT1197 and J82 cells), which is equivalent to the influence of the chemotherapy drug cisplatin, and they did so in a dose-dependent way. Qin *et al.* (2018) assessed the *in vitro* anticancer activity of ethanol extracts of total steroidal saponins from rhizomes (TSSRs) and total steroidal saponins from above-ground parts (TSSAPs) of *P. polyphylla* in five human carcinoma cell lines. TSSAPs exhibit a lower cytotoxic effect than TSSRs *in vitro* when tested on the HL-60, A-594, SMMC-7721, MCF-7, and SW480 cell lines. The IC_{50}s for TSSAPs and TSSRs ranged from 8.12 to 12.61 and 1.75 to 6.62 μg/mL, respectively.

Besides the crude extracts and fractions, the pure compounds, particularly various types of steroidal and triterpenoid saponins isolated from *P. polyphylla* rhizome and above-ground parts, were determined for their *in vitro* and *in vivo* anticancer activities.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Collection of plant species and used plant materials

Piper longum was collected from the natural habitat of the Rupandehi district (Tilottama-1) at an elevation of 170 meters above sea level during February 2020, and *Paris polyphylla* was collected from the Baglung district (Galkot-8) at an elevation of 2100 meters above sea level during November 2020 (Fig. 4). They were authenticated by comparing herbarium specimens deposited in the National Herbarium and Plant Laboratories (KATH) and Tribhuvan University Central Herbarium (TUCH), and voucher specimens were deposited in TUCH, Central Department of Botany, Kirtipur (voucher no. of *P. longum* 135 and 136, and voucher no. of *P. polyphylla* C15). Some plants were cultivated in the field of the Central Department of Botany, T.U., Kirtipur for tissue culture purposes.

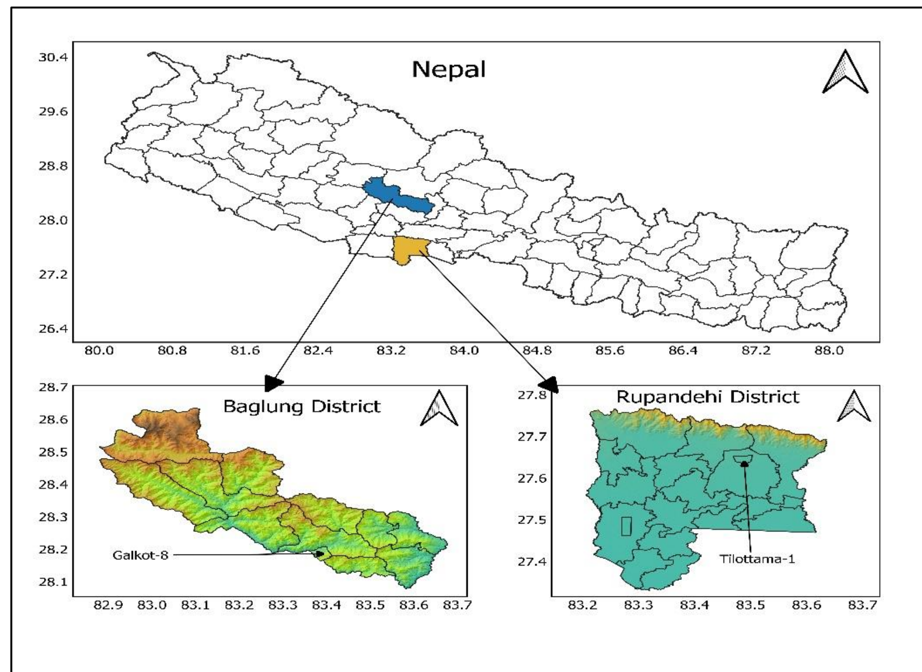


Figure 4: Location map for collection area of *P. longum* and *P. polyphylla*

The plant materials (explants) used for the *in vitro* propagation, suspension culture, genetic homogeneity test, and phytochemical analysis in this study are as follows.

(1) Nodal explants were utilized for the *in vitro* propagation of *P. longum.*

(2) Leaf and nodal explants were utilized for indirect organogenesis and somatic embryogenesis in *P. longum*.

(3) Leaf explant was used for the callus induction, proliferation, and differentiation in *P. polyphylla.*

(4) *In vitro*-raised calli were used for the suspension culture of *P. longum* and *P. polyphylla* using precursor (phenylalanine) and elicitor (salicylic acid).

(5) Wild-grown mother plants and *in vitro*-raised plants were used for the test of genetic homogeneity in *P. longum*.

(6) *In vivo*-grown roots, stems, leaves and fruits as well as *in vitro*-raised callus of *P. longum* were used for extract preparation for the chemical and bioactivity analysis.

(7) *In vivo*-raised rhizomes and *in vitro*-raised callus of *P. polyphylla* were used for extract preparation for the chemical and bioactivity analysis.

3.2 In vitro propagation of *Piper longum* and *Paris polyphylla*

3.2.1 Sterilization of glassware, metal instruments, and working place

Glassware such as petri dishes, culture tubes, culture jars, pipettes, beakers, conical flasks, and watch glasses was dipped in detergent solution for 24 hours and rinsed with running tap water, and the final wash was done with distilled water. Glassware and metal instruments were subjected to dry heat sterilization in a hot air oven at 150-200ºC for 1 hour before their use. Metal instruments like forceps, scalpels, and surgical blades were wrapped with aluminium foil before keeping inside the hot air oven for sterilization. Once they were sterilized in a hot air oven, they were autoclaved at 121ºC for 15 min at 15 lb/sq. inch pressure.

The laminar airflow chamber was sterilized by wiping it with spirit or cotton dipped in 70% ethyl alcohol. The culture tubes/jars holding medium, sanitized equipment, and glassware were subjected to UV rays for 45 minutes to eliminate any potential contaminants within and surrounding the transfer region. After switching off the UV light, the blower continued to run during the inoculation procedure.

3.2.2 Media preparation for *in vitro* propagation

In the present research, various strengths of MS medium (Murashige and Skoog, 1962) such as full-strength MS (FMS), half-strength MS (HMS), and quarter-strength MS medium (QMS), as well as the MS media together with different plant growth regulators (PGRs) and coconut water (5% and 10% as additives) were prepared for *in vitro* culture of leaves and nodal segments.

Preparation of nutrient stock solutions

For the preparation of MS media, various types of stock solutions such as stock A (macronutrients), stock B (micronutrients), stock C (Iron-EDTA), and stock D (vitamins) were prepared separately. During the preparation of each stock solution, all the required chemicals were weighed accurately and mixed completely in distilled water. To dissolve the chemicals more readily, the solutions were stirred with a magnetic stirrer. The final volume was made up to 1 liter for stock A, and 100 mL for stocks B, C, and D. Due to light sensitivity, all stock solutions were kept in clean brown bottles and were preserved in the refrigerator at 4ºC. The basic composition of MS media has been provided in Table 1.

Table 1: Basic components of Murashige and Skoog medium (MS) for *in vitro* culture.

Components	mg/L	Stock solution	Volume to be taken for 1-litre medium
Macronutrients (Stock A)		**(10X) g/L**	100 mL/L
Potassium nitrate (KNO_3)	1900	19.0	
Ammonium nitrate (NH_4NO_3)	1650	16.50	
Calcium chloride ($CaCl_2.2H_2O$)	440	4.40	
Magnesium sulphate ($MgSO_4.7H_2O$)	370	3.70	
Pot. dihydrogen phosphate (KH_2PO_4)	170	1.70	
Micronutrients (Stock B)		**(100X) mg/100 mL**	
Manganese sulphate ($MnSO_4.4H_2O$)	22.3	2230	1 mL/L
Zinc sulphate ($ZnSO_4.4H_2O$)	8.6	860	
Boric acid (H_3BO_4)	6.2	620	
Potassium iodide (KI)	0.83	83	
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25	25	
Cobalt chloride ($CoCl_2.6H_2O$)	0.025	2.5	
Copper sulphate ($CuSO_4$)	0.025	2.5	
Iron-EDTA (Stock C)		**(10X) mg/100 mL**	

Sod. ethylene diamine tetra acetate (Na_2EDTA)	37.3	373	10 mL/L
Ferrous sulphate (FeSO4.7H2O)	27.8	278	
Vitamin (Stock D)		**(100X) mg/100 mL**	
Glycine	2.0	200	1 mL/L
Nicotinic acid	0.5	50	
Pyridoxin HCl	0.5	50	
Thiamine HCl	0.1	10	
Myo-inositol (Fresh)	100.0	10,000	
Carbon source (Sucrose)			30 g/L
Gelling agent (Agar)			8 g/L

Potassium iodide (KI) was made separately in 100 mL of distilled water. Similarly, 100 mg of myo-inositol was freshly prepared and added in 1 L of media.

Plant growth regulators (PGRs) used for the *in vitro* culture experiment

(A) Auxins: α-Naphthalene acetic acid (NAA)**,** Indole-3-acetic acid (IAA)**,** Indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D).

(B) Cytokinins: Kinetin (KN)**,** 6-Benzylaminopurine (BAP), and Thidiazuron (TDZ).

(C) Gibberellins: Gibberellic acid (GA_3).

Preparation of hormone stock solution

All stock solutions were prepared 1000 times more concentrated than the concentration to be used in the medium. For this, 50 mg of IBA and 2,4-D (auxins) were dissolved first in about 1-2 mL of 95% ethanol, but the NAA was dissolved in 1-2 mL of 1 N of NaOH and added sterile distilled water to make the final volume of 50 mL. It was the stock solution of 1000 ppm (1 mg/1 mL). Similarly, 50 mg of BAP, KN, and TDZ (cytokinins) were dissolved first in about 1-2 mL of 0.5/or 1.0 N of NaOH and added sterilized distilled water to obtain the final volume of 50 mL, which was the stock solution of 1000 ppm. However, 50 mg of GA_3 was added in 50 mL of sterilized distilled water to obtain the 1000 ppm stock solution.

Preparation of 1 liter MS (Murashige and Skoog) media

The following steps were performed to prepare 1 liter of MS medium.

(1) At first 100 mL stock A, 1 mL stock B, 10 mL stock C, and 1 mL stock D were mixed in a clean 1 liter measuring cylinder or volumetric flask.

(2) 1 mL of freshly prepared KI and 100 mg of myo-inositol were added to the media.

(3) 30 g sucrose was weighed in an electric balance and dissolved in distilled water. It was poured and mixed in the measuring cylinder.

(4) Volume was adjusted to 1 liter by adding additional distilled water in the measuring cylinder.

(5) Media was balanced to pH 5.8 by adding 1N NaOH or 1N HCl before autoclaving and solidified with 0.8% agar.

(6) Following the preparation of full-strength MS, half- and quarter-strength MS media were made by diluting the media with distilled water. In callus suspension culture, a liquid MS medium was used.

(7) To make semi-solid MS media enriched with different concentrations of PGRs, 100 mL of MS media was added to a jar, followed by measured amounts of PGRs such as 0.25 mg/L, 0.5 mg/L, 1.0 mg/L, and so on, and 800 mg agar.

(8) The media was heated up to boiling to melt the agar. When the solution became transparent, about 16 mL medium per culture tube and 30 mL medium per culture jar were dispensed into sterile culture tubes and jars, and each tube/jar was sealed with aluminium foil.

(9) The culture tubes/jars containing medium were autoclaved at 121°C and pressure at 15lb/sq inch for 15 min. After cooling down, tubes were taken out and kept in a slanting position in the culture room.

3.2.3 Sterilization of plant materials in *P. longum* and *P. polyphylla*

The leaves, rhizome, and nodal parts were washed in flowing tap water with a few drops of liquid detergent Tween-20 for half an hour and again rinsed with sterile water three times. The leaf explants were sterilized with 70% ethyl alcohol for 30-60 seconds

and then 0.1% $HgCl_2$ for 3-4 minutes. However, the nodal explant was surface sterilized using 2% bavistin for 15 min, 70% ethyl alcohol for 1 min, and then 0.2% mercuric chloride solution for 5-7 minutes followed by three thorough washes with sterile water to eliminate any remnants of mercuric chloride.

3.2.4 Shoots initiation and elongation from nodal explants (direct organogenesis) in *P. longum*

Nodal explants (1.0 cm) were first grown in MS media in the absence of PGRs and coconut water and then with the supplementation of 0.5-3.0 mg/L BAP alone, 0.5-3.0 mg/L KN alone, 0.5-3.0 mg/L TDZ alone and with a combination of 0.5-3.0 mg/L BAP and at a constant concentration of 0.25 or 0.5 mg/L NAA and 5% and 10% coconut water. The culture was maintained in a 16 h light period, 28.57-42.86 μmol/m^2/s cool-white fluorescent light, and 26±2°C temperature in the culture room.

3.2.5 Callus induction and proliferation in *P. longum* and *P. polyphylla*

For callus induction MS medium in the absence of plant growth regulators (PGRs) or in the presence of PGRs, such as 0.25-3.0 mg/L kinetin alone, 0.25-5.0 mg/L 2,4-D alone, 0.25-5.0 mg/L NAA alone and in combinations with 0.25-5.0 mg/L 2,4-D + 0.25-3.0 mg/L KN, 0.25-5.00 mg/L NAA+ 0.25-3.0 mg/L KN, and 10% coconut water were utilized using leaf disc explants (0.8-1.0 cm diameter). The calli were induced after the 12 weeks of culture at 25±2°C, 16 hours photoperiod, and 3000-4000 lux cool-white fluorescent light. Then, the calli were weighed in sterilized filter papers inside the inoculation chamber, and the weighed amount of calli was subcultured in the MS medium for callus growth and proliferation with supplemented various PGRs such as 0.5-2.0 mg/L BAP alone, 0.5-2.0 mg/L KN alone, 0.5-2.0 mg/L TDZ alone, and 0.5-2.0 mg/L 2,4-D alone as well as in combination such as 0.5-2.0 mg/l NAA+ 0.5-2.0 mg/L BAP, 0.5 mg/L NAA+ 0.5-2.0 mg/L BAP+ 2.0 mg/L GA_3, and 0.5-2.0 mg/L BAP+0.5-2.0 mg/L KN+ 2.0 mg/L GA_3, and 10% CW. The culture was maintained at a 16 h photoperiod, 28.57-42.86 μmol/m^2/s cool-white fluorescent light, and 25±2°C temperature in the culture room.

The calli were harvested after the 8 weeks of subculture for phytochemical analysis and other purposes. The callus was weighed and air-dried, and the percentage of moisture content, as well as the percentage of growth index, were analyzed utilizing the formulae.

$$\text{The moisture content of callus (\%)} = \frac{\text{Fresh callus wt.- Dry callus wt.}}{\text{Fresh callus wt.}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots \ (1)$$

$$\text{Increase in callus wt. (\%) or Growth index} = \frac{\text{Fresh callus wt.-Fresh callus explant wt.}}{\text{Fresh callus explant wt.}} \times 100 \ldots \ (2)$$

3.2.6 Shoot differentiation from callus (indirect organogenesis) in *P. longum* and *P. polyphylla*

The callus induced from leaf and nodal explants were subcultured in MS medium in the absence of PGRs and coconut water, and supplemented with 0.25-3.0 mg/L TDZ alone, 0.25-3.0 mg/L KN alone, and 0.25-3.0 mg/L NAA alone with 5% and 10% coconut water. The culture was maintained in a 16 h photoperiod, 28.57-42.86 μmol/m^2/s cool-white fluorescent light, and 25±2°C temperature in the culture room.

3.2.7 Somatic embryogenesis in *P. longum*

Nodal (0.5 cm) and leaf (0.5 cm^2) explants were cut off and grown in MS media in the absence of PGRs and coconut water, and supplemented with 0.25-4.5 mg/L 2,4-dichlorophenoxyacetic acid alone, 0.25-4.5 mg/L α-naphthaleneacetic acid (NAA) alone, and in combination with 0.25-4.5 mg/L 2,4-D + 0.25 or 1.0 mg/L kinetin (KN), 5-10% coconut water (v/v), 3% sucrose (w/v), and 0.8% agar (w/v) for nodular calli development from leaf and nodal segments. Initially, cultures were grown in a culture room under a 16/8 h light and dark period, and the light was provided by cool-white fluorescent tubes (28.57-42.86 μmol/m^2/s) at 25 ± 2ºC and then incubated under the dark condition to form friable callus in culture.

For somatic embryo (SE) differentiation, development, and maturation, calli produced from both leaf and nodal explants were weighed in sterilized filter papers inside the inoculation chamber, and the weighed amount of calli was transferred to MS media fortified with 0.25-3.5 mg/L thidiazuron (TDZ) and 10% coconut water. The cultures were managed at 25±2 °C, 16 h photoperiod with cool-white fluorescent tube light (28.57-42.86 μmol/m^2/s). At intervals of 30 days, subcultures into new media were carried out. The SEs that had formed on the callus surfaces of each subculture were separated and transferred to full-strength, 1/2-strength, and 1/4-strength MS medium without growth regulators solidified with 0.8% agar to encourage germination or embryo conversion into seedlings.

3.2.8 Root formation from *in vitro* shoots in *P. longum* and *P. polyphylla*

The *in vitro* shoots were grown in MS medium in the absence of PGRs and with supplemented 0.25-2.0 mg/L IBA, 0.25-2.0 mg/L IAA, and 0.25-2.0 mg/L NAA. The culture was maintained in a 16 h photoperiod, 28.57-42.86 μmol/m^2/s cool-white fluorescent light, and 25±2ºC temperature in the culture room.

3.2.9 Histological studies

Embryogenic calli and somatic embryos were dipped in 1% acetocarmine for 12 h, heated in test tubes until boiling, squashed in glass slides, and observed under a compound microscope. Similarly, serial thin sections of embryogenic calli and morphogenic calli were cut with a fine blade, stained with safranin for 2 min, mounted in 10% glycerin water, and examined under a compound microscope. Photographs were taken using the image capture software ScopeImage 9.0 H1C.exe of the digital microscope (LABOMED-121, INC., USA).

3.3 Suspension culture using precursor and elicitor in *P. longum* and *P. polyphylla*

Production and enhancement of secondary metabolites using elicitor (salicylic acid) and precursor (phenylalanine) in suspension callus culture was done by following Vergara Martinez *et al.* (2017) with some modifications. At first, the calli were induced from the leaf explants in MS medium containing 0.25-5.0 mg/L 2,4-D + 0.25-3.0 mg/L KN + 10% CW in both *P. longum* and *P. polyphylla*, and harvested after the 8 weeks of culture. The callus was then subcultured in MS + 0.25-2.0 mg/L TDZ + 1.5 mg/L NAA for four weeks in *P. longum* and 0.25-2.0 mg/L BAP + 1.5 mg/L NAA in *P. polyphylla* to produce friable calli. The calli were weighed in sterilized filter paper inside the inoculation chamber, and the fresh calli (4.5 to 5.0 g) were grown in MS liquid medium added with 15 mg/L and 30 mg/L salicylic acid, 50 mg/L and 100 mg/L phenylalanine, and in MS liquid media as well as MS semi-solid medium in the absence of salicylic acid (SA) and phenylalanine (PHE) as a control in a rotary incubator at 25±2ºC in the dark at 100 rpm. Biomass of calli was collected after 15 and 30 days of culture and weighed.

The callus biomass was used to prepare 90% methanol (methanol 9: water 1) extracts after air-drying in the shade (a detailed process is given in section 3.6.3 below). The antioxidant, TPC, and TFC of methanol extracts of callus were determined. The

detailed protocol for determining antioxidant, TPC, and TFC of callus treated with SA and PHE, and untreated callus (control) is described in sections 3.7.2, 3.7.3, and 3.7.4 below.

3.4 Chemicals and equipment for phytochemical analysis

Alpha-glucosidase, acarbose, p-Nitrophenyl-beta-D-glucopyranoside (pNPG), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), and gallic acid were purchased from Merck Company, Germany. Quercetin, methanol (MeOH), ethanol (EtOH), dichloromethane (DCM), nutrient agar (NA), nutrient broth (NB), Mueller Hinton Agar (MHA), aluminum chloride, ascorbic acid, Folin–ciocalteau reagent (FCR), hexane, ferric chloride ($FeCl_3$), and ferrous chloride ($FeCl_2$) were purchased from HiMedia Laboratories, India. Porcine pancreatic α-amylase, Eagle's minimum essential medium (EMEM), and 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) were purchased from Sigma-Aldrich (USA). All the chemical reagents and glassware used in the study were obtained commercially and were of analytical grade.

A rotary evaporator (EYELA Co. Ltd., USA) and separating funnels were used to prepare the dry methanolic extracts/fractions. Synergy LX/96-well microplate reader (BioTek, USA) was used for spectrophotometric measurements for quantitative phytochemical analysis, such as antioxidant, antimicrobial, antidiabetic, and anticancer assays. A rotary incubator, an electronic balance, a laminar airflow chamber, etc. were also used for *in vitro* culture of plant materials.

3.5 Plant extract preparation

3.5.1 Drying and pulverizing

Leaves stems, roots, and fruits of *Piper longum* and rhizome of *P. polyphylla* were collected and rinsed with water to eliminate any dirt or additional materials before air drying at room temperature in the shade. It was pulverized in an electric grinder soon after it had completely dried. For later use, the powdered plant materials were kept in an airtight container at temperatures below 4°C.

3.5.2 Crude extract preparation

About 200 g powder was macerated in 2 L conical flasks containing 800-1000 mL 90% methanol (methanol 9: water 1) for 48 hours (Bhattarai *et al.*, 2022). It was stirred thoroughly with glass rods frequently. This process was repeated three times. Before final filtration, the residue was mixed with 90% methanol and warmed in the water bath at 50°C for one hour, and then it was filtered with the help of cotton and filter papers. The filtrate was concentrated at 37°C in a rotary evaporator. The crude extract was weighed and collected in vials and stored at 4°C in the refrigerator for further chemical analysis.

3.5.3 Callus extract preparation

The crude callus extract was prepared using the standard protocol (Park *et al.,* 2018). The fresh callus (150 g), grown in MS medium fortified with various plant growth regulators (2,4-D, KN) and 10% coconut water using leaf explant of *P. longum* and *P. polyphylla*, was air-dried and about 25 g dry callus, was crushed with 50 mL 90% methanol (methanol 9: water 1) in mortar and pestle. The mixture was poured into a 500 mL conical flask and 200 mL methanol (90%) was added. It was macerated for 48 hours in the dark stirring thoroughly with a glass rod frequently. Then, the mixture solution was filtered with the help of filter paper. The filtrate was concentrated at 37°C in a rotary evaporator. The crude extract was collected in vials and stored at 4°C in the refrigerator for further chemical analysis.

3.5.4 Fractionation of crude extracts

Fractionation of all the crude extracts was done by the liquid-liquid partition method (Bhattarai *et al.*, 2022) using serial nonpolar to polar solvents like hexane, dichloromethane (DCM), and methanol. At first, 11-43 g of crude extract (for *in vivo* parts) was mixed in 100 mL distilled water and filtered with the help of filter papers, and then the filtrate was mixed with hexane in a 1:2 ratio in a separating funnel. The mixed solution was shaken in a separating funnel and the upper layer of hexane and the lower aqueous layers were separated. The same process was repeated with hexane three times and the hexane layer was separated from the aqueous layer. Then, the aqueous layer was again mixed with DCM in a 1:2 ratio in a separating funnel, shaken well, and the upper aqueous and the lower DCM layers were separated. The same process was repeated with DCM three times and the DCM layer was separated from the aqueous

layer. The aqueous layer was concentrated in a rotary evaporator to obtain an aqueous extract, which was dissolved in absolute methanol. It was filtered with filter paper to obtain the methanol layer. All the hexane, DCM, and methanol layers were concentrated separately (at 37°C) in a rotary evaporator to obtain hexane, DCM, and methanol fractions. Each fraction was weighed, kept in vials, and stored in the refrigerator at 4°C for further chemical analysis. The hexane fraction was disregarded as it did not show a significant bioactivity.

The methanol crude extract of callus was fractionalized with hexane, DCM, and methanol, as previously described. However, all of the fractions were disregarded because their bioactivity was insignificant in the tested assays. The flow chart for the extraction and fractionation is shown in Fig. 5.

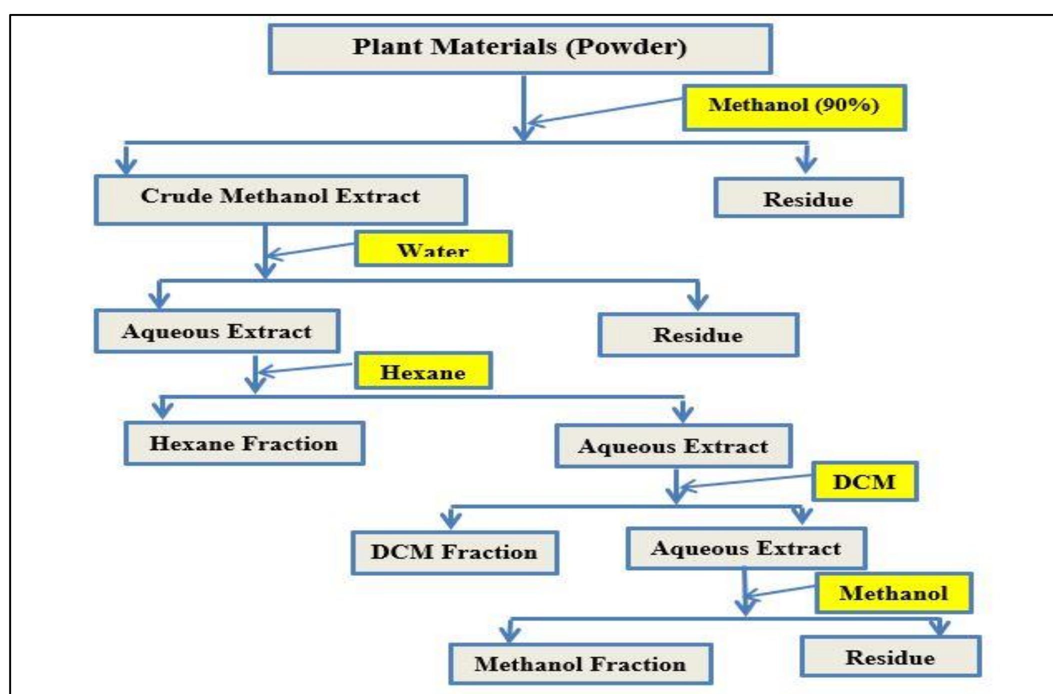


Figure 5: Flow chart of liquid-liquid extraction

3.6 Qualitative and quantitative analysis of plant extracts

3.6.1 Screening of phytochemicals

Standard qualitative procedures (Wagner and Bladt, 1996; Harborne, 1998) were used to detect key phytochemicals present in crude extracts of wild-grown plants and *in vitro*-raised callus of *Piper longum* and *Paris polyphylla*. The following assays were performed to identify significant phytochemicals in plant extracts.

3.6.1.1 Detection of alkaloids

Mayer's Test

A small aliquot (1 mL) of plant sample was mixed with 1 mL of Mayer's reagent (Potassium mercuric iodide solution) in a test tube. The presence of alkaloids was assessed by the appearance of a white creamy precipitate on a shaking well.

Wagner's test

A few drops of Wagner's reagent (Iodine-potassium iodide solution) were poured into 3 mL of crude extract to check for the presence of a reddish-brown precipitate.

Dragendorff's test

When 3 mL of crude extract solution is combined with a few drops of Dragendorff's reagent (Potassium iodide-bismuth nitrate), a reddish-brown or orange-red precipitate forms.

3.6.1.2 Detection of tannins

Ferric chloride test

A small aliquot (1 mL) of plant crude extract combined with one mL of ferric chloride (5% $FeCl_3$). The existence of tannins is indicated by the appearance of a dark blue or greenish-black color.

Gelatin test

A 10% NaCl solution is combined with a 1% gelatine solution. The gelatine solution is then mixed with a 1% tannin solution. If tannins exist, they will trigger gelatine precipitation from the solution, resulting in a white-colored precipitate.

3.6.1.3 Detection of glycoside

Borntrager's test

After boiling in the water bath, 2 mL of 5% H_2SO_4 was combined with the extract solutions and filtered. After that, the collected filtrate was combined with the same volume of $CHCl_3$ and allowed for five minutes. Half of the bottom layer of $CHCl_3$ was combined with diluted NH_4. Anthraquinone glycosides seem rose pink to red.

Keller-Kiliani test

The plant extract (2 mL) is combined with 1 mL of glacial acetic acid with 1 mL of a freshly prepared $FeCl_3$ solution. The liquid is thoroughly shaken before being slowly poured down the sides of a test tube holding concentrated H_2SO_4. The presence of cardiac glycosides is shown by the occurrence of a brown-colored ring at the junction.

3.6.1.4 Detection of flavonoids

Ferric chloride test

A few drops of neutral 5% $FeCl_3$ solution were mixed into the sample solution (2 mL). The existence of flavonoids is indicated by the emergence of a dark green hue.

Alkaline test

A small aliquot (two mL) of plant extract was added to the one mL of 2N sodium hydroxide (NaOH). The existence of flavonoids is indicated by an appearance of yellow color.

Shinoda test

A small aliquot (5 ml) of 95% ethanol is added to the dry extract, followed by a few drops of concentrated HCl and 0.5 g magnesium ribbons in the solution. The presence of flavones is indicated by the appearance of pink, orange-red, or violet color.

3.6.1.5 Detection of terpenoids

Liebermann-Burchard test

3 mL extracts, 2 mL chloroform, and some drops of acetic anhydride in test tubes were warmed in water baths and then rapidly cooled in cold water. When conc. H_2SO_4 was combined from the test tube wall, and a brown ring formed at the intersection of the two liquids. The existence of steroids was indicated by the top layer turning green, while the presence of terpenoids was shown by the appearance of a dark red tint.

3.6.1.6 Detection of phenol

Ferric chloride test

Small aliquots (one mL) of plant sample were added to the 2 mL of pure water and a few drops of 10% $FeCl_3$. The existence of phenols is marked by the appearance of a dark green color.

3.6.1.7 Detection of steroids

Salkowski test

A small aliquot (0.50 mL) of plant sample, two mL of $CHCl_3$, and one mL of H_2SO_4 were mixed. The existence of steroids is indicated by the appearance of a reddish-brown ring at the contact.

3.6.1.8 Detection of Saponin

Frothing test

A small aliquot (two mL) of distilled water was added to the two milli liters of plant extract in a graduated cylinder for 15 minutes longitudinally. The existence of saponins is indicated by the formation of a one cm thickness of foam.

3.6.1.9 Detection of carbohydrate

Fehling's test

The same quantities of Fehling "A" and Fehling "B" solutions (2 mL each) were mixed, and trace amounts of crude extracts were added to the mixture. Moderate heating produces a reddish brick coloring on the bottom of the tube, showing the presence of sugar.

Benedict's test

A small amount (1 mL) of the plant sample was added to the small amount of Benedict reagent before being warmed in water baths for two minutes. The appearance of reddish-brick precipitates indicates the existence of reducing sugar.

3.6.2 Evaluation of antioxidant Activity by DPPH assay

The free radical scavenging activities of the extracts/fractions were assessed by utilizing the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Blois, 1958;

Desmarchelier *et al.*, 1997). An antioxidant compound donates the electrons to DPPH thus causing its reduction and in the reduced form its color changes from deep violet to yellow. The % of free radical scavenging activities was computed by utilizing the following formula.

$$\% \text{ Inhibition} = \frac{Abs_1 - Abs_2}{Abs_1} \times 100 \quad \ldots\ldots (3)$$

Where Abs_1= Control reaction absorbance (DPPH + methanol)

Abs_2= Sample reaction absorbance (DPPH + plant extract)

The IC_{50} (50% inhibitory concentration) value is indicated as the effective concentration of the sample that is necessary to scavenge 50% of the free radicals.

3.6.2.1 Preparation of DPPH solution

The 0.1 mM solution of DPPH was made by mixing 3.94 mg of DPPH (molecular wt. 394.32 g/mol) in methanol and the final volume was made to 100 mL. It was stored in a dark place for further use.

3.6.2.2 Preparation of stock solution of sample extract

At first, 1 mg samples to be tested were dissolved in 1 mL methanol to get the stock solution of one mg/mL. Different concentrations of test samples of 25 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL were prepared from the stock solution by serial dilution method.

3.6.2.3 Preparation of stock solution of Ascorbic acid

Ascorbic acid (1 mg) was dissolved in 1 mL of MeOH to make a stock solution of one mg/mL, and then serial dilution was done to prepare the required concentrations of 50 µg/mL, 40 µg/mL, 30 µg/mL, 20 µg/mL, 10 µg/mL, and 5 µg/mL respectively.

3.6.2.4 Measurement of DPPH free radical scavenging activity

An amount of 100 µL of each plant extract and 100 µL DPPH reagents were loaded in 96 well plates in triplicate. It was incubated for up to 30 min in the absence of light and measurements of all samples were calculated at 517 nanometers using a microplate reader after the conversion of violet 1,1-Diphenyl-2-picrylhydrazyl (DPPH) to pale yellow or colorless 1,1-Diphenyl-2-picrylhydrazine (Fig. 6).

For positive control, various concentrations of ascorbic acid (5-50 µL) were used in place of plant extracts, and its measurement was taken at 517 nm. The absolute methanol was utilized as a negative control.

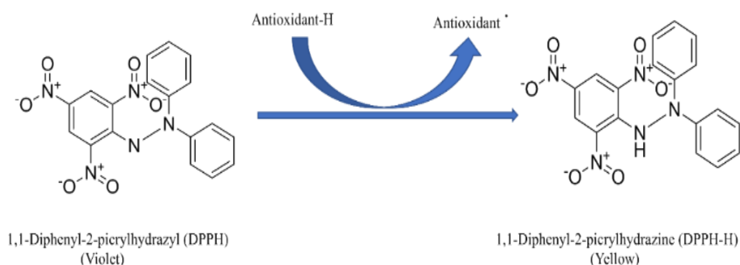


Figure 6: Reduction of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) to 1,1-Diphenyl-2-picrylhydrazine

The IC_{50} was calculated using a linear regression line (Chou, 1976). The most basic method to determine IC_{50} is to plot x-y and fit the data with a straight line (linear regression). The fitted line was subsequently utilized for calculating the IC_{50} value.

$Y = m * x + b$ (Chou, 1976)

$IC_{50} = (0.5\text{-}b)/m$.. (4)

where, Y = The response (e.g., percentage inhibition)

X = The inhibitor concentration (or log of the concentration)

m = The slope of the line

b = The Y-intercept

3.6.3 Determination of total phenolic contents (TPC)

The TPC of all plant samples was evaluated utilizing Folin-Ciocalteau reagent (FCR) involving gallic acid as standard. The TPC was assessed according to Zhang *et al.* (2006).

Preparation of reagents

To prepare 1 M sodium carbonate, 5.29 g of Na_2CO_3 was mixed in 50 mL pure water, and 10 mL of Folin Ciocaltau reagent (FCR) was diluted in 100 mL pure water to make a 1:10 (v/v) FCR solution.

3.6.3.1 Preparation of gallic acid stock solution

The stock solution of gallic acid was made by mixing one mg of gallic acid in one mL of methanol. Then, various concentrations of gallic acid such as 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 60 µg/mL were prepared from the stock solution.

3.6.3.2 Evaluation of total phenolic contents (TPC)

The total assay mixture containing 20 µL of standard gallic acid (10 to 60 µg/mL) or 20 µL of extracts (1mg/mL), 100 µL of FCR (1:10), followed by 80 µL of Na_2CO_3 (1 M) in a 96-well plate were separately incubated in the absence of light for 15 min and the measure was taken at 765 nm. Each sample was measured in triplicate. The result was manifested as milligrams of gallic acid equivalent per gram of dry weight (mg of GAE/g dry extract) of the extract utilizing a gallic acid standard curve.

3.6.4 Assessment of total flavonoid contents (TFC)

An aluminum chloride complex-forming assay was used to assess the total flavonoid contents (TFC) of the extracts. Quercetin was used as standard and the TFC was assessed as milligram of quercetin equivalent per gram dry weight (Chang *et al*., 2002).

Preparation of Reagents

To prepare 10% aluminum trichloride ($AlCl_3$), 1 g $AlCl_3$ was mixed in 10 mL of pure water, and 0.98 g of CH_3COOK was mixed in 10 mL of water to make 1 M potassium acetate.

3.6.4.1 Making of standard quercetin stock solution

It (1 mg/mL) was made by mixing one mg of quercetin in one mL MeOH. Various concentrations of quercetin such as 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL, 70 µg/mL, and 80 µg/mL were prepared from the stock solution.

3.6.4.2 Evaluation of Total Flavonoid Contents (TFC)

The whole assay mixture containing 130 µL of standard quercetin (10-80 µg/mL) or 130 µL of extracts (1 mg/mL), 60 µL EtOH, 5 µL 10% aluminium chloride, and 5 µL CH3COOK (1 M) in a 96-well plate were incubated in the absence of light for 30 min and measurement was taken at 415 nm. The total phenolic content of the extract was manifested as milligrams of quercetin equivalent per gram of dry weight (mg of QE/g dry extract) of the extract utilizing the quercetin standard curve.

3.6.5 Assessment of antibacterial activity

The capacity of plant extracts or compounds to prevent or halt the growth of hazardous germs is referred to as antibacterial activity.

3.6.5.1 Materials required for antibacterial test

Conical flasks, beakers, petri dishes, test tubes, forceps, oven, autoclave, refrigerator, laminar air flow cabinet, micropipettes, tips and tips holder, Eppendorf tubes, cork borer, methanol, cotton swabs, bacterial strains, ciprofloxacin, inoculation loop, nutrient media, saline water, permanent markers, etc.

3.6.5.2 Bacterial strains used in antibacterial activity test

The standard cultures of microorganisms (ATCC) under examination were obtained from the National Public Health Lab (NPHL) in Kathmandu, Nepal. The three gram-negative strains such as *Acinetobacter baumannii* (ATCC 19606), *E. coli* (ATCC MA 35218), and *Pseudomonas aeruginosa* (ATCC 9027), and two gram-positive strains *Staphylococcus aureus* (ATCC baa977), and *Bacillus subtilis* (ATCC 6051-U) were utilized for antibacterial activity.

3.6.5.3 Media preparation

Nutrient Broth (NB) media

A small amount (13 g) of powdered nutrient broth was weighed and dissolved in one L of pure water. It was heated to boiling to dissolve the medium before being autoclaved for 15 minutes at 15 lbs pressure and 121°C temperature. The pH was adjusted to 7.4.

Nurient Agar (NA) media

A small amount (28 g) of powdered nutrient agar was weighed and dissolved in one L of pure water. It was heated to boiling to dissolve the medium before being autoclaved for 15 minutes at 15 lbs pressure and 121°C temperature. It was cooled to 45-50°C, mixed well, and dispensed into sterile petri dishes.

Mueller Hinton Agar (MHA) media

A small amount (38 g) of powdered Mueller Hinton agar was weighed and dissolved in 1 L of distilled water. It was heated to boiling to dissolve the medium before being autoclaved for 15 minutes at 15 lbs pressure and 121°C temperature. It was cooled to 45-50°C, mixed well, and dispensed into sterile Petri dishes.

3.6.5.4 Preparation of extract stock solution

To make a 100 mg/mL stock solution, 100 mg extract was weighed and diluted in 1 mL of pure methanol. Then, using the serial dilution method, 5, 10, 20, 40, and 60 mg/mL solutions were prepared.

3.6.5.5 Preparation of 0.5 McFarland standard solution

First, 1% anhydrous barium chloride ($BaCl_2$) and 1% sulfuric acid (H_2SO_4) solutions were prepared. The 0.5 McFarland standard solution was then prepared by combining 0.05 mL $BaCl_2$ and 9.95 mL H_2SO_4.

3.6.5.6 Preparation of ciprofloxacin (antibiotic) stock solution

A small amount (1 mg) of pure ciprofloxacin (Lomus Pharmaceuticals, Kathmandu, batch no. CPH/1109265) was diluted in distilled water to form a 1 mg/mL stock solution, and then 10, 20, 30, 40, 50, and 60 µg/mL solutions were prepared using the serial dilution method.

3.6.5.7 Determination of zone of inhibition (ZoI)

With slight changes, the agar well diffusion assay was utilized to determine antibacterial activity (Mosquera *et al.,* 2004; Lokhande *et al*., 2007). To revive the bacteria and produce a pure culture, all of the microbial strains were carpet-cultured on a nutrient agar petri plate and cultured at 37°C for 24 h. After that, 1.5×10^8 CFU/mL of bacteria were generated by transferring a few comparable bacterial colonies into normal saline and diluting them to 0.5 McFarland standard turbidity. Single bacterial strains were inoculated onto Mueller Hinton Agar (MHA) plates using a sterilized cotton swab from the diluted bacterial solutions. On the MHA plates, 6 mm-diameter wells were drilled with a sterilized cork borer. After that, the Mueller Hinton Agar plate was left to incubate for 24 hours at 37°C containing 35 µL of extracts in every well. The antibacterial effect was measured using the zone of inhibition (ZoI) in mm around the use site of each extract solution—5, 10, 20, 40, and 60 mg/mL. Ciprofloxacin (50 µg/mL) was utilized as a +ve control, and pure methanol was utilized as a -ve control. The average ZoI was calculated by averaging triplicate readings of extracts.

3.6.5.8 Determination of relative percentage inhibition (RPI)

By evaluating the zones of inhibition of positive (ciprofloxacin) and negative (methanol) controls, the relative percentage inhibition of bacterial strains at each

concentration of the extract was determined using the following formula (Mayirnao and Bhat, 2017).

Relative percentage inhibition of test extract= $\frac{(a-b)}{(c-b)}\times 100$……………………….(5)

Where, a= Plant extract (samples) inhibition zone

b= Methanol (negative control) inhibition zone

c= Ciprofloxacin (positive control) inhibition zone

3.6.5.9 Minimum inhibitory concentration and minimum bactericidal concentration assays

Those extracts that showed an inhibitory zone with specific bacterial strains were used to determine minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) values. The MIC of several samples was assessed using the micro-broth dilution method (Abu-Shanab *et al*., 2006) with some changes. The extracts were further diluted in methanol at concentrations of 1.0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, and 50 mg/mL using serial dilution method. Each extract dilution (1 mL) was mixed with a 0.05 mL solution of the examined microbial strain before incubating at 37°C for 24 h. The negative control was plant extracts (0.05 mL) of each estimated concentration in 2 mL of nutrient broth, while the positive control was bacterial suspension in 2 mL of nutrient broth. The minimum inhibitory concentration was visually identified as the smallest amount of extract (mg/mL) that stopped bacteria from developing in a mixture of nutrient broth, and the MBC for every extract was measured by inoculating each concentration of the sample solution beginning from the MIC in Mueller Hinton Agar media and incubating for 24 hours at 37°C. The MBC is the lowest amount of an antibacterial agent (extracts) required to eliminate a specific bacterium.

3.6.6 Determination of cytotoxic activity

3.6.6.1 Brine shrimp lethality bioassay

Preparation of salt water (artificial seawater)

To make salt water (seawater), 3.5 g of NaCl was weighed and mixed in 100 mL of pure water.

Preparation of plant extract stock solution

To make a 5 mg/mL stock solution, 5 mg extract was weighed and diluted in 1 mL saline water. Then, in salt water, a solution of 20-1250 µg/mL was prepared using the serial dilution method.

Process of brine shrimp lethality bioassay

The brine shrimp lethality assay is an easy and inexpensive bioassay used to assess the cytotoxicity of phytochemicals present in the plant extracts. It was performed using the protocols given by Meyer *et al.* (1982) and Fatope *et al.* (1993). About 10 mg eggs of brine shrimp (*Artemia salina*) were incubated in artificial seawater for 48 hours with the temperature (80-watt bulb) adjusted at 23ºC and eggs were hatched into Nauplii (larvae). A stock solution of various parts (root, stem, leaf, fruits, and rhizome) and *in vitro*-grown callus of *P. longum* and *P. polyphylla* were made by mixing 10 mg samples in 1 mL of distilled water. Various concentrations (20-2500 µg/mL) were made from the stock solution by serial dilution method. A volume of 100 µl of each concentration of plant extracts and 100 µl seawater with 10 live Nauplii were added in a 96-well microplate. The microplates were kept in artificial light for 24 hours. As a negative control, salt water was utilized instead of plant extract, while potassium dichromate was used as a positive control. Each experiment was performed in triplicate. After 24 hours total live Nauplii were counted and LC_{50} was calculated for each plant extract.

$$\text{Percentage mortality} = \frac{\text{No.of dead Nauplli (Larvae)}}{\text{Initial No.of live Nauplli}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

3.6.6.2 Determination of the cytotoxicity of extracts (MTT Assay).

Preparation of phosphate-buffered saline (PBS) in 500mL

To prepare the PBS solution, 4 g NaCl, 0.1 g potassium phosphate dibasic (K_2HPO_4), and 0.72 g sodium phosphate monobasic (NaH_2PO_4) were weighed, diluted in 500 mL distilled water, and adjusted to 7.4 pH.

Preparation of MTT stock solution

To make a 5 mg/mL stock solution, 5 mg MTT powder was weighed and diluted in 1 mL of PBS solution. It was filtered using a Seitz filter paper (0.22 µm) before being diluted to the required concentrations (0.5 mg/mL).

Preparation of 10% fetal bovine serum (FBS) in 100 mL

To make a 10% FBS solution, 10 milliliters of FBS were poured into the 90 mL of EMEM medium.

Preparation of sample/extract stock solution

A small amount (1 mg) of plant extract was weighed and mixed in 0.1% DMSO to make a 1 mg/mL stock solution, which was then various concentrations of solution (400 µg/ml, 200 µg/mL, 100 µg/mL, and 50 µg/mL) in EMEM media was made by serial dilution method.

Collection of human carcinoma cell lines

The cervix cancer cells (HeLa), breast cancer cells (MCF-7), osteosarcoma cell lines (U2 OS), and normal epithelial cell lines were taken from the Shikhar Biotech Pvt. Ltd, Nepal.

Process of MTT assay

The cytotoxic activity of samples was determined on 96-well flat-bottomed plates (Corning) utilizing the standard MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method with slight modifications (Joshi *et al*., 2020; Pant *et al*., 2021). Two types of human carcinoma cell lines, such as HeLa (cervical carcinoma cells) and MCF-7 (breast carcinoma cells), and normal epithelial cells (dermal and fibroblast in origin) were cultured in T25 flasks in EMEM medium (Eagle's minimum essential medium) added with 10% FBS (fetal bovine serum), 1% streptomycin, and 1% L-glutamine and set at 37°C in a 5% carbon dioxide incubator. All the cells were grown in 96-well plates (2×10^4 cells/well) in 100 µL of medium and set at 37°C for 24 hours in a 5% carbon dioxide incubator. The cells were subjected to treatment with 100 µL of plant extracts (50, 100, 200, and 400 µg/mL) diluted in 0.1% DMSO (dimethyl sulfoxide) for 48 hours after the cell attachment, and the cells had expanded to encompass the entire growth surface area of the culture vessel, developing a continuous monolayer (cell confluence). After 48 hours, the supernatant was substituted in each well with 125 µL of media comprising 25 µL of MTT. After 4 hours of incubation, a purple formazan crystal of living cells developed from yellow MTT in the presence of mitochondrial dehydrogenase enzyme (Fig. 7). For dissolving the formazan crystals, 150 µL of 0.1% DMSO was poured onto the plates, which were then

left to incubate for 15 minutes at the ambient temperature. At a wavelength of 595 nm, the absorbance of each sample was measured using a microplate reader. Cisplatin, an anticancer medicine, was used as a +ve control. The percentage of cytotoxic activity was calculated using a simple formula:

$$\% \text{ Cytotoxic Activity} = \frac{Ab_{Control} - Ab_{Sample}}{Ab_{Control}} \times 100 \quad (6)$$

Where $Ab_{Control}$ refers to cell absorbance without the presence of plant extracts and Ab_{Sample} refers to the absorbance of cells in the presence of plant extracts.

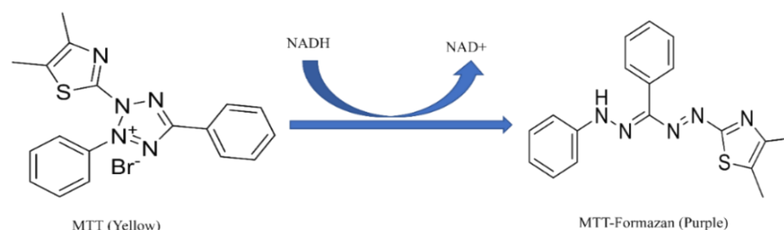


Figure 7: Reduction of MTT to MTT-Formazan

3.6.7 Determination of antidiabetic activity

3.6.7.1 *In vitro* alpha-glucosidase inhibition assay

Preparation of 0.1 M phosphate buffer solution

To make a 0.1 molar phosphate buffer solution with a pH of 6.8, 0.796 g of K_2HPO_4 and 0.739 g of KH_2PO_4 were weighed and mixed in 100 mL of pure water.

Preparation of alpha-glucosidase enzyme solution

For making 0.5 unit/mL α-glucosidase enzyme, 0.5 mg of α-glucosidase enzyme (100 units/mg) was dissolved in 100 milliliters of phosphate buffer (0.1 M, pH 6.8).

Preparation of 5 mM pNPG solution

To make 5 mM pNPG, 0.0753 g of pNPG (Mol. wt. 301.3 g/mol) was dissolved in 50 mL of phosphate buffer.

Preparation of sample stock solution

A small amount (5 mg) of the sample was mixed in one mL of phosphate buffer solution (0.1 M, pH 6.8) to make a 5 mg/mL stock solution, which was then made the requisite concentrations of 1200, 600, 300, 150, and 75 µg/mL solution by serial dilution method.

Process of *in vitro* alpha-glucosidase inhibition assay

The anti-diabetic activities of crude extracts and fractions were evaluated using the α-glucosidase inhibition assay (Fouotsa *et al.*, 2012). A 96-well plate was filled with 20 μL of different extract concentrations (75-1200 μg/mL), 20 μL of α-glucosidase enzyme (0.5 unit/mL), and 60 μL of phosphate buffer (6.8 pH, 0.1 M). After pre-incubating the mixed solution at 37ºC for 15 minutes, the initial value of absorbance at 405 nm was calculated using a microplate reader. The mixture solution was then combined with 40 μL of p-NPG (5 mM) and set at 37°C for 15 min in the incubator. The reaction was stopped with the mixing of 60 μL of 1 M Na_2CO_3, and the amount of p-nitrophenol released by p-NPG was estimated utilizing the final value of absorbance recorded at 405 nm. Acarbose was used as a +ve control, and phosphate buffer was used as a -ve control. The following equation was utilized to compute the percentage of α-glucosidase inhibition, and the needed amount of extract for inhibiting 50% of α-glucosidase activity (IC_{50}) was calculated using a linear regression equation.

$$\%\ \text{Inhibition} = \frac{A_1 - A_2}{A_1} \times 100 \quad (7)$$

Where, A_1=Absorbance of enzyme-substrate reaction with phosphate buffer

A_2= Absorbance of enzyme-substrate reaction with various extracts of the plant

3.6.7.2 *In vitro* alpha-amylase inhibition assay

Preparation of 50 mM sodium phosphate buffer solution

At 6.9 pH, 29.99 mg monobasic sodium phosphate (NaH_2PO_4) and 359.4 mg dibasic sodium phosphate (NaH_2PO_4) were weighed and mixed in 100 mL of pure water.

Preparation of 1 mM 2-Chloro-4-Nitrophenyl-α-D-Maltotrioside (CNPG$_3$) solution

To prepare a 1 mM CNPG$_3$ (Molecular weight 660.12 g/mol) solution, 66.012 mg of CNPG$_3$ was dissolved in 100 mL of sodium phosphate buffer.

Preparation of alpha-glucosidase enzyme solution

To prepare a 1.5 unit/mL solution of the alpha-amylase enzyme, 15 μL of the 10 unit/mg stock enzyme was diluted to a final volume of 1 mL using sodium phosphate buffer.

Process of alpha-amylase inhibition assay

The α-amylase inhibition was assessed by using the method of Senger *et al.* (2012) with some changes. The different extract concentrations (20 µL) were combined with 80 µL of the enzyme (1.5 units/mL) in 50 milli molar sodium phosphate buffer (pH 6.9, 0.9% NaCl) followed by preincubation at 37°C for 10 minutes. The resultant mixture was set at 37 °C for 15 min in the incubator after 100 µL of $CNPG_3$ (1.0 mM) was added as the substrate. Acarbose was utilized as the positive control and sodium phosphate buffer as the -ve control. The measurement of the sample was determined in a spectrophotometer at 405 nm, and the enzyme inhibitory effect was estimated utilizing the above-mentioned formula (equation 7).

3.7 LC-HRMS analysis of plant extracts/callus extracts

3.7.1 LC-HRMS Analysis

For analysis, the methanolic crude extracts of callus and DCM fractions of root and rhizome were mixed in pure MeOH at a concentration of 0.2 mg/mL. An UltiMate 3000 HPLC system combined with MaXis-4G equipment (Bruker Daltonics, Bremen, Germany) was used to perform the Liquid Chromatography/High-resolution Electron Spray Ionization Mass Spectrometry (LC/HRESI-MSMS) measurements. The mobile phase consisted of 0.1% HCOOH in water (solvent 1) and HPLC-grade methanol (solvent 2). The planned HPLC technique was a range of 10% solvent 2 to 100% solvent 2 eluted in 40 minutes, ending with 100% solvent 2 for another 15 minutes, with a flow rate of 0.3 mL/minute, 5 µL injected volume, and UV detector (UV/VIS) wavelength monitoring at 210, 254, 280, and 360 nm. To aid separation, a Phenomenex Luna Omega Polar C18 (3 µm, 15×3 mm) column was combined with an MS acquisition range of m/z 50-1800. The capillary voltage was set to 4500 V, the nebulizer gas pressure (Nitrogen) was set to 2 (1.6) bar, the ion source temperature was set to 200°C, the dry gas flow was set to 9 L/min source temperature, and the spectrum rates were set to 3 Hz for MS1 and 10 Hz for MS2. The 10 most energetic ions per MS1 were chosen for subsequent collision-induced dissociation (CID) with the recommended stepwise CID energies to yield MS/MS fragmentation (Hegazi *et al.*, 2020). For mass calibration, sodium format was immediately infused before each sample measurement.

3.7.2 MS-based dereplication of putative compounds

To ensure proper ionization of chemical compounds, the LC-HRMS results were first processed with Bruker Compass Data Analysis 4.0 software. The Bruker MetaboScape 3.0 software was used to process only the positively ionized HRMS data, leaving out the poorly ionized negative mode data. Data were evaluated to group all molecular ions into a single file. During organizing, some specific parameters were adjusted, including intensity threshold (counts); 5000, minimum peak length (spectra); 4, minimum peak length (recursive); 3, EIC correlation; 0.8, and mass calibration; true. The adduct ions were confirmed $[M+H]^+$ as the primary ion, $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$, $[2M+Na]^+$, $[M+2H]^{2+}$, and $[M+2Na]^{2+}$ as seed ions, and $[M-H_2O+H]^+$, $[M+H_2O+H]^+$, $[2M+H_2O+H]^+$, and $[2M-H_2O+H]^+$ as common ions during processing. The retention time range of 5-35 minutes and molecular mass range of 200-1500 m/z were taken into account from the raw MS chromatogram for developing the bucket list of various compounds using these criteria. Following organizing/grouping, the software entered the molecular ions detected in the HRMS chromatograms of their particular extract sources (callus, root, and rhizome) into a column with their corresponding retention time, adduct ions or parent ions, molecular weight, and abundance (relative ion intensity). The grouping list of these molecular ions was exported as mascot generic format (MGF) files, which were then converted into five various file formats, including Excel and GNPS files. The GNPS file format was submitted via the GNPS platform for molecular networking, in which the GNPS web server groups tandem mass compounds in a molecular network based on a minimum matched fragment ion count of 5 and a minimum cosine score of 0.7. Molecular networking produced a list of known and potential unknown metabolites organized by relative abundance. In addition, these compounds were validated utilizing CSI: FingerID analysis of individual raw MS data in the SIRIUS 4 platform utilizing molecular structure database search with m/z threshold 20. Additional databases including ChemDraw, PubChem, Dictionary of Natural Products, ChemSpider, and METLIN, were utilized for searching and assigning molecular formulas and structures of compounds.

3.8 Statistical analysis

Each of the experiments was repeated three times to obtain three replicates of data, and all results were presented as mean ± SD. The 50% inhibition concentrations (IC_{50}) and 50% lethal concentrations (LC_{50}) were calculated using a linear regression line equation in Microsoft Excel 10. One-way ANOVA, coupled with Duncan's test, and two-way ANOVA were used for variance analysis in SPSS version 20 and R-program version 4.0.5, and $p<0.05$ was taken to be statistically significant.

LC-HRMS data were analyzed with Bruker Compass Data Analysis 4.0 and the Bruker MetaboScape 3.0 software as well as GNPS databases. Additionally, putative compounds were also validated utilizing CSI: FingerID analysis of individual raw MS data (MFG files) in the Sirius 5.6.3 platform.

CHAPTER 4

4. RESULTS AND DISCUSSION

The findings and discussion of this study are described below.

(A) Study of in vitro propagation in *Piper longum* and *Paris polyphylla*.

4.1 Assessment of best explants, culture type, and media composition for direct organogenesis, indirect organogenesis, and somatic embryogenesis in *Piper longum* and *Paris polyphylla*

Micropropagation of *P. longum* was obtained by utilizing nodal explants through direct organogenesis, as well as using nodal and leaf explants by indirect organogenesis and somatic embryogenesis. Similarly, micropropagation of *P. polyphylla* was obtained utilizing leaf explants through indirect organogenesis. It is explained below.

4.1.1 Micropropagation of *P. longum* utilizing nodal explants through direct organogenesis

Multiple shoot buds and adventitious roots were generated directly from nodal explant in MS medium added with varying concentrations of TDZ alone, BAP alone, and a combination of BAP+NAA and 10% CW. MS+1.0 mg/L TDZ produced more shoot buds per explant (5.33±1.15) and longer shoots (6.16±0.65 cm) compared to MS+BAP (3.33±0.57) or MS+BAP+NAA (4.0±1.0) (Table 3). However, the MS media without PGRs and 10% CW (control) produced fewer shoots (1.00±00) and shorter shoot lengths (1.83±0.26 cm). When BAP+NAA was coupled with MS media at a constant concentration of 0.25 mg/L or 0.5 mg/L, the average number of shoot buds (4.0±1.0) and the length of shoots (5.2±0.30 cm) were observed to be higher at 0.25 mg/L of NAA than at 0.5 mg/L of NAA.

Table 2: The average shoot number and length at different PGR concentrations in *P. longum.*

Plant Growth Regulators (PGRs) (mg/L)				Average shoot number per explant (M±SE)	Average shoot length in cm (M±SE)
BAP	TDZ	NAA	CW (%)		
0	0	0	0	1.0±0.0$^{bccb/*zzyx}$	1.83±0.28$^{bcdc/*yzzz}$
0.5	0	0	10	1.33±0.57$^{a/*y}$	2.76±0.25$^{a/*x}$
1	0	0	10	2.33±0.57$^{b/*x}$	3.8±0.7$^{b/*x}$
2	0	0	10	3.33±0.57$^{a/*w}$	5.46±0.87$^{a/*w}$
3	0	0	10	2.33±0.57$^{a/*wx}$	2.7±0.72$^{b/*x}$
0	0.5	0	10	1.66±0.57$^{a/*y}$	3.26±0.68$^{a/*x}$
0	1	0	10	5.33±1.15$^{a/*w}$	6.16±0.65$^{c/*w}$
0	2	0	10	3.66±0.57$^{a/*x}$	3.96±0.50$^{b/*x}$
0	3	0	10	2.33±0.57$^{a/*y}$	2.93±0.45$^{b/*y}$
0.5	0	0.25	10	1.0±0.0$^{a/*y}$	2.83±0.30$^{a/*y}$
1	0	0.25	10	2.33±0.57$^{b/*x}$	3.76±0.25$^{b/*x}$
2	0	0.25	10	4.0±1.0$^{b/*w}$	5.2±0.30$^{a/*w}$
3	0	0.25	10	2.66±0.57$^{a/*x}$	3.8±0.43$^{a/*x}$
0.5	0	0.5	10	1.66±0.57$^{a/*w}$	3.23±0.15$^{a/*x}$
1	0	0.5	10	2.33±0.57$^{b/*w}$	4.53±0.35$^{b/*w}$
2	0	0.5	10	2.0±1.0$^{a/*w}$	2.7±0.2$^{c/*y}$
3	0	0.5	10	1.33±0.57$^{a/*w}$	2.33±1.5$^{b/*y}$

Note: The letters 'a', 'b', 'c', and 'd' in the column denote significant differences at the 0.05 significance level with respect to control and the various combinations of PGR groups at each concentration. The alphabets '*w', '*x', '*y', and '*z' in the column denote significant differences at 0.05 significance level with the control and to the various concentrations of PGRs on each PGR. Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter. (n=57)

The one-way ANOVA test for the impact of the different PGRs (BAP, TDZ, BAP+NAA 0.25, and BAP+NAA 0.5) and coconut water at each concentration versus the number of shoots and the length of shoots, as well as the impact of different PGR concentrations (0.5, 1.0, 2.0, and 3.0 mg/L) on each PGR versus number of shoots and the length of shoots, proved to be significant ($p > 0.05$) (Table 3). Moreover, the two-way ANOVA test for the impact of PGRs at different concentrations versus the number of shoots generated proved to be significant at the 0.05 level (p-value of PGRs vs shoot number was <0.001, concentration vs shoot number was <0.001, and the interaction between PGRs and concentration was 0.002). Similarly, the two-way ANOVA test for the impact of different PGRs at various concentrations on shoot length proved to be

significant at the 0.05 level (p-value of PGRs vs shoot length was 0.001, concentration vs shoot length was <0.001, and the interaction between PGRs and concentration was<0.001).

Result of the Two-way ANOVA test between the various PGRs and their concentrations on the regeneration of shoot numbers					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	57.813[a]	15	3.854	7.708	.000
Intercept	285.187	1	285.187	570.375	.000
PGRs	12.563	3	4.188	8.375	.000
Concentration	27.729	3	9.243	18.486	.000
PGRs * oncentration	17.521	9	1.947	3.894	.002
Error	16.000	32	.500		
Total	359.000	48			
Corrected Total	73.813	47			
a. R Squared = .783 (Adjusted R Squared = .682)					

Result of Two-way ANOVA test between the various PGRs and their concentrations on the increase in shoot length					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	56.173[a]	15	3.745	15.658	.000
Intercept	663.053	1	663.053	2772.348	.000
PGRs	5.233	3	1.744	7.294	.001
Conc	26.182	3	8.727	36.490	.000
PGRs * Conc	24.758	9	2.751	11.502	.000
Error	7.653	32	.239		
Total	726.880	48			
Corrected Total	63.827	47			
a. R Squared = .880 (Adjusted R Squared = .824)					

After four weeks of growth in similar media, the *in vitro*-regenerated shoots were cut off and transplanted to MS media fortified with 0.25 to 2.0 mg/L of IAA, IBA, or NAA. After 4 weeks of culture, MS medium with 1.0 mg/L of IBA (7.0±1.0) and 1.5 mg/L of IAA (4.66±0.57) regenerated the greatest number of roots and the longest roots (5.53±0.25 cm and 3.9±0.23 cm, respectively) (Table 4 and Fig. 8). The NAA failed to produce new roots from *in vitro* shoots at lower concentration, i.e., 0.25 and 0.5 mg/L.

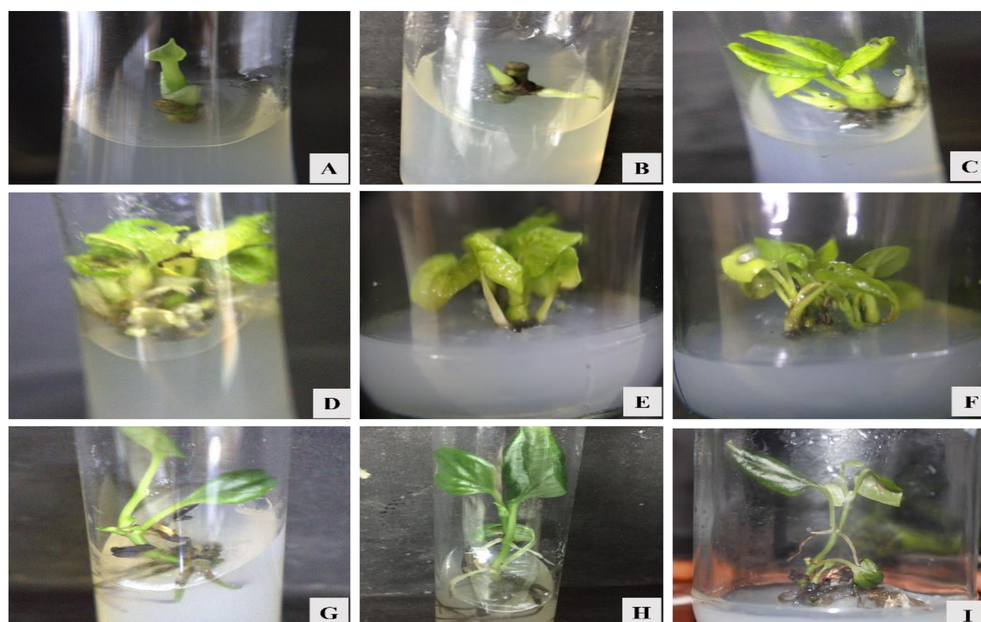


Figure 8: Different stages of shoot and root development in P. longum from node explants: (A-B) MS+ 2.0 mg/L BAP+ 0.25 mg/L NAA; (C) MS+2.0 mg/L BAP; (D-F) MS+1.0 mg/L TDZ; (G) MS+2.0 mg/L NAA; (H) MS+1.5 mg/L IAA (I) MS+1.0 mg/L IBA.

Table 3: The average number of roots and root length at various concentrations of PGRs in *P. longum*.

Concentrations of PGRs (mg/L)			**Average no. of roots per explant (M±SE)**	**Average root length in cm (M±SE)**
IAA	IBA	NAA		
0	0	0	0.0	0.00
0	0.25	0	3.00±1.0^{b}	1.46±0.25^{a}
0	0.5	0	3.66±0.57^{b}	1.8±0.26^{a}
0	1	0	7.00±1.0^{a}	5.53±0.25^{a}
0	1.5	0	5.66±0.57^{ab}	3.8±0.2^{a}
0	2	0	4.33±0.47^{ab}	3.4±0.3^{a}
0.25	0	0	2.0±1.0^{a}	1.43±0.15^{a}
0.5	0	0	3.00±1.0^{a}	2.06±0.37^{a}
1	0	0	5.33±1.15^{a}	2.3±0.2^{a}
1.5	0	0	4.66±0.57^{a}	3.9±0.23^{a}
2	0	0	4.33±0.94^{a}	3.23±0.30^{a}
0	0	0.25	0.0^{c}	0.00^{b}
0	0	0.5	0.0^{c}	0.00^{b}
0	0	1.0	3.0±1.0^{bc}	1.66±0.20^{ab}
0	0	1.5	3.66±0.57^{ab}	2.2±0.25^{ab}
0	0	2.0	4.0±0.81^{a}	4.0±0.43^{a}

Duncan's Multiple Range Test (α=0.05) indicated no significant difference between means in columns with the same letter. (n=54)

P. longum is a valuable medicinal plant that needs *ex-situ* conservation due to excessive harvesting of the roots and fruits for conventional medicinal uses, loss of habitat due to climate change, and a lack of information about how to effectively conserve plants in their natural environments. Due to the existence of less viable seeds and the short duration of seed germination, vegetative reproduction by seeds is difficult in *P. longum* (Sarasan *et al*., 1993). As a result, *in vitro* culture techniques help multiply true-to-type plants and their *ex-situ* conservation. In this study, plants were regenerated in MS media by using nodal explants (direct organogenesis). However, micropropagation from nodal explants via direct organogenesis is difficult in *P. longum* due to the existence of systemic endogenous bacteria, which often lead to contamination in culture (Bhat *et al*., 1995; Parida and Dhal, 2011; Sathelley *et al*., 2016), as well as the browning of media caused by the release of metabolic byproducts. Direct organogenesis was found in the MS medium in the absence of PGRs (BAP, NAA, and TDZ) and CW as a control, resulting in a single shoot from each nodal explant. MS treatment with 1.0 mg/L TDZ and 10% CW resulted in the maximum number of shoot buds (5.33±1.15) and the longest shoot length (6.16±0.65 cm) from nodal explants (Table 3). According to Guo *et al.* (2011) TDZ has both cytokinin- and auxin-like actions, and smaller concentrations stimulate axillary shoot regeneration while greater concentrations induce adventitious shoot development. At first 5% CW was utilized instead of 10% CW in MS media treated with BAP alone, NAA alone, TDZ alone, or a mixture of BAP and NAA, however, this failed to result in multiple shoot formation. According to Gnasekaran *et al.* (2010) coconut water behaves like a growth regulator (cytokinins) having a variety of nutrients and other growth regulator components, including diphenyl urea, and promotes cell division, growth, and shoot differentiation in culture. In comparison to BAP alone or BAP+NAA, TDZ in MS media produced higher outcomes for multiple shoot differentiation, multiplication, and longer shoot length development (Table 3). This research corroborates the results of Nautiyal *et al.* (2022) who described the *in vitro* regeneration of numerous shoots in *Oryza sativa*. They reported that the number of shoot buds regenerated from TDZ-treated seedlings in media exceeded that of BAP-treated seedlings. In addition, it was found that TDZ produced a greater effect than BAP in peanut shoot bud regeneration (Victor *et al.*, 1999; Gairi and Rashid, 2004). MS medium added with 2.0 mg/L BAP+0.25 mg/L NAA+10% CW resulted in more shoots (4.0±1.0) and longer shoots (5.2±0.30 cm) compared to 2.0 mg/L BAP+10% CW (Table 3). This could be due to the synergistic

action of auxin (NAA) and cytokinin (BAP). Furthermore, MS media supplied with 0.5-3.0 mg/L BAP in a constant amount of 0.25 mg/L NAA produced more shoots and longer shoots than MS media supplied with 0.5-3.0 mg/L BAP in a constant amount of 0.5 mg/L NAA (Table 3). It could be because the higher concentrations of NAA (0.5) in combination with various quantities of BAP have a lower impact on shoot regeneration.

Furthermore, shoots regenerated *in vitro* from nodal explants were transplanted to MS media enriched with IBA, IAA, or NAA to promote adventitious root formation. *In vitro* development of plantlets with numerous roots is essential for the successful establishment of newly formed plants in soils (Ohyama, 1970). Roots did not regenerate in MS media in the absence of PGRs (IBA, IAA, and NAA). However, MS medium fortified with 1.0 mg/L IBA formed the greatest number of roots (7.0±1.0) and longest roots (5.53±0.25 cm). This finding was corroborated by the observation that IBA produced roots in *in vitro* shoots of *P. longum* (Soniya and Das, 2002; Sharon and Maurya, 2004; Rani and Dantu, 2012). Similarly, in the present research, MS medium with 1.5 mg/L IAA resulted in more roots (5.33±1.15) and longer roots (3.8±0.2 cm). This result is supported by the finding that IAA regenerated roots from *in vitro* shoots in MS media containing IAA in *P. longum* (Bhat *et al.*, 1992). This study additionally demonstrated the ability to produce roots from *in vitro* shoots in MS medium enriched with 1.0-2.0 mg/L NAA. The observations of Philip *et al.* (1992) in *P. nigrum* and Sianipar *et al.* (2016) in *P. crocatum* corroborated this finding.

4.1.2 *In vitro* propagation of *P. longum* using leaf and nodal explants through indirect organogenesis

Micropropagation of *P. longum* through indirect organogenesis was carried out in two ways: multiple shoots regeneration from the calli and somatic embryo regeneration from the nodular callus. In both cases, the callus was induced from leaves and nodal explants.

4.1.2.1 Callus induction and proliferation from leaf explants

By inoculating sterile leaf segments (0.8-1.0 cm) in MS media both in the absence and the presence of various dosages of 2,4-D and KN (0.25, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/L) along with 10% coconut water, callus induction and its proliferation were achieved. All the media were not responding well to calli induction. In the absence of PGRs, FMS

(full MS), ½ MS, and 1/4 MS media induced calli; however, FMS medium (45%) induced larger calli than 1/2 MS (30%) and 1/4 MS (25%) media. MS medium in combination with KN alone was not effective for the production of calli. Soniya and Das (2002) also found that MS media enriched with KN alone was not effective for the production of calli as well as for direct plant regeneration in leaf explants. However, they were able to generate calli from leaf explants in MS medium enriched with 2.0-5.0 mg/L 2,4-D alone or in combination with KN.

The callus is an undifferentiated, loosely organized, thin-walled parenchymatous tissue that arises over an injured surface of plants. Calli are formed naturally due to injuries, tumor-producing bacterium (Ti genes), and genomic tumors (Bhatia, 2015), but this may also be generated artificially by culturing leaves or nodal explants in an appropriate culture media. Generally, both auxins in single as well as together with cytokinin produce callus in a variety of plant species. An intermediate proportion of auxin and cytokinin stimulates callus production; a high proportion of auxin-to-cytokinin stimulates root development; and a higher proportion of cytokinins to auxins stimulates shoot regeneration (Skoog and Miller, 1957). In certain plants, ABA and brassinosteroids promote calli production and may replace auxin or cytokinin (Hu *et al*., 2000). But auxins and cytokinins are well-investigated and utilized hormones in the contexts of calli production, organ formation, and metabolite synthesis. The calli are kept alive and held in culture over an infinite duration via in new medium.

At twelve weeks of primary culture, the most effective callusing media were MS + 1.0 mg/L 2,4-D + 2.0 mg/L KN (75%), followed by 0.25 mg/L 2,4-D + 1.0 mg/L KN (65%), 0.25 mg/L2,4-D + 2.0 mg/L KN (65%), 0.5 mg/L 2,4-D + 1.0 mg/L KN (55%), etc. (Fig. 9). Similarly, MS media treated with greater dosages of 2,4-D (3.0- 5.0 mg/L) were observed to be suitable for calli induction (Table 5). Hussain *et al.* (2011) also generated calli in *P. nigrum* leaf explants using MS medium with greater concentrations of 2,4-D (2.0-3.0 mg/L). It demonstrates that small to large amounts of 2,4-D (2.0-5.0 mg/L) in MS medium stimulate calli development in *P. longum*. Furthermore, raising the amount of 2,4-D in MS medium between 0.25-1.0 mg/L while maintaining an unchanged KN of 1.0-2.0 mg/L boosted callus formation and growth and then decreased (Table 5). During primary culture, most of the callus was compact and light white. The calli may be friable or compact, it could be made up of somatic embryos (embryonic callus), roots (rooty callus), or shoots (shooty callus), depending on the

explants, growth regulators, growth conditions, and the type of plants (Bhatia, 2015). Previous research showed that MS medium fortified with 1.0 mg/L IAA + 1.0 mg/L BAP (Sathelly *et al.*, 2016), 0.5 mg/L TDZ (Prajapati *et al.*, 2019), 1.5 mg/L BAP + 1.0 mg/L 2,4-D (Malthi *et al.*, 2016), and 1.0-2.0 mg/L 2,4-D + 1.0 mg/L BA (Sarasan *et al.*, 1993) were the best conditions for callus induction and growth from leaf segments in *P. longum*. Similarly, the most effective condition for induction of callus and growth from leaf segment was MS media enriched with 3.0 mg/L NAA + 0.05 mg/L BAP in *P. betel* (Johri *et al.,*1996); with 0.5 or 1.5 mg/L BA + 1.0 mg/L NAA in *P. nigrum* (Ahmad *et al.*, 2010); with 1.0 mg/L BA + 0.5 mg/L GA_3 in *P. nigrum* (Ahmad *et al.*, 2013); with 2.0 mg/L 2,4-D + 1.5 mg/L KN in *P. auritum* (Dominguez, 2006). It indicates that callus induction takes place from leaf explants in MS media enriched with various PGRs. Callus induction in plants is influenced by factors such as explant type (leaf, stem, or rhizome), position, PGRs, age of the plant and its explants, media structure, physiological situation, temperatures, culture conditions, and source plant type (Klimek-Chodacka *et al.*, 2020). However, effective callus development is dependent on the plant species.

Table 4: Callus induction in different concentrations of 2,4-D and Kinetin in *P. longum.*

2,4-D/→ KN ↓	**0.0 mg/L**	**0.25 mg/L**	**0.5 mg/L**	**1.0 mg/L**	**1.5 mg/L**	**2.0 mg/L**	**3.0 mg/L**	**4.0 mg/L**	**5.0 mg/L**
0.0 mg/L	-	-	-	-	-	SC	MC	LC	LC
0.25 mg/L	-	-	-	-	SC	SC	SC	MC	SC
0.5 mg/L	-	-	MC	MC	MC	MC	MC	SC	SC
1.0 mg/L	-	LC	LC	LC	MC	SC	-	-	-
1.5 mg/L	-	MC	MC	MC	MC	SC	-	-	-
2.0 mg/L	-	LC	LC	LC	MC	SC	-	-	-
3.0 mg/L	-	LC	LC	MC	MC	SC	-	-	-
In vitro condition: MS media, 25 ± 2 °C, 12-16 h Photoperiods, 12 weeks									

Abbreviations: SC=Small callus (fresh wt. <0.5 g), *MC=Moderate callus (fresh wt. 0.5-2.0 g), *LC=Large callus (fresh wt. >2.0 g) (n=189)

The calli induced from 1.0 mg/L 2,4-D + 2.0 mg/L KN continued to develop by subculturing in MS media containing the same concentrations of 2,4-D and KN. The calli were then subcultured in MS media with various PGR combinations for callus proliferation. Callus proliferation was observed at all concentrations, however, the greatest callus proliferation was observed in MS + 2.0 mg/L BAP alone, as indicated

by the calli's growth index after 8 weeks of culturing (Table 6). After 8 weeks of culturing, this concentration resulted in 2.149±0.521 g fresh weight callus from an initial fresh weight of 0.219 ± 0.030 g callus explant, representing a 674% increase. Following the cultures, the calli structure and color altered from compact light white to slightly friable yellow, compact yellow, and compact green. Callus in MS medium that contained auxin (2,4-D) were slightly friable yellow, whereas those in MS medium supplied with cytokinins (KN and BAP) were compact green and compact yellow. Changes in callus structure and color after culture might be related to genetic variation in callus and hormone concentration in the medium. Explants with various genotypes do not respond equally in tissue culture under the same conditions (Nehara *et al.*, 1990). Plant growth regulators influence cellular development, differentiation, and metabolite production (Lian *et al.*, 1991). When the concentration of all hormones, such as 2,4-D, KN, and BAP, was reduced in MS media, callus proliferation decreased.

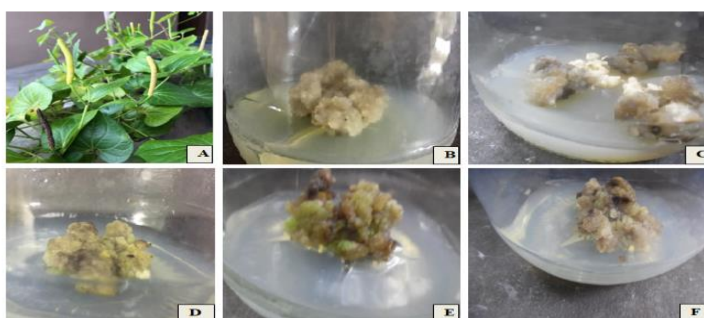


Figure 9: (A) *Piper longum* plant (B-F) Callus of *P. longum*:

Callus: (B) MS + 1.0 mg/L 2,4-D + 2.0 mg/L KN, (C), MS + 0.25 mg/L 2,4-D + 1.0 mg/L KN, (D) MS + 0.5 mg/L 2,4-D + 1.0 mg/L KN, (E) MS + 0.5 mg/L 2,4-D + 2.0 mg/L KN, (F) MS + 1.0 mg/L 2,4-D + 1.0 mg/L KN.

This study showed that cytokinin (Kinetin and BAP) was more efficient than auxin (2,4-D) for callus proliferation in MS medium in *P. longum*. The two-way ANOVA analysis of callus growth (g) vs PGRs (at p-value<0.05, p=0.0116) and different concentrations of 2,4-D, KN, and BAP (at p-value<0.05, p= 0.0289) revealed significant differences. It demonstrates that callus proliferation is dependent on the kinds of plant growth regulators and their concentrations added in MS medium. Callus proliferation favors raising the concentrations of 2,4-D, KN, and BAP in MS medium from 0.5 to 2.0 mg/L.

Table 5: Effects of various PGRs (2, 4-D, KN, and BAP) on the growth and development of callus after secondary culture in *P. longum.*

2,4-D (mg/L)	KN (mg/L)	BAP (mg/L)	Fresh wt. of callus explant (g.)	Fresh wt. of callus at 8 weeks (g)	Dry wt. of callus at 8 weeks (g)	Growth index of callus
0.5			0.220±0.029 CLW	0.401±0.015 FY	0.102±0.013	182^{c}
1.0			0.232±0.070 CLW	0.544±0.057 FY	0.122±0.020	234^{b}
1.5			0.235±0.073 CLW	0.571±0.025 FLY	0.129±0.015	243^{b}
2.0			0.227±0.078 CLW	0.950±0.093 FLY	0.213±0.047	419^{a}
	0.5		0.222±0.076 CLW	1.006±0.082 CY	0.262±0.059	312^{c}
	1.0		0.213±0.033 CLW	1.223±0.330 CG	0.227±0.013	359^{b}
	1.5		0.224±0.029 CLW	1.141±0.071 CG	0.189±0.012	352^{b}
	2.0		0.209±0.053 CLW	1.677±0.021 CG	0.317±0.019	543^{a}
		0.5	0.236±0.030 CLW	0.702±0.090 CY	0.139±0.028	209^{d}
		1.0	0.226±0.077 CLW	1.057±0.113 CG	0.204±0.013	324^{c}
		1.5	0.257±0.041 CLW	1.751±0.262 CG	0.346±0.048	430^{b}
		2.0	0.219±0.030 CLW	2.149±0.521 CG	0.367±0.106	674^{a}
***In vitro* condition: MS media, 25 ± 2 °C, 12-16 h light periods, 8 weeks**						

Abbreviations: CLW: compact light white, CG: compact green, CY: compact yellow, FLY: friable light yellow, FY: friable yellow. Data were collected from 12 combinations and repeated three times (for a total of 36 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter. (n=36)

Similarly, after 8 weeks of culturing, MS + 0.5 mg/L NAA + 2.0 mg/L BAP produced the best callus proliferation in terms of growth index (Table 7). After 8 weeks of culturing, 1.843±0.134 g fresh weight callus was found from a callus explant weighing 0.316 ± 0.011 g. After culturing, all of the calli changed morphology from compact light white to compact white brown, compact yellow, compact green, and slightly friable light yellow, which could be attributed to secondary metabolite release, and hormonal content changes in MS medium. As the concentration of NAA (auxin) in MS media increased from 0.5 to 2.0 mg/L but the concentration of BAP (cytokinin) remained constant, callus growth decreased. It demonstrates that increased concentrations of auxins combined with constant cytokinin do not promote callus proliferation in *P. longum*.

Table 6: The combined effects of NAA and BAP on the proliferation of callus following secondary culture in *P. longum.*

NAA (mg/L)	BAP (mg/L)	Fresh wt. of callus explants (g.)	Fresh wt. of callus at 8 weeks (g.)	Dry wt. of callus at 8 weeks (g.)	Growth index of callus
0.5	0.5	0.379±0.047 CLW	2.089±0.185 CWB	0.541±0.067	551^{b}
1.0	0.5	0.340±0.042 CLW	1.732±0.266 CG	0.431±0.078	509^{c}
1.5	0.5	0.309±0.055 CLW	0.856±0.079 CY	0.206±0.028	277^{f}
2.0	0.5	0.363±0.061 CLW	0.727±0.045 CY	0.171±0.008	200^{h}
0.5	1.0	0.331±0.037 CLW	1.642±0.150 CG	0.426±0.049	496^{c}
1.0	1.0	0.394±0.014 CLW	1.178±0.052 CG	0.290±0.039	298^{f}
1.5	1.0	0.338±0.023 CLW	0.716±0.176 CG	0.162±0.027	211^{h}
2.0	1.0	0.386±0.015 CLW	0.630±0.071 FLY	0.156±0.023	172^{i}
0.5	1.5	0.344±0.007 CLW	1.465±0.098 CG	0.361±0.045	425^{d}
1.0	1.5	0.314±0.017 CLW	1.132±0.147 CY	0.258±0.011	360^{e}
1.5	1.5	0.308±0.020 CLW	0.744±0.096 FLY	0.183±0.029	241^{g}
2.0	1.5	0.387±0.014 CLW	0.628±0.120 FLY	0.157±0.040	162^{i}
0.5	2.0	0.316±0.011 CLW	1.843±0.134 CWB	0.428±0.004	583^{a}
1.0	2.0	0.363±0.011 CLW	1.561±0.064 CY	0.390±0.051	430^{d}
1.5	2.0	0.397±0.025 CLW	0.644±0.131 CY	0.158±0.042	162^{i}
2.0	2.0	0.344±0.045 CLW	0.543±0.104 CG	0.146±0.031	157^{i}

Abbreviations: CLW: compact light white, CG: compact green, CY: compact yellow, FLY: friable light yellow, CWB: compact white brown. Data were collected from 16 combinations and repeated three times (for a total of 48 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter. (n=48)

The inclusion of organic additives (10% coconut water) in the MS medium was beneficial for inducing and promoting callus formation in the *P. longum* leaf explants. The control (MS media in the absence of PGRs and coconut water) media had a lower callus induction rate of 45% (full MS), 30% (1/2 MS), and 25% (1/4 MS) than MS media treated with 10% CW and PGRs. Gnasekaran *et al.* (2010) showed that coconut water functions like a cytokinin, having various nutrient and hormone ingredients that promote the division of cells and development in cultures.

4.1.2.2 Callus differentiation and plant regeneration

Aggregate compact calli were developed in MS media supplied with 1.5 mg/L 2,4-D + 1.0 mg/L KN + 10% CW from leaf segments, whereas it was produced in MS medium

supplied with 2.0 mg/L NAA + 10% CW from nodal segments of *P. longum* (Fig. 10). The callus was sub-cultured in MS media enriched with TDZ alone and a combination of BAP + NAA and 10% CW for the regeneration of multiple shoots.

Table 7: The average shoot number and average shoot length regenerated in various PGRs from callus in *P. longum.*

Plant Growth Regulators (PGRs) and additives		**Average shoot number/(±SE)**	**Average shoot length in cm/(±SE)**
TDZ	**NAA**		
0	0	0.00	0.00
0.25	0	15.33±1.52[a]	4.33±0.28[a]
0.5	0	12.00±2.00[a]	3.66±0.57[a]
1	0	8.33±1.52[b]	3.66±0.57[a]
1.5	0	5.00±2.00[bc]	3.33±0.57[a]
2	0	3.66±2.08[c]	2.00±1.00[b]
0	0.25	0.00[d]	0.00[c]
0	0.5	0.00[d]	0.00[c]
0	1	2.00±1.00[c]	2.33±1.52[b]
0	1.5	4.66±0.57[b]	3.00±1.00[b]
0	2	8.33±1.50[a]	4.66±0.57[a]

Data were collected from 11 combinations and repeated three times (for a total of 33 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

This study showed that multiple shoots and roots were produced from the calli that were developed from the leaf and nodal explants (Table 8, 9, and Fig. 10). Multiple shoot buds were developed from the callus in MS + 0.25-2.0 mg/L TDZ + 10% CW after the 8 weeks of culture. In the absence of PGRs and coconut water, the MS media failed to differentiate shoots in the callus. The use of 5% CW and PGRs in MS media also did not differentiate shoots in callus. Coconut water is often used in tissue culture due to its unique chemical makeup, which includes carbohydrates, vitamins, minerals, amino acids, and plant growth regulators, such as auxin, 1,3-diphenylurea, and cytokinin (Yong et al., 2009). However, the best media for shoot differentiation from the callus was MS + 0.25 mg/L TDZ + 10% CW. The maximum number of shoot buds (15.33±1.52) and maximum length of shoots (4.33±1.52 cm) were found in MS + 0.25 mg/L TDZ + 10% CW in callus regenerated using the leaf explants. The MS medium containing BAP + NAA + 10% CW could not differentiate shoots from the callus.

However, the callus that had grown from the node regenerated multiple shoots in MS + 1.0-2.0 mg/L NAA + 10% CW (Table 8 and Fig. 10C). In this case, the highest number of shoot buds (8.33±1.50) and maximum length of shoot (4.66±0.57 cm) were obtained in MS + 2.0 mg/L NAA + 10% CW. The anatomical study of young shoots differentiated from the callus showed the meristem dome and the leaf primordia (Fig. 11).

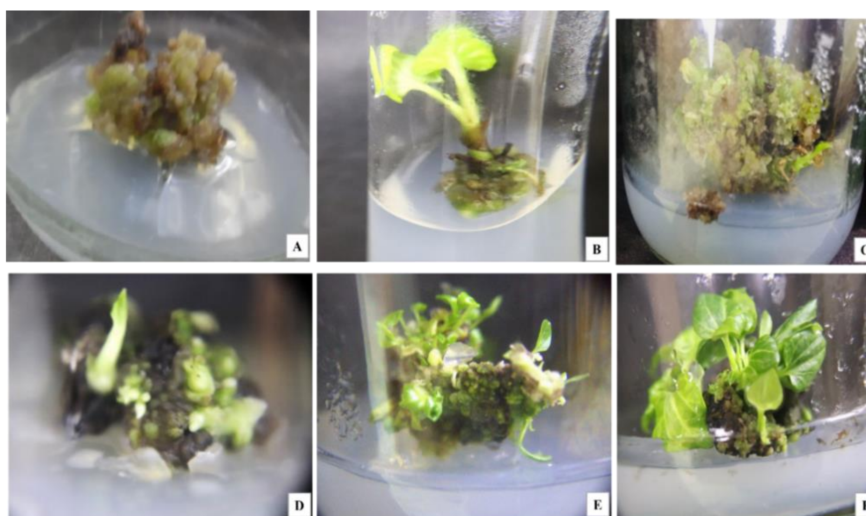


Figure 10: Various stages of shoot differentiation from the callus:

(A) Callus from the leaf segment at MS + 1.5 mg/L 2,4-D + 1.0 mg/L KN; (B) Callus from the nodal explant at MS + 2.0 mg/L NAA; (C) Initial Multiple shoot buds from the node callus at MS+2.0 mg/L TDZ; (D-E) Multiple shoot buds at MS + 0.25 mg/L TDZ.

The two-way ANOVA test for the influence of PGRs (TDZ and NAA) at different concentrations (0, 0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) on shoot number development revealed statistically a significant effect at the 0.05 level. The p-values for PGRs vs. shoot number was 5.37×10^{-11}, concentrations of PGRs vs. shoot number was 1.41×10^{-8}, and the interaction influence of PGRs and concentrations on shoot number was 4.39×10^{-12}. It demonstrates that the number of shoot differentiation/development in callus is dependent on the types of PGRs and their concentrations used in nutrient media. Similarly, the two-way ANOVA test for the influence of PGRs (TDZ and NAA) at different concentrations (0, 0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) on the increase in length of shoots showed statistically a significant effect at the 0.05 level. The p-values for PGRs vs. shoot length were 6.04×10^{-5}, concentrations of PGRs vs. shoot length were 0.008915, and the interaction influence of PGRs and concentrations on shoot length was 7.61×10^{-7}. It demonstrates that the increase in shoot length is dependent on the types of PGRs and their concentrations used in nutrient media.

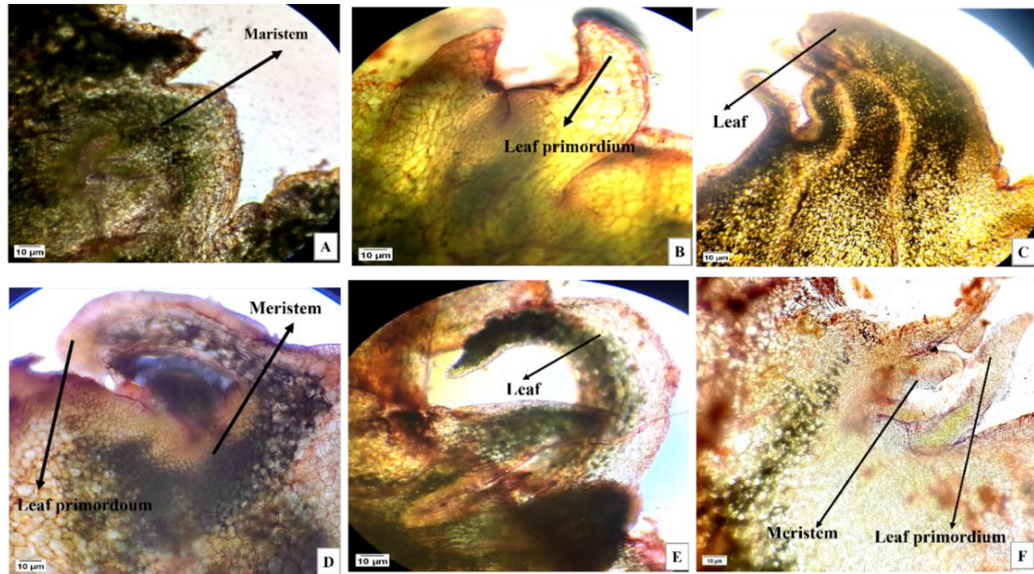


Figure 11: Anatomical study of differentiated shoot apex from the callus:

(A-F) A longitudinal section of shoot apex through the callus with leaf primordia and meristem dome.

The two-way ANOVA analysis for the influence of PGRs (IBA and NAA) at various concentrations (0, 0.5, 1.0, 1.5, and 2.0 mg/L) on root number development was analyzed statistically at the 0.05 significance level. The p-values for PGRs vs. root number were found to be 1.0 (>0.05), concentrations of PGRs vs. root number were found to be 9.51×10^{-8} (<0.05), and the interaction effect of PGRs and concentrations on root number was found to be 2.88×10^{-10} (<0.05). This shows that the number of root development from *in vitro* shoots was not dependent on the types of PGRs, however, it depended on the concentrations of PGRs used in nutrient media. Moreover, the effect of PGR types on the formation of root numbers is different at different concentrations. Similarly, the two-way ANOVA analysis for the influence of PGRs (IBA and NAA) at various concentrations (0, 0.5, 1.0, 1.5, and 2.0 mg/L) on the increase in root length showed a statistically significant effect at the 0.05 level. The p-values for PGRs vs. root length were found to be 0.000557, concentrations of PGRs vs. root length were found to be 4.82×10^{-11}, and the interaction effect of PGRs and concentrations on root length was found to be 2.86×10^{-10}. This shows that the increase in root length is dependent on the types of PGRs and their concentrations used in nutrient media.

When shoot buds associated with some callus masses were sub-cultured into MS media enriched with 0.25-2.0 mg/L of IBA and NAA, roots were produced from the shoots and calli (Table 9 and Fig. 12), but roots were not regenerated in MS medium fortified

with IAA. The maximum number of roots (8.33±1.52) and the longest roots (6.26±1.07 cm) were regenerated in MS + 0.5 mg/L IBA in the shoots and callus induced from leaf explant. Similarly, the maximum number of roots (10.66±1.52) and the longest root length (5.63±0.80 cm) were regenerated in MS + 2.0 mg/L NAA in the shoots and calli produced from the nodal explants. When individual shoots that were not associated with the callus mass were cultured on rooting media, root regeneration was found to be troublesome. A longitudinal and transverse section of roots through the callus showed the root tip meristem and other tissues under the compound microscope (Fig. 13).

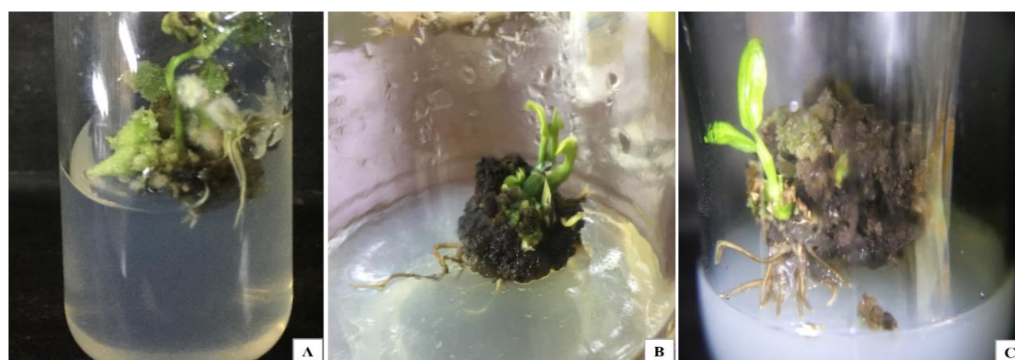


Figure 12: Root differentiation from the callus:

(A) MS + 1.5 mg/L IAA; (B) MS + 1.0 mg/L IBA; (C) MS + 2.0 mg/L NAA.

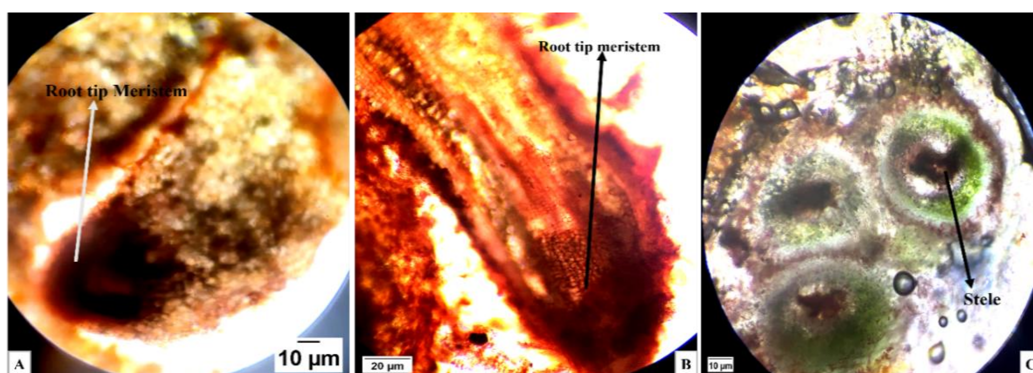


Figure 13: Anatomical study of differentiated root apex from the callus:

(A and B) A longitudinal section of root apex; (C) A transverse section of three roots through the callus.

Indirect organogenesis is a multi-stage process in which callus tissue is formed as an intermediary step in the process of regeneration, allowing the differentiation of multiple shoots or roots from the callus. Micropropagation from nodal explant through direct organogenesis is challenging in *P. longum* due to the presence of systemic endogenous bacteria that frequently cause contamination in culture ((Bhat *et al*., 1995; Parida and

Dhal, 2011; Sathelley *et al*., 2016) and due to the browning of media with the release of metabolites. In this context, the indirect organogenesis through the induction of callus from the leaf explant might be easier and safer for culture management and plant regeneration. In this investigation, multiple shoots were obtained from callus regenerated both from the leaf and nodal explants in MS + 0.25 to 2.0 mg/L TDZ + 10% CW. This work was supported by the finding that TDZ regenerated shoot buds from an *in vitro* callus in *P. longum* (Prajapati *et al*., 2019). However, Sathelly *et al.* (2016) regenerated shoots from an *in vitro* callus generated from leaf segments in MS media enriched with BAP and kinetin; Sarasan *et al*., (1993) generated shoots in MS media added with BA in *P. longum.* This study also found that callus regenerated from nodal explants regenerated multiple shoot buds at higher (2.0 mg/L) concentrations of NAA (Fig. 10C). It could be because of its properties such as increasing cell division, encouraging cell differentiation, counteracting the effects of inhibitory hormones (ABA), and triggering genetic and metabolic alterations in cells. However, the TDZ was more effective than NAA in regenerating multiple shoots from the callus in *P. longum*.

Table 8: The average root number and average root length regenerated in various PGRs from callus in *P. longum.*

Plant Growth Regulators (PGRs)		Average root number/±SE	Average root length in cm/±SE
IBA	NAA		
0	0	0	0
0.5	0	8.33±1.52^{a}	6.26±1.07^{a}
1	0	5.00±1.00^{b}	4.76±0.66^{b}
1.5	0	3.00±1.00^{bc}	3.93±0.51^{bc}
2	0	1.66±1.15^{c}	3.13±0.55^{c}
0	0.5	0.00^{c}	0.00^{c}
0	1	3.00±1.00^{b}	3.16±0.47^{b}
0	1.5	4.33±1.52^{b}	5.00±0.5^{a}
0	2	10.66±1.52^{a}	5.63±0.80^{a}

Data were collected from 9 combinations and repeated three times (for a total of 27 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

Furthermore, *in vitro* shoots regenerated using nodal explants or leaf explants were transferred in an MS medium containing IBA, IAA, or NAA for the regeneration of adventitious roots. The highest number of roots (8.33±1.52) and longest roots

(6.26±1.07 cm) were obtained in MS + 0.5 mg/L IBA. This finding was supported by the observation that IBA induced roots in *in vitro* shoots in *P. longum* (Soniya and Das, 2002; Sharon and Maurya, 2004; Rani and Dantu, 2012). However, other researchers, such as Bhat et al. (1992 and 1995), Parida and Dhal (2011), and Padhan (2015), regenerated roots in IAA from *in vitro* shoots of *P. longum.* Similarly, a greater number of roots (10.66±1.52) and longer roots (5.63±0.80 cm) were also developed in MS + 2.0 mg/L NAA. This finding was further supported by the finding that roots were regenerated in NAA from *in vitro* shoots of *P. longum* (Deca and Kalita, 2007; Sathelly *et al.*, 2016; Malthi *et al.*, 2016; Fonseka Wickramaarachchi, 2018). Roots were not regenerated from the isolated *in vitro* shoot in MS medium supplemented with NAA, but roots were regenerated when the *in vitro* shoots were still associated with callus mass (Fig. 12C). It could be because callus plays a supporting role in nutrient supplementation as well as producing endogenous hormones for root development.

4.1.3 *In vitro* propagation of *P. longum* using leaf and nodal explants through indirect organogenesis (somatic embryogenesis)

4.1.3.1 Induction of embryogenic and nodular calli from leaf and nodal explants

Formation of embryogenic calli from explants is the initial step in the production of somatic embryos. Embryogenic and nodular callus was not induced in MS media in the absence of PGRs and coconut water. However, MS medium supplied with 2,4-D alone, NAA alone, or together with 2,4-D + KN, and 10% CW, both leaf and nodal segments produced an embryogenic mass of callus in *P. longum* (Fig. 14A B). In a subsequent subculture using the identical media and culture condition, the embryogenic callus transformed into a nodular aggregate callus with various stages of embryos (Fig 16D); however, in the case of nodal segments, the nodular callus was generated by subculturing the embryogenic callus in the same media in dark conditions (Fig 16C).

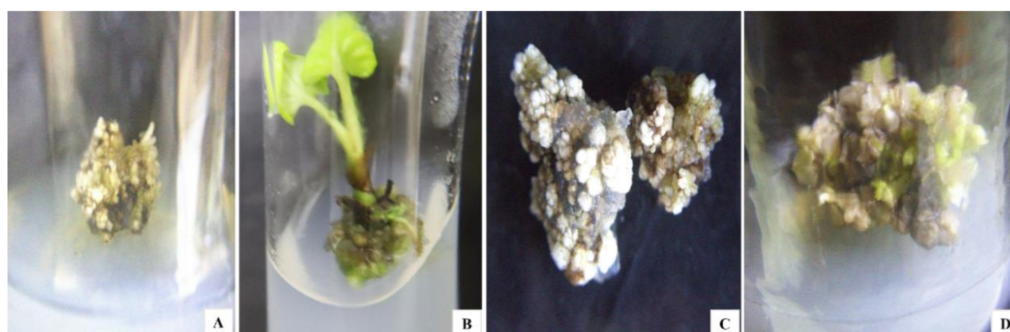


Figure 14: Embryogenic and nodular calli in *P. longum*:

(A) Embryogenic calli from leaf, (B) Embryogenic calli from nodal explant, (C) Nodular calli from node explant at dark, and (D) Nodular calli from leaf explant at light.

The nodular aggregate calli from leaf explants developed on MS + 1.5 to 4.5 mg/L 2,4-D + 10% CW alone, and together with 1.0 to 3.5 mg/L 2,4-D + 0.25 to 1.0 mg/L KN + 10% coconut water (Tables 10 and 11). The initial subculture on MS + 3.5 mg/L 2,4-D + 10% CW resulted in the highest frequency of nodular callus development (50%) and number of embryos (16.66±2.08) per 0.2 to 0.3 g fresh weight embryogenic callus.

Table 9: The frequency of embryogenic/nodular calli and somatic embryos (SEs) formation from leaf segments on MS + 2,4-D + 10% CW and in control (MS only) in *P. longum.*

Media composition/Treatment MS + 2,4-D (mg/L)	Embryogenic calli (%)	No. of countable SEs/callus ± SE
-	-	0.00
0.25	-	0.00^{b}
0.5	-	0.00^{b}
1.0	-	0.00^{b}
1.5	16.6	4.00 ± 1.00^{b}
2.5	33.3	13.66 ± 4.50^{a}
3.5	50.0	16.66 ± 2.08^{a}
4.5	33.3	6.33 ± 1.52^{b}

Data were collected from 8 combinations and repeated three times (for a total of 24 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

In the first subculture on MS + 1.5 mg/L 2,4-D + 1.0 mg/L KN + 10% CW, the leaf segments produced the highest frequency of nodular calli (66.66%) and number of embryos (28.33±3.511) per 0.2 to 0.3 g fresh weight embryogenic callus (Table 11).

Table 10: The frequency of embryogenic/nodular calli and somatic embryos (SEs) development from leaf segment on MS + 2,4-D + KN + 10% CW, and control (MS + 2,4-D + KN) in *P. longum*.

Media composition/Treatment		Embryogenic callus (%)	No. of countable SE/callus ± SE
2,4-D (mg/L)	KN (mg/L)		
0.25	0.25	-	-
0.25	0.25	-	0.00[d]
0.5	0.25	-	0.00[d]
1.0	0.25	33.33	14.33±1.52[bc]
1.5	0.25	50.00	16.33±2.51[bc]
2.5	0.25	33.33	8.66±1.52[cd]
3.5	0.25	16.66	7.00±2.00[cd]
0.25	1.0	-	0.00[d]
0.5	1.0	-	0.00[d]
1.0	1.0	16.66	10.00±2.64[bc]
1.5	1.0	66.66	28.33±3.05[a]
2.5	1.0	50.0	19.66±2.08[b]
3.5	1.0	33.33	12.33±2.51[bc]

Data were collected from 13 combinations and repeated three times (for a total of 39 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

Furthermore, nodular aggregate calli from stem explants were generated in MS medium fortified with 0.5 to 4.5 mg/L NAA + 10% CW (Table 12). However, nodular callus was not generated on MS + 2,4-D only or together with 2,4-D + KN. The first subculture on a media added with 1.0 mg/L NAA exhibited the maximum frequency of embryogenic calli production (50%) and the maximum number of embryos (12.66±2.51) per 0.2 to 0.3 g fresh weight embryogenic callus.

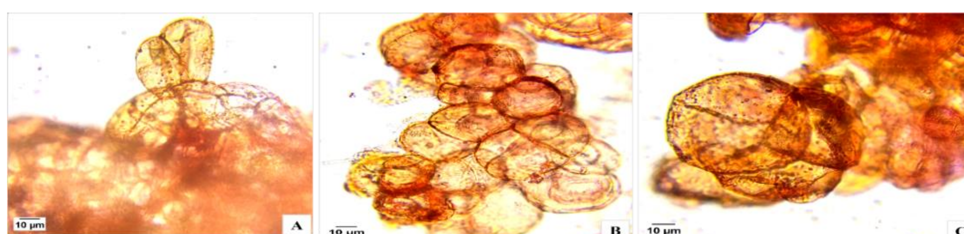
Table 11: The frequency of embryogenic/nodular calli and somatic embryo (SE) development from the nodal segment on MS + NAA + 10% CW, and in control (MS only) in *P. longum*.

Media composition /Treatment NAA (mg/L)	Embryogenic callus (%)	No. of countable SEs/callus ± SE
-	-	-
0.25	-	0.00[c]
0.5	16.66	6.66±2.51[ab]
1.0	50.00	12.66±2.51[a]
1.5	16.66	8.00±3.00[ab]
2.5	33.33	9.00±2.00[ab]
3.5	33.33	10.33±0.57[ab]
4.5	16.66	4.33±1.52[bc]

Data were collected from 8 combinations and repeated three times (for a total of 24 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

4.1.3.2 Somatic embryo development, maturation, and conversion into plantlets

The nodular callus developed into somatic embryos through 2-celled, 4-celled, 8-celled, globular, and torpedo stages on further subculture in the MS media fortified with 2, 4-D alone, NAA alone, and in combination with 2, 4-D + KN + 10% CW (Fig. 15). Morphogenic differentiation of somatic embryos (whole plantlets/seedlings or shoots only), maturation, and conversion into seedlings were obtained when the nodular/embryogenic calli with somatic embryos, induced in the leaf explants, were transferred to the MS + 0.25−2.5 mg/L thidiazuron (TDZ) + 10% CW while those induced in the nodal explants were transferred to the MS + 0.25−3.5 mg/L + 10% CW (Table 13). However, seedlings were also differentiated from nodular calli with somatic embryos in MS media in the absence of PGRs.



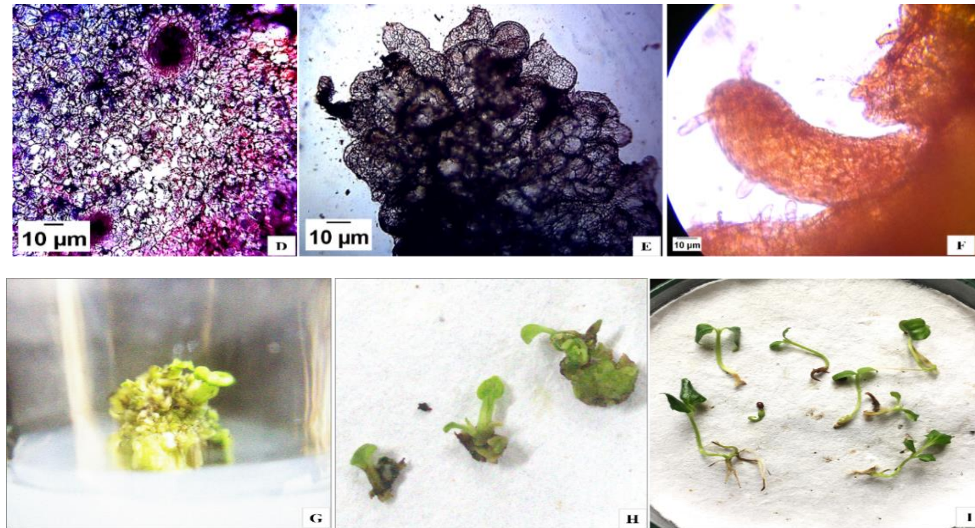


Figure 15: Stages of somatic embryo development in *P. longum*:

(A) two cells, (B) four cells, (C) eight cells, (D) Globular stage. (E) Globular and torpedo stages. (F) Torpedo stages. (G) Multiple shoots from nodular callus. (H and I) Mature isolated seedlings with roots.

In the case of nodular callus produced from the leaf explant, the highest frequency of shoot differentiation (83.33%) and entire plantlets (16.67%) occurred from the various stages of embryos, which was present in nodular callus, in MS + 0.5 mg/L TDZ + 10% CW, and this was not observed to be differentiated into roots only or callus only in any treatments (Table 13). However, in the case of the nodular callus induced from the nodal segment, the maximum frequency of the entire plantlet/seedling differentiation (66.66%) occurred in the MS + 2.5 mg/L TDZ + 10% CW, and the differentiation of shoots only (33.34%) also occurred from the various stages of the embryos in the MS + 2.5 mg/L TDZ + 10% CW while differentiation into roots only was not noticed in any treatments. The nodular callus induced from the nodal segments was not differentiated into whole plantlets and shoots only in the MS + 0.25−0.5 mg/L TDZ + 10% CW but produced callus only in this treatment.

Table 12: The frequency of morphogenic response from leaf and nodal segments in MS media enriched with TDZ and 10% CW in *P. longum.*

Explants/ Nodular calli	Media composition/ Treatment	Morphogenic response (%)			
	TDZ (mg/L)	Shoots only	Roots only	Callus only	Whole plantlets
Leaf	-	-	-	-	-
Leaf	0.25	66.66[a]	-	-	8.33[b]
Leaf	0.5	83.33[a]	-	-	16.67[a]
Leaf	1.5	33.33[b]	-	-	0.00[a]
Leaf	2.5	16.66[b]	-	-	0.00[a]
Nodal	-	-	-	-	-
Nodal	0.25	0.00[c]	-	66.66[a]	0.00[b]
Nodal	0.5	0.00[c]	-	33.33[b]	0.00[b]
Nodal	1.5	0.00[c]	-	0.00[c]	16.66[b]
Nodal	2.5	33.34[a]	-	0.00[c]	66.66[a]
Nodal	3.5	20.66[b]	-	0.00[c]	50.00[a]

Data were collected from 11 combinations and repeated three times (for a total of 33 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

(Table 11). It showed that every sub-cultured nodular callus with various stages of embryos did not differentiate into shoots only and plantlets only, but also into calli. It revealed that in *P. longum*, a lower amount of TDZ (0.25-0.5 mg/L) promotes the differentiation of embryos into shoot only, whereas a higher TDZ concentration (2.5 mg/L) promotes the conversion into seedlings from the various stages of embryos that were present in nodular callus.

This study showed that the stem-induced embryos, that were present in the nodular callus, were converted into a maximum number of whole plantlets (shoots with roots) than the leaf-induced embryos, which were present in the nodular callus, in MS + TDZ + 10% CW. The leaf-induced embryos in nodular callus produced a maximum number of shoots only than the whole plantlets. A few somatic embryos that were developed into shoots only from an embryo in nodular callus, when transferred to MS media, roots were regenerated (Fig. 16A). Moreover, roots were regenerated from the shoots that were differentiated from the nodular calli regenerated from leaf segments in MS + 0.25-2.5 mg/L TDZ and nodal segments in MS + 2.5-3.5 mg/L TDZ when transferred to the

MS media in the absence of PGRs (see Figure 4A). Similarly, some nodular calli containing somatic embryos were also differentiated directly into seedlings after being transferred to the growth regulator-free full-strength MS media (data not shown in the table). For the acclimatization process, seedlings were grown in various substrates (Fig. 16B and 16C).

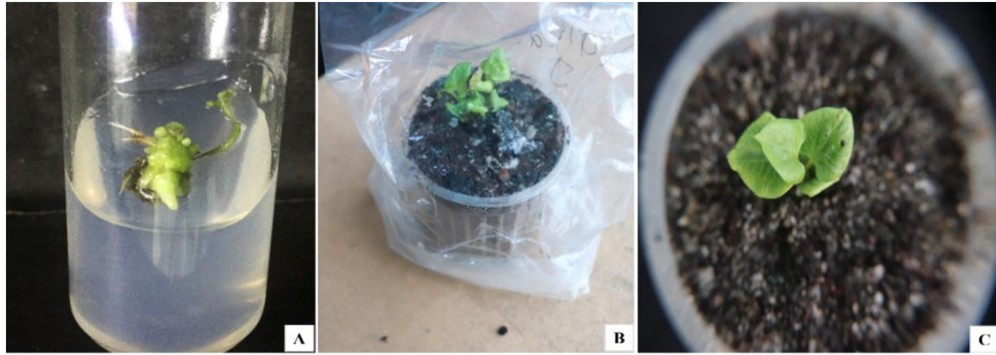


Figure 16: Embryo conversion in *P. longum*:

(A) rooting seedling, (B) and (C) Process of acclimatization of plantlets.

Sexual reproduction in *P. longum* through seed production is problematic due to a lack of viable seeds and a shorter duration of seed germination (Sarasan *et al.*, 1993). Therefore, propagation by somatic embryogenesis may be crucial for the *ex-situ* management of *P. longum*. According to Williams and Maheswaran (1986) indirect embryogenesis produces somatic embryos through the production of calli. The formation of embryogenic calli from tissue/explant is the initial step in the development of a somatic embryo. At a later stage of development, the embryogenic callus can transform into a nodular callus, which is characterized by compact and distinct spherical structures or nodules inside a callus mass (Ferreira *et al.*, 2022). In the present research, embryogenic and nodular calli were not produced in MS medium supplemented with BAP + 10% CW as well as 2,4-D + BAP + 10% CW and BAP + NAA + 10% CW. The combination of MS medium with 1.5 mg/L 2,4-D, 1.0 mg/L KN, and 10% CW exhibited the highest proportion of embryogenic callus (66.66%) and somatic embryos (28.33±3.05) in leaf explants compared to MS +3.5 mg/L 2,4-D. It might be because the combination of 2,4-D and KN has a synergistic influence on somatic embryogenesis, increasing cell proliferation, differentiation, and the establishment of proper hormonal balance. The combination of 2,4-D + KN is widely utilized to stimulate somatic embryogenesis in various kinds of plant species (Zhang *et al.*, 2004; Joshee *et al.*, 2007; Mazri *et al.*, 2017). Several other studies showed that

somatic embryo was developed on MS media in combination with KN + 2,4-D in some plants such as *Centella asiatica* (Joshee *et al.*, 2007), *Epipremnum aureum* (Zhang *et al.*, 2004), and *Phoenix dactylifera* (Mazri *et al.*, 2017). Similarly, the 2,4-D is an auxin that induces somatic embryogenesis, but auxins may require another auxin or cytokinin in association to induce somatic embryos (Yong-Wook, 2000). A lower concentration of 2,4-D only as well as together with KN did not induce nodular callus and somatic embryos in this study. Therefore, this study showed that induction of nodular callus and somatic embryos in the leaf segment prefers a higher proportion of 2,4-D and a lower proportion of KN in MS medium. Some researchers also developed somatic embryos from leaf segments in other combinations of PGRs such as MS + BA + KN in *P. colubrinum* (Yusuf *et al.*, 2001) and MS + NAA + BAP in *P. aduncum* (De Sousa *et al.*, 2020) demonstrating that effective somatic embryos can develop in combination of both cytokinins as well as auxins and cytokinin.

Moreover, the maximum frequency of embryogenic calli induction (50%) and the no. of somatic embryos (12.66±2.51) were found in MS + 1.0 mg/L NAA alone +10% CW in the callus induced from the nodal explants of *P. longum* in this study. This finding is supported by the results of Xu *et al.* (2019) in *Ranunculus scleratus*, where they induced somatic embryos from stem, leaf, and roots at higher concentrations of NAA. Mazri *et al.* (2017) and Szewczyk-Taranek and Pawlowska (2015) also developed somatic embryos in *Phoenix dactylifera* and *Hepatica nobilis* respectively. NAA can stimulate somatic embryogenesis by boosting cell division and differentiation, activating embryogenic pathways, restoring hormonal balance, initiating dedifferentiation, and encouraging the organogenesis process (Bhatia, 2015). However, Venkatachalam *et al.* (1999) found the 2,4-D to be a more effective auxin for the induction and production of somatic embryos than NAA in *Arachis hypogaea*.

Another crucial step during somatic embryogenesis is the differentiation, maturation, and transformation of somatic embryos into seedlings/plantlets from the various stages of embryos that were present in the nodular callus. If the nodular calli formed from both the leaf and nodal explants were further subcultured in the same composition of media, it differentiated into somatic embryos ranging from 2-celled to torpedo stage embryos (Fig. 21), but embryo differentiation into multiple shoots or whole plantlets did not occur in the absence of TDZ and 10% CW. Nhut *et al.* (2006) observed that TDZ, whether alone or together with other PGRs, can stimulate somatic embryogenesis

in a tissue culture media. Murthy *et al.* (1998) found that altering the auxin to cytokinin proportion *in vitro* results in somatic embryogenesis in somatic cells, while TDZ only can also cause somatic embryogenesis in many species. Sreenivasu *et al.* (1998) utilized TDZ alone to stimulate somatic embryogenesis in *Azadirachta indica* and *Cajanus cajan*. Moreover, coconut water might act as a natural and useful ingredient in tissue culture media for somatic embryo differentiation, supplying important nutrients, growth hormones, osmotic management, and antioxidant protection to aid in the growth and development of embryos. The maximum percentage of somatic embryo conversion (83.33% shoots only and 16.67% whole plantlet) was observed in MS + 0.5 mg/L TDZ + 10% CW from nodular calli produced from the leaf explants, while the maximum percentage of somatic embryo conversion (33.33% shoots only and 66.66% whole plantlets) was observed in MS + 2.5 mg/L TDZ + 10% CW from nodular callus induced from the nodal explants. Thus, the percentage of whole plantlets conversion was higher in the nodular callus developed from the nodal explants than that developed from the leaf explants. It may be due to the residual effect of NAA in somatic embryos that was induced from nodal explants for *in vitro* rooting. On the other hand, after embryo maturation, all the embryos developed into seedlings when they were grown in full-strength MS media in the absence of PGRs. This result was endorsed by the findings of Mazri *et al.* (2017) in *Phoenix dactylifera*. However, Simos *et al.* (2010) showed that not all embryos were transformed into seedlings (plantlets), but a small percentage were converted into roots only and callus only in MS medium without the addition of PGRs. In some species of *Piper*, including *P. nigrum*, somatic embryogenesis was reported from micropylar tissues or zygotic embryos in Schenk and Hildebrandt (SH) media in the absence of hormones at dark (Nair and Gupta, 2003, 2006; Sasi and Bhat, 2016).

4.1.4 *In vitro* propagation of *Paris polyphylla* using leaf explants through indirect organogenesis

4.1.4.1 Callus induction in MS media from leaf explants

Inoculation of *P. polyphylla* leaf discs on MS media with various dosages of 2,4-D and KN (0.25, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/L) along with 10% CW resulted in callus formation. According to Trigiano and Gray (2000) the majority of tissues in plant tissue culture require a combination of specific PGRs to produce the desired growth response.

MS media at full, half, and quarter strength did not produce callus in the absence of PGR supplements and 10% coconut water. Similarly, applying different concentrations of KN alone, 2,4-D alone, or together with NAA and KN to MS media did not produce a callus in *P. polyphylla*. Soniya and Das (2002) observed that in MS medium fortified with KN alone, *Piper longum* leaf explants did not form callus. They were able to develop calli from leaf explant using MS media fortified with 2,4-D + KN. Kinetin is not always required, however exogenous auxin is necessary to initiate tissue callus formation (Okazawa *et al.*, 1967). Kinetin is commonly used in tissue culture to induce callus formation (in combination with auxin) and to generate shoots from callus (at lower auxin concentrations) (Duszka *et al.*, 2009).

The findings of this study exhibited that the most efficient medium for inducing callus was the MS enriched with 0.25 mg/L 2,4-D+0.5 mg/L KN (85%), then 0.25 mg/L 2,4-D+0.25 mg/L KN (70%), 0.25 mg/L 2,4-D+1.0 mg/L KN (60%), 0.25 mg/L 2,4-D+1.5 mg/L KN (55%), and 0.5 mg/L 2,4-D+0.26 mg/L KN (50%) at 12 weeks of initial culture (primary culture) (Fig. 17 and Table 14). When 2,4-D and KN concentrations were almost the same or KN concentration was more than that of 2,4-D, callus induction and growth in *P. polyphylla* was shown to be more favorable. Calli occur on plants naturally as a result of wounds, microorganisms that produce tumors (the Ti gene), and genetic tumors (Bhatia, 2015). Calli can, however, be intentionally produced *in vitro* using a leaf or other explant in the appropriate nutrient medium. Auxin, alone or together with cytokinin, often causes the induction of calli in several plants. Initiation of callus formation takes place when the proportion of auxin to cytokinin is intermediate; root differentiation takes place when the proportion of auxin to cytokinin is high; and shoot differentiation takes place when the proportion of cytokinin to auxin is high (Skoog and Miller, 1957). In some plants, ABA and brassinosteroids can produce calli in the absence of auxin or cytokinin (Hu *et al.*, 2000).

This study also found that callus induction and growth increased first, then decreased, when KN concentration was increased from 0.25-1.0 mg/L in MS medium with a constant 0.25 mg/L 2,4-D concentration (Table 12). Similarly, raising the dosages of 2,4-D in MS media from 0.25-1.5 mg/L reduces callus induction and development while keeping the KN concentration constant. In the primary culture, all calli were somewhat compact and light white. Based on the explants, growth regulators, growth conditions, and types of plants, the callus might or might not include somatic embryo

(embryonic calli), roots (rooty calli), and shoots (shooty calli), as well as be friable or compact. When calli were subcultured in the same concentrations of 2,4-D + KN, their morphology altered from compact to friable. When the calli were subcultured in cytokinins such as BAP, KN, and TDZ, the morphology of the callus changed from friable to more compact and greener. Organogenesis may have begun in the callus, which can alter its morphology. Previous studies generated calli using leaf segments in MS medium containing 2,4-D alone, NAA alone, Indole-3-butyric Acid (IBA) alone, or a combination of 2,4-D+KN in *Melaleuca alternifolia* (Kiong *et al.*, 2007), with a combination of IAA+BAP in *Piper longum* (Sathelly *et al.,* 2016), with a combination of 2,4-D+KN in *Piper auritum* (Dominguez, 2006), and with a combination of NAA+BAP in *Bergenia ciliata* (Shrestha and Pant, 2011). The overall data suggest that callus induction can occur from leaf explants in MS medium added with auxins, either alone or together with cytokinin, therefore it is dependent on plant genotype. Furthermore, within a plant species, callus induction is affected by the type of explants, their orientation, plant growth regulators, the age of the explant (young or elderly), media formulation, the plant's physiological state, temperature, growing environments, and the kind of source plant (Klimek-Chodacka *et al.*, 2020).

Table 13: Induction of callus in varied concentrations of Kinetin and 2,4-dichlorophenoxy acetic acid in *P. polyphylla*.

KN/→ 2,4-D ↓	**0.0 mg/L**	**0.25 mg/L**	**0.5 mg/L**	**1.0 mg/L**	**1.5 mg/L**	**2.0 mg/L**	**3.0 mg/L**
0.0 mg/L	-	-	-	-	-	-	-
0.25 mg/L	-	LC	LC	LC	MC	MC	SC
0.5 mg/L	-	MC	MC	MC	MC	MC	-
1.0 mg/L	-	MC	MC	SC	SC	SC	-
1.5 mg/L	-	SC	SC	SC	SC	-	-
2.0 mg/L	-	-	-	-	-	-	-

Abbreviations: SC=Small callus (fresh wt. <0.5 g), *MC=Moderate callus (fresh wt. 0.5-2.0 g), *LC=Large callus (fresh wt. >2.0 g), (n=126).

4.1.4.2 Callus proliferation in MS medium

The callus produced in MS medium with 2,4-D + KN and 10% coconut water were grown by culturing in an identical amount of 2,4-D and KN, after which the callus was sub-cultured in MS medium enriched with various amounts of BAP alone, KN alone, and TDZ alone, as well as NAA+BAP+GA_3, BAP+KN+GA_3, and 10% coconut

water. As the concentration of all PGRs, such as BAP, KN, and TDZ, in MS media increased, so did the development of callus. Although callus proliferated in all PGR concentrations and combinations, the most efficient proliferation was obtained in MS medium containing 2.0 mg/L KN, as indicated by the growth index of callus following 8 weeks of subculture (Table 14). Following eight weeks of culture, a callus explant with an initial fresh weight of 0.326±0.041 g produced 6.523±0.347 g of callus, resulting in a 647.33% increase in callus growth (Growth Index) at 2.0 mg/L of KN. Similarly, a callus formed on MS medium with BAP+KN+GA_3 showed a higher growth index than a callus grown on MS medium with NAA+BAP+GA_3 (Tables 15 and 16). The maximum callus growth index (589.03%) was found in MS medium containing 1.0 mg/L BAP, 2.0 mg/L KN, and 2.0 mg/L GA_3 from 0.350±0.086 g callus explant (Table 14), whereas the highest callus growth index (570.58%) was obtained in 2.0 mg/L BAP+0.5 mg/L NAA+2.0 mg/L GA_3 from 0.366±0.062 g callus explant (Table 15). It might be because two cytokinins, BAP and KN, work together to stimulate the proliferation and development of calli. Similarly, following eight weeks of culture in MS media, callus proliferation, and development increased in regards to fresh wt or callus growth index when BAP (cytokinin) concentrations raised from 0.5-2.0 mg/L while NAA (auxin) concentrations were held constant (Table 15). It indicates that raising cytokinin concentrations in the presence of auxin helped to promote callus proliferation in *P. polyphylla*. Furthermore, as the concentrations of BAP and KN were raised, the proliferation of calli cultured in MS medium enriched with BAP+KN+GA_3 increased in terms of fresh weight or growth index but somewhat decreased after the KN concentration of 1.0 mg/L (Table 16). It may be related to the saturation level of BAP+KN for *P. polyphylla* callus growth.

The callus morphology altered after subcultures, changing from slightly compact light-white to white, yellow, and greenish. The calli produced in combination with cytokinin and auxin (BAP+NAA) were compact light yellow and compact white, but the calli in MS media containing cytokinins, either alone or in combination, were compact light green and white. Genetic heterogeneity in the callus, metabolite release by the callus, and PGR concentration in the media may all contribute to the change in callus morphology after subculture. In tissue culture, explants of different genotypes respond differentially to the same growing environment (Nehara *et al.,* 1990), and PGRs

influence metabolite synthesis, organogenesis, and cell development (Liang *et al.*, 1991).

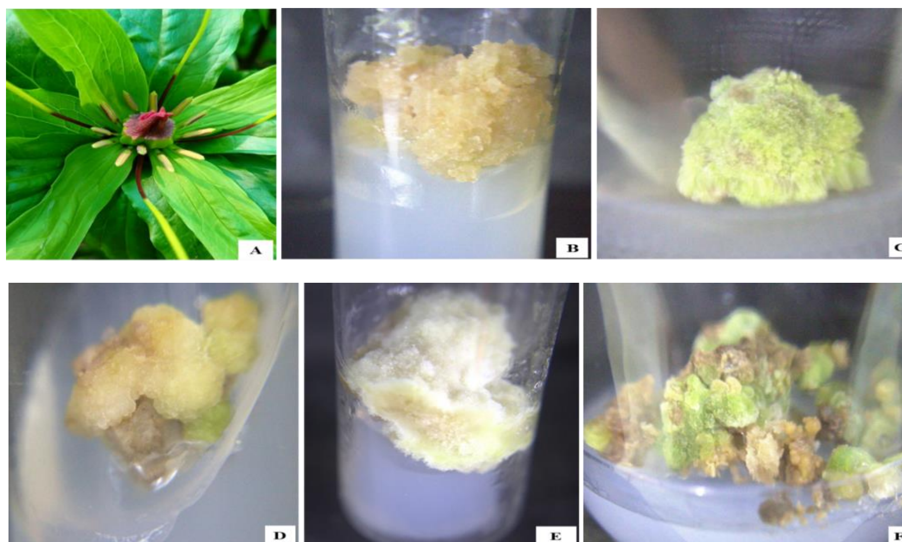


Figure 17: Callus development from leaf segment of *P. polyphylla*.

(A-F) Callus: (B) MS + 0.25 mg/L 2,4-D+0.5 mg/L KN, (C) MS +2.0 mg/L KN, (D) MS + 1.0 mg/L TDZ, (E) MS + 2.0 mg/L BAP + 0.5 mg/L NAA, (F) MS + 1.0 mg/L BAP+2.0 mg/L KN.

The p-value of PGRs (BAP, KN, and TDZ) at various dosages (0.5, 1.0, 1.5, and 2.0 mg/L) versus callus growth (g) or growth index showed a significant difference at $p<0.005$ level (p-value=7.91×10^{-26} and 6.55×10^{-16}, respectively). It reveals that callus proliferation in terms of callus growth (g) or growth index was determined by the kinds of PGRs used and the concentrations supplied to the MS medium.

Table 14: Independent impacts of BAP, KN, and TDZ on the proliferation and growth of calli after secondary culture in *P. polyphylla*.

BAP (mg/L)	KN (mg/L)	TDZ (mg/L)	Callus explants fresh wt. (g)	Callus fresh wt at 8 weeks (g)	Callus dry wt at 8 weeks (g)	Growth index (%) at 8 weeks
0.5			0.333±0.039	1.191±0.139 CLW	0.376±0.042	91.19^{d}
1.0			0.304±0.070	2.422±0.230 CLW	0.882±0.121	229.65^{c}
1.5			0.303±0.018	3.708±0.125 CY	1.544±0.056	362.67^{b}
2.0			0.332±0.036	4.572±0.257 CG	2.104±0.164	449.95^{a}
	0.5		0.318±0.039	2.564±0.380 CY	0.792±0.096	243.98^{d}
	1.0		0.348±0.025	3.762±0.172 CLW	1.304±0.062	366.98^{c}
	1.5		0.311±0.032	5.243±0.276 CG	2.148±0.083	518.42^{b}
	2.0		0.326±0.041	6.523±0.347 CG	2.956±0.145	647.33^{a}
		0.5	0.344±0.055	3.549±0.174 CLW	1.122±0.042	345.22^{c}

	1.0	0.314±0.062	5.882±0.011 CG	2.825±0.095	582.30[a]
	1.5	0.321±0.039	4.332±0.379 CG	1.043±0.157	425.77[b]
	2.0	0.316±0.049	3.439±0.183 CY	0.826±0.067	334.77[c]

Abbreviations: CLW: compact light-white, CG: compact light greenish, CY: compact light yellow. Data were collected from 12 combinations and repeated three times (for a total of 36 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

Table 15: Cumulative effects of different combination of BAP and NAA on the proliferation and growth of callus following secondary culture (2.0 mg/L GA_3) in *P. polyphylla*.

BAP (mg/L)	NAA (mg/L)	Callus explants fresh wt. (g.)	Callus fresh wt at 8 weeks (g.)	Callus dry wt at 8 weeks (g.)	Growth index (%) at 8 weeks
0.5	0.5	0.330±0.077	1.965±0.670 CY	0.711±0.261	179.68[h]
1.0	0.5	0.370±0.084	2.532±0.111 CLW	0.986±0.018	238.59[fg]
1.5	0.5	0.368±0.066	2.859±0.070 CLW	1.314±0.006	273.08[e]
2.0	0.5	0.366±0.062	5.769±0.185 CLW	2.749±0.054	570.58[a]
0.5	1.0	0.340±0.061	2.690±0.224 CLW	0.997±0.109	256.41[ef]
1.0	1.0	0.354±0.058	3.428±0.295 CY	1.470±0.025	332.54[d]
1.5	1.0	0.377±0.049	3.739±0.107 CY	1.719±0.014	363.87[c]
2.0	1.0	0.352±0.079	4.662±0.157 CLW	2.331±0.104	458.68[b]
0.5	1.5	0.390±0.033	1.302±0.061 CY	0.469±0.035	100.32[i]
1.0	1.5	0.364±0.071	2.549±0.098 CLW	1.019±0.018	240.66[fg]
1.5	1.5	0.371±0.035	2.796±0.023 CY	1.286±0.037	266.36[e]
2.0	1.5	0.338±0.033	3.287±0.154 CY	1.383±0.274	318.41[d]
0.5	2.0	0.351±0.034	1.017±0.118 CY	0.371±0.031	67.15[j]
1.0	2.0	0.376±0.040	1.446±0.105 CY	0.564±0.052	118.55[i]
1.5	2.0	0.350±0.035	2.439±0.100 CLW	1.122±0.064	229.52[g]
2.0	2.0	0.368±0.062	2.745±0.112 CLW	1.300±0.089	261.13[ef]

Abbreviations: CLW: compact light white, CY: compact light yellow. A total of 36 explants were used, and three times experiments were repeated to collect data. Data were collected from 16 combinations and repeated three times (for a total of 48 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

The addition of an organic additive (10% CW) to the MS media effectively induced callus in *P. polyphylla* leaf explants. Induction of Calli was observed in MS media treated with 2,4-D+KN and 10% coconut water, but not in media without 10% coconut water. According to Gnasekaran *et al.* (2010) coconut water promotes cell division and

growth in culture by functioning as a cytokinin-like PGR and contains a variety of nutrients and hormone elements.

Table 16: Cumulative effects of different combinations of BAP and KN on the proliferation and growth of calli after secondary culture (2.0 mg/L GA_3) in *P. polyphylla*.

BAP (mg/L)	KN (mg/L)	Callus explants fresh wt. (g.)	Callus fresh wt at 8 weeks (g.)	Callus dry wt at 8 weeks (g.)	Growth index (%) at 8 weeks
0.5	0.5	0.375±0.043	2.092±0.130 CLW	0.753±0.051	191.27^{i}
1.0	0.5	0.394±0.044	2.333±0.052 CLW	0.933±0.034	216.46^{h}
1.5	0.5	0.331±0.056	2.404±0.023 CG	0.985±0.016	226.65^{gh}
2.0	0.5	0.399±0.031	2.521±0.043 CG	1.262±0.020	236.33^{gh}
0.5	1.0	0.379±0.082	2.620±0.044 CLW	0.943±0.015	247.59^{fg}
1.0	1.0	0.330±0.045	2.747±0.052 CY	1.135±0.022	262.73^{ef}
1.5	1.0	0.357±0.059	2.829±0.055 CG	1.216±0.023	270.31^{ef}
2.0	1.0	0.326±0.075	2.898±0.017 CG	1.333±0.021	278.59^{e}
0.5	1.5	0.350±0.071	3.218±0.032 CLW	1.148±0.025	310.98^{d}
1.0	1.5	0.366±0.062	3.342±0.040 CY	1.359±0.044	323.26^{d}
1.5	1.5	0.360±0.068	3.590±0.009 CG	1.675±0.050	348.95^{c}
2.0	1.5	0.345±0.037	3.676±0.037 CY	1.813±0.028	358.19^{c}
0.5	2.0	0.456±0.207	5.183±0.065 CG	1.796±0.023	509.48^{b}
1.0	2.0	0.350±0.086	5.949±0.048 CG	2.840±0.133	589.03^{a}
1.5	2.0	0.365±0.051	5.802±0.018 CG	2.613±0.008	573.96^{a}
2.0	2.0	0.348±0.057	5.747±0.098 CY	2.740±0.178	568.63^{a}

Abbreviations: CLW: compact light white, CG: compact light green, CY: compact light yellow. Data were collected from 16 combinations and repeated three times (for a total of 48 samples). Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

4.1.4.3 Callus differentiation in MS media

The calli, which were produced in MS media supplied with 2,4-D and KN, was subcultured in MS media fortified with various amounts of KN (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L) and 10% coconut water. Mini rhizomes with root primordia were generated in the MS media fortified with 2.5 mg/L KN+ 10% CW, and adventitious roots were developed from the mini-rhizomes (Fig. 18). Kinetin, a synthetic plant growth regulator, is mainly responsible for shoot initiation and development. However, exogenous auxins (2,4-D) together with kinetin and 10% coconut water were applied initially during callus induction from the leaf explant, which may have stimulated

rooting. In tissue culture, the artificial auxin 2,4-D is commonly utilized to encourage somatic embryogenesis. However, it is rarely used commercially for inducing roots. Kinetin promotes callus development, implying that the addition of kinetin to the media would most likely aid exogenous auxin-induced activation of callus growth. Cytokinin such as kinetin can encourage axillary buds to differentiate and regenerate (Le Bris, 2017). A medium with a low auxin concentration is required for roots to develop in culture (Okazawa *et al*., 1967).

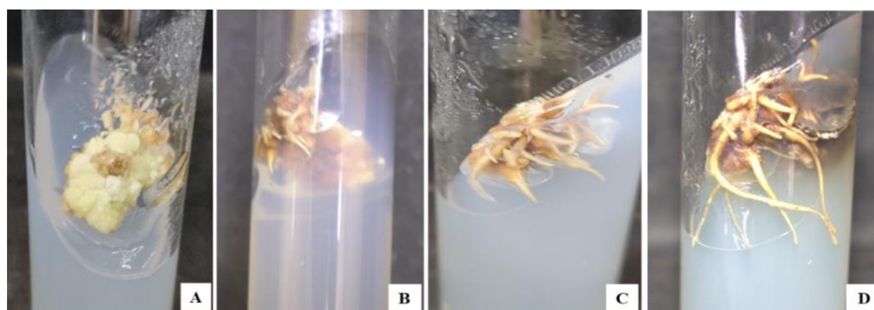


Figure 18: Various stages of mini rhizomes and root initiation in MS medium fortified with 2.5 mg/L KN from callus in *P. polyphylla.*

(B) Bioactivity analysis, suspension culture, and screening of phytochemical constituents of *in vitro* callus and wild-grown parts of *Piper longum* and *Paris polyphylla*

Methanolic crude extracts of *in vitro*-raised callus and wild-grown various parts such as root, stem, leaf, and fruit of *P. longum*, and rhizome of *P. polyphylla* were evaluated for their antioxidant, total phenolic contents, total flavonoid contents, antibacterial, cytotoxic, and anti-diabetic properties. Similarly, phytochemical screening of metabolites and identification of putative compounds of *in vitro*-raised callus and wild-grown various parts of *P. longum* and *P. polyphylla* were also carried out in this study.

Yield of crude extracts and fractions in *P. longum*

The maximum percentage of crude extract was found in fruit (21.54%), which was followed by leaf (11.07%), stem (6.17%), root (5.75%), and callus (2.05%) (Table 18). Similarly, the highest percentage of fraction was obtained in methanol, which was followed by hexane, and DCM in tested parts, such as root, stem, leaf, fruit, and callus. According to Abubakar *et al.* (2017) plant material characteristics (e.g., species, part of the plant used), solvent polarity, extraction technique (percolation, maceration, solvent extraction), environmental conditions (climate, type of soil, growing

condition), harvesting procedures (timing, methods), post-harvest storage (drying, curing, storage condition), methods of processing (grinding, milling), etc. may all have an impact on the percentage yield of crude extracts and fractions. Moreover, polar solvents (such as methanol) produce higher yields since they are capable of dissolving a wider range of chemical compounds.

Table 17: Yield of crude extract and fractions (Hexane, DCM, and Methanol) in various parts of *P. longum*.

Plant parts	Extracts/Fraction	Solvent used	Yield (%)
Stem	Crude	90% Methanol	6.17%
	Hexane fraction	Hexane	2.20%
	DCM fraction	DCM	1.78%
	MeOH fraction	100% Methanol	78.77%
Root	Crude	90% Methanol	5.75%
	Hexane fraction	Hexane	11.09%
	DCM fraction	DCM	0.57%
	MeOH fraction	100% Methanol	16.19%
Fruit	Crude	90% Methanol	21.54%
	Hexane fraction	Hexane	3.50%
	DCM fraction	DCM	2.05%
	MeOH fraction	100% Methanol	35.66%
Leaf	Crude	90% Methanol	11.07%
	Hexane fraction	Hexane	38.00%
	DCM fraction	DCM	1.69%
	MeOH fraction	100% Methanol	12.77%
Callus	Crude	90% Methanol	2.05%
	Hexane	Hexane	3.89%
	DCM fraction	DCM	3.30%
	MeOH fraction	100% Methanol	42.80%

Yield of crude extract and fractions in *P. polyphylla*

The crude extracts were prepared by using a cold and hot maceration process in 90% methanol while the hexane, DCM, and methanol fractions were prepared by using the liquid-liquid partition method. The highest percentage of crude extract was obtained in the rhizome (18.32%) than in the callus (11.65%).

Table 18: Yield of crude extract and fractions (Hexane, DCM, and Methanol) in various parts of *P. polyphylla*.

Plant parts	Extracts/Fraction	Solvent used	Yield (%)
Rhizome	Crude	90% Methanol	18.32%
	Hexane fraction	Hexane	1.11%
	DCM fraction	DCM	0.55%
	MeOH fraction	100% Methanol	21.07%
Callus	Crude	90% Methanol	11.66%
	Hexane fraction	Hexane	1.98%
	DCM fraction	DCM	0.84%
	MeOH fraction	100% Methanol	10.67%

Similarly, the highest percentage of fraction was obtained in methanol, which was followed by hexane, and DCM in both rhizome and callus (Table 19). Plant material properties, solvent polarity, extraction method, environmental conditions, harvesting practices, post-harvest storage, processing techniques, etc. may affect the percentage yield of crude extracts and fractions (Abubakar *et al.*, 2017). Moreover, polar solvents (such as methanol) produce higher yields since they are capable of dissolving a wider range of chemical compounds.

4.2 Assessment of the *in vitro* antioxidant, total phenolic content (TPC), total flavonoid contents (TFC), antibacterial, cytotoxic, and antidiabetic activities of *in vitro*-raised callus and wild-grown parts of *Piper longum* and *Paris polyphylla*

Crude extracts and their fractions such as DCM and methanol were utilized for the test of various types of bioactivities in *P. longum* and *P. polyphylla*. However, the hexane fraction of all the *in vivo* parts and all the fractions (DCM, hexane, and methanol) of *in vitro* callus were disregarded due to their insignificant bioactivity.

4.2.1 Assessment of the *in vitro* antioxidant activity, TPC, and TFC of *in vitro*-raised callus and wild-grown parts of *P. longum*

4.2.1.1 Antioxidant activity by DPPH assay

Because of its stability in the radical form and the ease of the test, DPPH is a popular substrate for quickly determining the free radical scavenging activity of plant extracts (Bozin *et al.*, 2008). The effect of antioxidants on DPPH was thought to be owing to their capacity to provide hydrogen (Brighente *et al.*, 2007). In this assay, the absorbance

value falls when the concentration of antioxidants (such as plant samples or ascorbic acid) in the reaction mixture rises.

Among the tested crude extracts of wild-grown parts and tissue culture-grown callus, the root extracts exhibited the maximum free radical scavenging activity, with an IC_{50} of 193.31±0.394 µg/mL, followed by callus extracts (IC_{50}=206.61±0.649 µg/mL) (Table 20). The greater value of IC_{50} denotes weaker antioxidant activity, whereas the lesser value of IC_{50} denotes stronger antioxidant activity. Similarly, the root had the maximum antioxidant activity among the tested methanolic crude extracts of wild-grown parts, followed by leaf, fruit, and stem. It might be due to the presence of a greater concentration of antioxidant compounds in the crude root extracts than in other extracts. However, Jobi and Seju (2020) found that the MeOH crude extract of the leaf had better antioxidant capacity and TPC than the fruits of *P. longum*. In this work, methanolic crude extracts of wild-grown parts of *P. longum* have been found to have a different capacity to scavenge free radicals than various crude extracts reported by earlier researchers (Parida and Dhal, 2011; Archana *et al.,* 2015; Sultana *et al.,* 2019). It may be influenced by factors such as the purity of the extraction solvent, the use of polar or non-polar solvents, location, the habitat in which the plant grows, and other environmental variables. Pure molecules isolated from diverse crude extracts and fractions, on the other hand, can have the potential to be powerful antioxidants.

Table 19: Antioxidant activities, total phenol, and flavonoid content of the various extracts and fractions of *P. longum.*

Plant parts	Crude Extract /Fraction	Antioxidant activity (IC_{50}: µg/mL)	Total phenol content (mg of GAE/g dry wt)	Total flavonoid content (mg of QE/g dry wt)
Root	Crude extract	193.31±0.39^{b}	137.33±0.12^{a}	113.18±0.53^{a}
	DCM fraction	134.81±1.16^{c}	41.22±0.50^{b}	73.41±0.53^{b}
	Methanol fraction	487.38±0.25^{a}	12.59±0.92^{c}	7.94±0.19^{c}
Leaf	Crude extract	311.72±0.49^{b}	118.32±0.10^{a}	98.17±0.09^{a}
	DCM fraction	186.11±0.48^{c}	27.62±0.84^{b}	24.15±0.49^{b}
	Methanol fraction	346.82±0.58^{a}	14.37±0.92^{b}	8.82±0.28^{b}
Fruit	Crude extract	391.74±0.57^{b}	113.94±0.19^{a}	82.10±0.05^{a}
	DCM fraction	194.89±0.51^{c}	22.25±0.16^{b}	20.75±0.31^{b}

	Methanol fraction	520.30±0.58^{a}	10.55±0.96^{b}	7.60±0.32^{b}
Stem	Crude extract	576.32±0.83^{a}	94.80±0.23^{a}	62.60±0.30^{a}
	DCM fraction	310.04±0.60^{b}	21.66±0.50^{b}	17.81±0.43^{b}
	Methanol fraction	584.28±0.49^{a}	9.59±0.57^{b}	7.10±0.28^{b}
Callus	Crude extract	206.61±0.649	115.87±0.58	95.81±0.15
Ascorbic acid (+ve control)		7.40±0.533	-	-

Among the examined fractions of wild-grown parts, the DCM fraction of root demonstrated the greatest antioxidant activity, with an IC_{50} of 134.81±1.162 µg/mL, followed by the DCM fraction of leaf (IC_{50}=186.11±0.487.60 µg/mL) in comparison to ascorbic acid (IC_{50}=7.40±0.533 µg/mL). This study found that fractions are more effective at scavenging free radicals than crude extracts. The overall content of phenols, which are known to have antioxidant properties, may be linked to this action. Several studies have revealed that the existence of bioactive molecules including phenols and flavonoids might be responsible for the plants' free radical scavenging ability, indicating that they might be exploited as a natural antioxidant source (Subedi *et al.*, 2014). Sannigrahi *et al.* (2010) reported that the EtOAc fraction of *Enhydra fluctuans* was more efficient than the methanolic crude extracts, as well as the chloroform and n-butanol soluble fractions, in terms of antioxidant activity. Silveira *et al.* (2017) found that fractions containing n-butanol, ethyl acetate, and DCM were more effective than the crude stem bark extract in *Tabernaemontana catharinensis*. The crude extract may contain impurities or contaminants such as lipids, carbohydrates, or other non-polar inactive compounds. Furthermore, this study shows that DCM fractions accumulate higher total phenolic and flavonoid contents, resulting in a greater antioxidant capacity than methanol fractions. According to Thapa *et al.* (2023) the dichloromethane fraction's significant antioxidant and bioactivity could be attributed to the synergistic interaction of polar and non-polar molecules, as it is capable of dissolving both polar and nonpolar organic compounds. Increasing the extract concentration from 25 to 400 µg/mL improved the percentage of free radical scavenging effect on DPPH.

A one-way ANOVA test revealed a significant difference (at p-value <0.05, p=$<2\times10^{-16}$, n=30) between crude extracts of *in vitro*-grown callus as well as extracts of various wild-grown plant parts such as root, stem, leaf, and fruit and their IC_{50} values or

antioxidant activity. This study demonstrated that the antioxidant activity of various crude extracts is dependent on plant parts. The existence of bioactive metabolites changes according to the plant part and amount accumulated (Pant *et al.*, 2021). Similarly, the correlation between antioxidant activity (IC_{50}) and TPC or TFC was negative (Pearson's correlation coefficient, r = -0.23 for IC_{50} and TPC, and r = -0.41 for IC_{50} and TFC). This suggests that extracts with higher antioxidant activity (lower IC_{50} values) had higher TPC and TFC values.

Furthermore, *in vitro*-grown callus exhibited higher antioxidant activity (IC_{50}=206.61±0.649 µg/mL) compared to methanolic crude extracts of some wild-grown parts (fruit, stem, and leaf). Based on some reports, the callus has substantial antioxidant properties. According to Sagharyan *et al.* (2020) antioxidants such as phenolic and flavonoid compounds are generated and stored in *in vitro*-grown calli to increase antioxidant activity. Other researchers found that *in vitro*-grown callus has a high potential for scavenging free radicals. In *Habenaria edgeworthii*, callus grown on half MS media enriched with 3.0 µM BAP (6-benzylamino purine) showed considerable phenolic content and increased antioxidant activity (Giri *et al.*, 2012). Likewise, MeOH extracts of both light- and dark-grown calluses from *Inula crithmoides* reduced the DPPH with better antioxidant activity (Bucchini *et al.*, 2013).

4.2.1.2 Total phenolic contents (TPC)

Using linear regression, the calibration curve for gallic acid was derived as y=0.018x and R^2=0.9807 (Fig. 19).

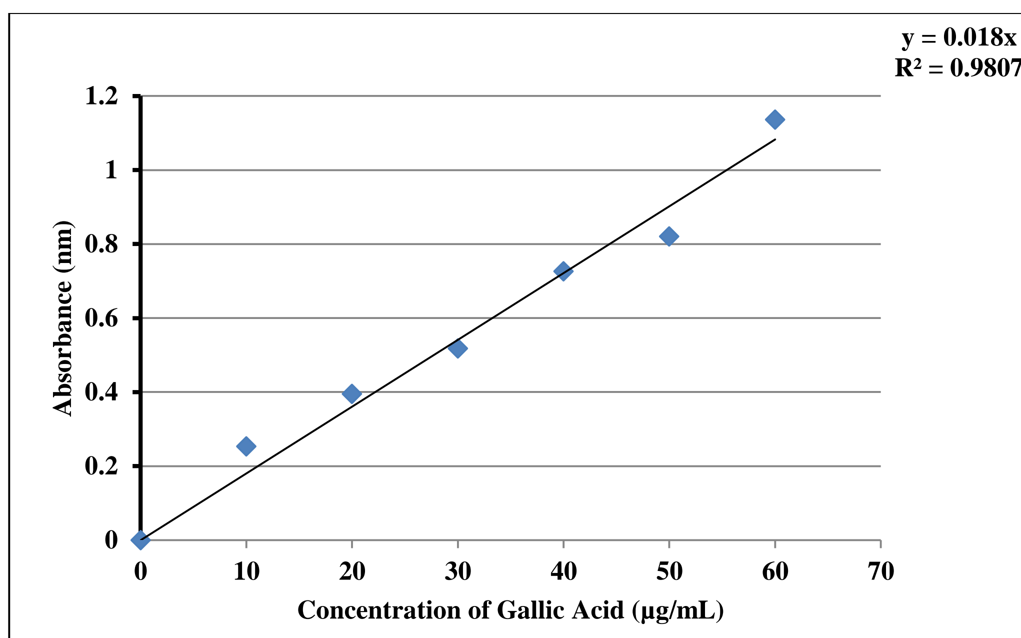


Figure 19: Calibration curve of standard Gallic acid.

Table 20 shows that the root extracts had the highest (137.33±0.120 mg of GAE/g dry wt), whereas the stem had the lowest (94.80±0.231 mg of GAE/g dry wt) TPC values among the tested methanolic crude extracts of *P. longum*, possibly due to the existence of higher no. of phenic compounds. I*n vitro*-grown calli exhibited the second-highest TPC (115.87±0.58 mg of GAE/g dry wt.). Similarly, among the tested fractions, the highest TPC was obtained in the DCM fraction of root (41.22±0.509 mg of GAE/g dry wt.) and the least was found in the methanol fraction of stem (9.59±0.570 mg of GAE/g dry wt.) (Table 20). The substantial free radical scavenging activity of the dichloromethane fraction is probably due to the high concentration of polyphenolics therein, which may have been refined and concentrated during the fractionation process. This could be because phenolic compounds have a higher affinity for dichloromethane than methanol. Phenolic compounds are antioxidants that break down chain reactions. The overall phenolic concentration of crude extracts and fractions has been linked to their ability to scavenge free radicals, with greater phenolic contents indicating better antioxidant activity.

4.2.1.3 Total flavonoid contents (TFC)

Using linear regression, the calibration curve for quercetin was derived as y=0.0256x and R^2=0.9838 (Fig. 20).

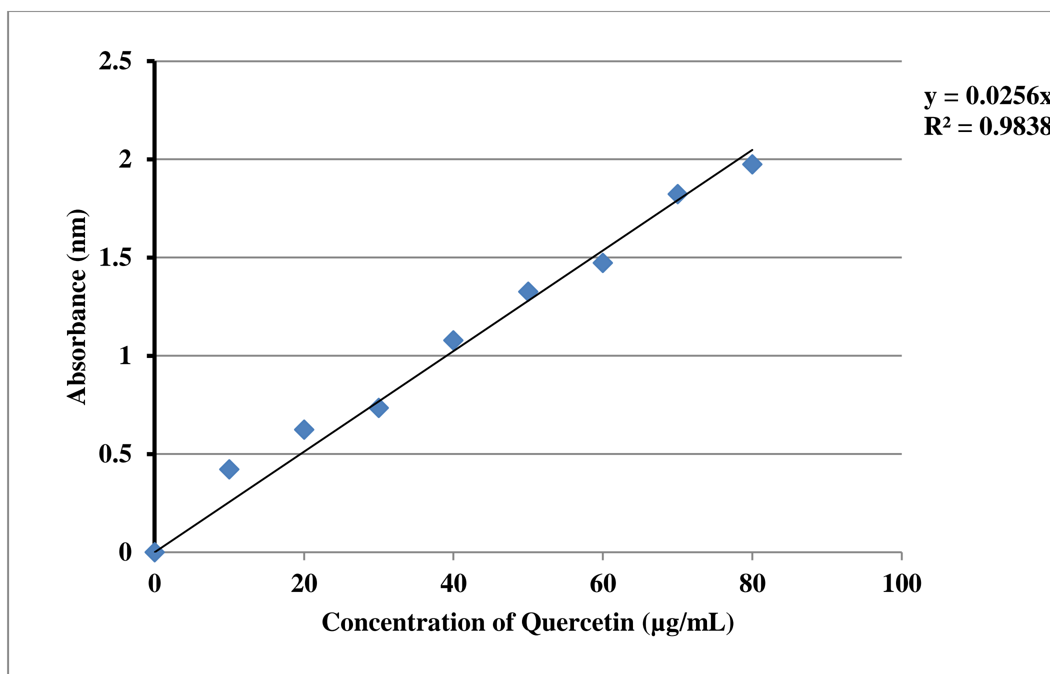


Figure 20: Calibration curve of standard Quercetin.

Among the tested methanolic crude extracts, the root had the highest TPC (113.18±0.532 mg of QE/g dry wt), whereas the stem had the lowest (62.6±0.301 mg of QE/g dry wt), possibly due to its higher antioxidant potential (Table 18). *In vitro*-grown callus had the second-highest TPC (105.81±0.156 mg of QE/g dry wt). Among the tested fractions, the dichloromethane fraction of root had the highest total flavonoid content (73.41±0.531 mg of QE/g dry wt) while the methanol fraction of stem had the lowest (7.10±0.281 mg of GAE/g dry wt). When compared to the methanol fractions, the dichloromethane fractions contained more flavonoids. This could be because flavonoid molecules have a higher affinity for dichloromethane than methanol. This study found that extracts and fractions with a higher total flavonoid content have greater antioxidant effects.

4.2.2 Assessment of the *in vitro* antioxidant activity, TPC, and TFC in *Paris polyphylla*

4.2.2.1 Antioxidant activity by DPPH assay

DPPH is a stable, nitrogen-centered free radical with a violet hue and an odd electron in its structure. Because of its stability in radical state and simplicity in the assay, the antioxidants are accountable for the decrease in DPPH radical absorbance since antioxidant molecules interact with free radicals to diminish or scavenge them by

giving hydrogen or electrons, as evidenced by the gradual fading of the hue from violet to yellow. The absorbance value fell as the plant extract and ascorbic acid concentrations increased.

Methanolic crude extracts of rhizome had a greater free radical scavenging capacity (339.60±0.680 μg/mL) compared to that of crude extract of callus (650.54±0.515 μg/mL) (Table 21). Similarly, the DCM fraction of rhizome had a greater free radical scavenging ability than the methanol fraction, with an IC_{50} of 197.06±0.635 μg/mL in comparison to the ascorbic acid (IC_{50}=7.40±0.875 μg/mL) (Table 21). DCM is an organic solvent used in numerous chemical and industrial processes because of its high volatility and capacity to dissolve wide varieties of non-polar and polar organic compounds, establishing both London-type interactions and dipole-dipole interactions (Rosberg *et al.*, 2006). The synergistic effect of both polar and non-polar molecules may account for the DCM fraction's high antioxidant and other bioactivities. The DCM fraction of *Apium graveolens* had a higher antioxidant activity than ethyl acetate and butanol fractions, and mother liquor (Emad *et al.*, 2022). This research also demonstrated that fractions had a stronger potential to scavenge free radicals than their methanolic crude extracts. This effect could be connected to the total amount of phenol, a chemical family recognized for its antioxidant characteristics. The ethyl acetate fraction, dichloromethane fraction, and n-butanol fraction were all more effective than the crude stem bark extract of *Tabernaemontana catharinensis* (Silveira *et al.*, 2017). It might be brought about by impurities/contaminants in the crude extract, such as carbohydrates, lipids, or a combination of other non-polar substances. Furthermore, DCM fractions were more effective at scavenging free radicals than methanol fractions, possibly due to the accumulation of more strong antioxidant compounds such as phenolic and flavonoids (Table 21). Increasing the extract concentration from 25 to 400 μg/mL increased the percentage of DPPH radical scavenging activity.

Table 20: Antioxidant activity, TPC, and TFC of the rhizome and callus extracts of *P. polyphylla*.

Plant parts	Extract /Fraction	Antioxidant activity (IC_{50}: μg/mL)	Total phenol content (mg of GAE/g dry wt)	Total flavonoid content (mg of QE/g dry wt)
Rhizome	Crude extract	339.60±0.68^{b}	28.38±0.71^{b}	13.07±0.23^{b}
	DCM fraction	197.06±0.63^{c}	43.72±0.61^{a}	26.40±0.61^{a}

	Methanol fraction	317.97±0.73^{b}	30.20±0.81^{ab}	16.81±0.84^{ab}
Callus	Crude extract	650.54±0.51^{a}	6.62±0.23^{b}	25.27±0.37^{b}
Ascorbic acid (Positive control)		7.40±0.875	-	-

Duncan's Multiple Range Test (DMRT) (α=0.05) indicated no significant difference between means in columns with the same letter.

Although the methanolic crude extract of rhizome demonstrated better antioxidant capacity (IC_{50}=339.60±0.680 µg/mL) than the methanolic crude extracts of callus (IC_{50}=650.54±0.515 µg/mL), *in vitro*-raised callus has been found to produce and retain antioxidants such as phenolic compounds. It was found that the callus regenerated from the *P. polyphylla* leaf had produced antioxidants in the culture media. Shrestha and Pant (2011) identified an antioxidant compound, bergenin (trihydroxybenzoic acid glycoside), from an *in vitro*-produced callus of a *Bergenia ciliata* leaf. In *Habenaria edgeworthii*, callus grown on 1/2 MS showed higher antioxidant properties and phenolic content that was cultured on an MS media with 3.0 µM BA (Giri *et al.*, 2012). Methanol extracts of dark-grown and light-grown calli reduced stable DPPH free radicals in *Inula crithmoides* (Bucchinia *et al*., 2013). Using the *in vitro*-raised callus from *P. polyphylla* leaves as an antioxidant and natural medication for disease treatment could help to ensure the long-term conservation of wild plant species.

Previous research found that the capacity and potency of crude rhizome extracts to scavenge free radicals differed between extracts, such as methanol extract (Mayirnao and Bhat, 2017; Lepcha and Chhetri, 2019) and ethanol extract (Devi *et al*., 2018) in *P. polyphylla*. The results of this research differed from those of the previous study, which might be attributed to the impact of solvent purity, extraction with polar or non-polar solvents, the plant's geographical location and habitat, and other environmental factors. In contrast, pure compounds isolated from diverse crude extracts and fractions may show significant antioxidant properties.

Moreover, the correlation between antioxidant activity (IC_{50}) and TPC or TFC was negative (Pearson's correlation coefficient, r = - 0.78 for IC_{50} and TPC, and r = -0.91 for IC_{50} and TFC). This suggests that extracts with higher antioxidant activity (lower IC_{50} values) had higher TPC and TFC values.

4.2.2.2 Total phenolic contents (TPC)

The TPC of the MeOH crude extract and fractions of crude extracts of *P. polyphylla* were analyzed using the FCR reagent in terms of gallic acids equivalent (mg of GAE/g of the dry wt of extract) utilizing the calibration curve of gallic acid (10 µg/mL-60 µg/mL).

The MeOH crude extract of rhizome had a greater total phenolic content (28.38±0.71 mg of GAE/g dry wt) compared to the methanolic crude extract of callus (25.27±0.37 mg of GAE/g dry wt). Similarly, the DCM fraction of the rhizome exhibited greater total phenolic content (43.72±0.61 mg of GAE/g dry wt) compared to the methanol fraction (30.20±0.81 mg of GAE/g dry wt) (Table 21). The high phenolic content in the DCM fraction could be attributed to phenolic purification and concentration during the fractionation process, which is most likely accountable for its substantial antioxidant activity. Furthermore, the DCM fractions of the rhizome contained more phenolics than the methanol fractions. It might be because the phenolic compounds have a stronger affinity for DCM than methanol in *P. polyphylla*. Plants possess phenolic compounds with redox properties that allow them to serve as antioxidants by interrupting chain reactions (Shoib and Shahid, 2015).

4.2.2.3 Total flavonoid contents (TFC)

The Calibration curve (Fig. 20) was produced using Quercetin (10-80 µg/mL) with the line of equation. Utilizing this equation, the flavonoid content of the different extracts of plants was determined.

The methanolic crude extract of rhizome had a greater total flavonoid content (13.07±0.23 mg of QE/g dry wt) compared to the methanolic crude extract of callus (6.62±0.23 mg of QE/g dry wt). Similarly, the DCM fraction of the rhizome had a greater total flavonoid content (26.40±0.61 mg of QE/g dry wt.) compared to the methanol fraction (16.81±0.84 mg of QE/g dry wt). (Table 21). The DCM fractions of rhizome had more flavonoids than the methanol fractions. It might be because the flavonoid compounds have a stronger affinity for DCM than methanol in *P. polyphylla.* The concentration of flavonoids is directly proportional to the degree of light absorption at that wavelength.

4.2.3 Assessment of the *in vitro* antibacterial activities in *P. longum*

The antibacterial activity of crude extracts and fractions was assessed using five different bacterial strains regarding the zone of inhibition (ZoI), relative percentage of inhibition (RPI), minimum inhibitory concentration, and minimum bactericidal concentration as stated below.

4.2.3.1 Zone of inhibition (ZoI) and relative percentage inhibition (RPI)

Methanolic crude extracts of root and fruit the growth inhibition of all (100%) Gram +ve (*S. aureus* and *B. subtilis*) and Gram -ve (*P. aeruginosa, E. coli,* and *A. baumannii*) bacteria at five different concentrations (5, 10, 20, 40, and 60 mg/mL); leaf and callus crude extract inhibited 80% of tested bacteria; and stem crude extract inhibited 60% of tested bacteria (Fig. 21). It had been compared to the antibiotic ciprofloxacin (50 μg/mL), which stopped the growth of all the tested bacterial strains. It could be due to that the crude root and fruit extracts contain a higher concentration of antibacterial compounds. The antibacterial test in the present study utilized two gram-positive and three gram-negative bacteria since their reactions to different extracts and drugs could differ. Gram -ve bacteria, for example, typically have an outer layer containing a lipopolysaccharide component that protects them from antibiotics, making them more resistant to the drugs than Gram +ve bacteria, and Gram -ve bacteria also produce more toxins than Gram +ve bacteria. Moreover, the crude extract of callus grown *in vitro* demonstrated antibacterial activity comparable to that of other wild-grown parts, inhibiting the growth of both Gram +ve (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram -ve (*Pseudomonas aeruginosa*) bacterial strains. The presence of antimicrobial compounds in callus synthesized *in vitro* is determined by the plant type and growth conditions, such as callus age, growth hormone type, and so on. Callus of *Canthium parviflorum* consisted of antimicrobial compounds such as phenol, 4H-pyran-4-one, 1,2-benzene dicarboxylic acid, and octadecanoic acid (Kala and Ammani, 2017). Further testing of the *in vitro*-grown callus of *P. longum* for antibacterial activity against other bacterial strains could produce promising results.

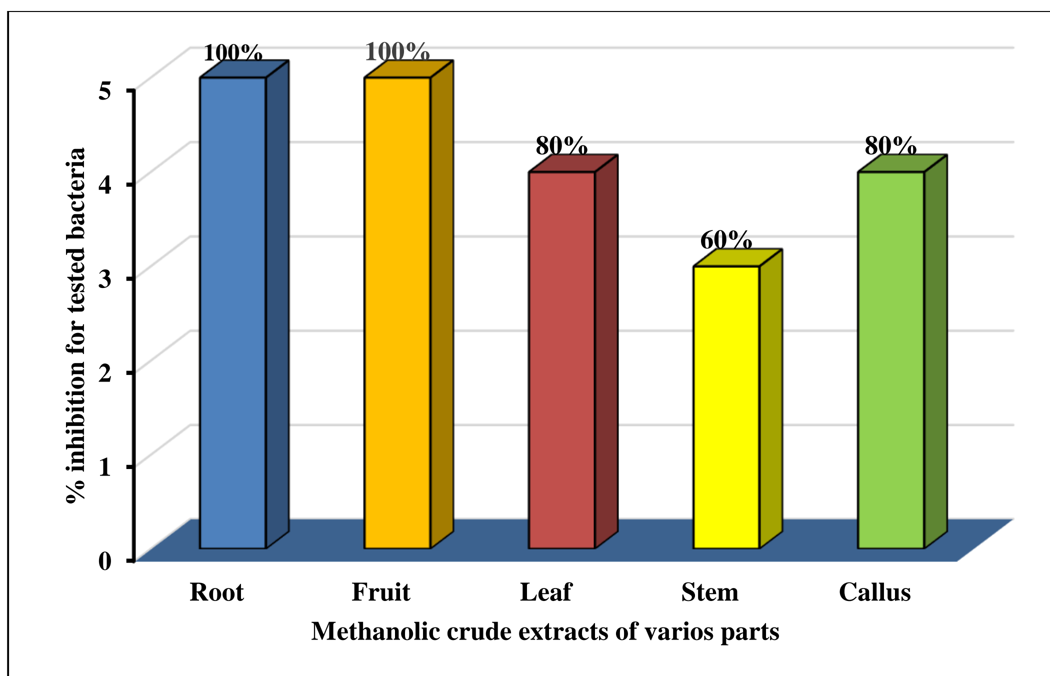


Figure 21: Percentage inhibition by crude extracts of *P. longum* on tested bacterial strains.

Likewise, the DCM fraction of root and fruit prevented the growth of 100% of tested bacterial strains; the DCM fraction of stem inhibited *B. subtilis, E. coli,* and *P. aeruginosa*; the DCM fraction of leaf inhibited *A. baumannii, E. coli,* and *P. aeruginosa*; the methanol fraction of root inhibited *A. baumannii, E. coli,* and *P. aeruginosa*; the methanol fractions of stem inhibited *Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa*; the methanol fraction of leaf inhibited *Escherichia coli,* and *Staphylococcus aureus*; and the methanol fraction of fruit inhibited *A. baumannii, B. subtilis, E. coli,* and *S. aureus* at five different concentrations (5, 10, 20, 40, and 60 mg/mL) (Fig. 22 and Table 22 and 23).

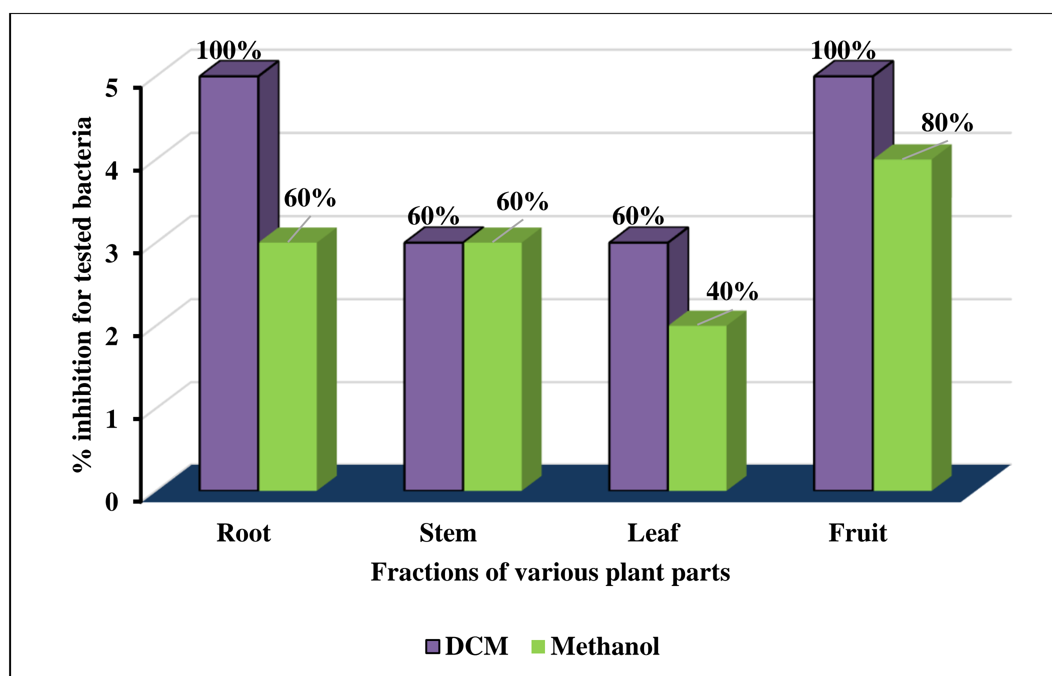


Figure 22: Percentage inhibition by fractions of various parts of *P. longum* on tested bacterial strains.

Gram -ve bacteria had a smaller zone of inhibition than Gram +ve bacteria and were more resistant to the tested extract and fractions because of the presence of their protective outer membrane. We observed that crude extracts and dichloromethane fractions of root and fruits were more effective than other extracts and fractions against the tested bacterial strains, possibly due to the presence of stronger antibacterial compounds. Furthermore, all of the extracts and fractions showed a zone of inhibition (ZoI) and relative percentage inhibition (RPI) that varied in concentration with the bacterial strains tested (Fig. 23).

Table 21: Inhibition zone (ZoI) and relative percentage inhibition (RPI) of methanolic crude extracts *of P. longum* on various bacterial strains.

Sample/ Extract	Test Bacteria	ZOI (Inhibition zone) in mm±SE/RPI (Relative percentage inhibition)				
		5 mg/mL	10 mg/mL	20 mg/mL	40 mg/mL	60 mg/mL
Root (Crude methanol)	**Ab*	2.0±1.52 (8.11%)	4.0±1.00 (16.22%)	6.0±2.00 (24.33%)	7.0±2.08 (28.38%)	9.0±2.64 (36.49%)
	**Bs*	4.0±2.00 (14.11%)	6.0±1.00 (28.17%)	7.0±2.64 (24.7%)	9.0±1.73 (31.76%)	15.0±1.52 (52.94%)
	**Ec*	6.0±2.08 (23.38%)	8.0±2.64 (31.17%)	10.0±1.00 (38.97%)	12.0±2.64 (46.76%)	14.0±1.00 (54.55%)
	**Pa*	3.0±2.00	5.0±1.00	7.0±1.15	10.0±2.64	12.0±1.00

		(10.84%)	(18.07%)	(25.30%)	(36.15%)	(43.38%)
	**Sa*	6.0±1.15 (18.37%)	7.0±1.52 (21.43%)	11.0±2.08 (33.68%)	13.0±1.52 (39.80%)	20.0±2.00 (61.23%)
Stem (Crude methanol)	**Ab*	-	-	-	-	-
	**Bs*	-	-	6.o±2.00 (21.17%)	8.0±1.00 (37.78%)	10.0±0.57 (35.29%)
	**Ec*	5.0±2.64 (19.48%)	6.0±1.52 (30.8%)	9.0±1.00 (35.97%)	10.0±2.00 (38.97%)	13.0±1.0 (50.66%)
	**Pa*	-	4.0±1.73 (14.46%)	6.0±1.00 (21.69%)	9.0±2.00 (32.52%)	11.0±1.52 (39.76%)
	**Sa*	-	-	-	-	-
Leaf (Crude methanol)	**Ab*	-	-	4.0±1.00 (16.22%)	5.0±1.52 (20.64%)	8.0±2.08 (32.44%)
	**Bs*	-	-	-	-	-
	**Ec*	-	6.0±2.00 (23.38%)	9.0±1.73 (35.07%)	11.0±0.57 (42.86%)	13.0±1.00 (50.66%)
	**Pa*	-	-	5.0±1.00 (18.07%)	8.0±0.57 (28.92%)	10.0±2.08 (36.15%)
	**Sa*	-	7.0±2.00 (21.43%)	10.0±0.57 (30.61%)	11.0±1.52 (33.68%)	19.0±2.08 (58.17%)
Fruit (Crude methanol)	**Ab*	1.0±0.57 (4.05%)	3.0±2.00 (12.16%)	5.0±1.00 (20.27%)	6.0±2.08 (24.33%)	8.0±1.52 (32.88%)
	**Bs*	3.0±1.73 (10.58%)	5.0±2.00 (17.64%)	6.0±1.00 (21.17%)	8.0±2.08 (28.23%)	14.0±1.52 (49.41%)
	**Ec*	5.0±2.00 (19.48%)	7.0±1.52 (27.27%)	9.0±1.00 (35.07%)	11.0±0.57 (42.86%)	13.0±1.52 (50.66%)
	**Pa*	2.0±1.00 (7.23%)	4.0±0.57 (14.46%)	6.0±2.08 (21.69%)	9.0±1.73 (32.53%)	11.0±2.00 (39.76%)
	**Sa*	5.0±0.57 (15.30%)	6.0±2.00 (18.37%)	9.0±1.00 (27.55%)	12.0±1.73 (36.74%)	15.0±1.52 (45.92%)
Callus (Crude methanol)	**Ab*	-	-	-	-	-
	**Bs*	-	6.0±2.00 (21.17%)	10.0±0.57 (35.29%)	14.0±1.73 (49.41%)	17.0±1.00 (60.0%)
	**Ec*	-	-	6.0±2.64 (23.38%)	8.0±1.52 (31.17%)	15.0±1.00 (58.45%)
	**Pa*	-	5.0±1.73 (18.07%)	7.0±1.73 (25.3%)	10.0±2.64 (36.15%)	15.0±1.00 (54.22%)

	*Sa	8.0±1.00 (24.49%)	10.0±0.57 (30.61%)	14.0±2.00 (42.86%)	19.0±1.73 (58.17%)	22.0±2.64 (67.30%)
Ciprofloxacin (50 µg/mL)	For positive control: *Ab*= 24.66 mm, *Bs*=28.33 mm, *Ec*=25.66 mm, *Pa*=27.66, *Sa*=32.66 mm					
Absolute Methanol	For negative control: *Ab*= 0.0 mm, *Bs*=0.0 mm, *Ec*=0.0 mm, *Pa*=0.0, *Sa*=0.0 mm					

**Ab=Acinetobacter baumannii, *Ec=E. coli, *Pa=Pseudomonas aeruginosa, *Sa=Staphylococcus aureus*, **Bs=Bacillus subtilis.*

Table 22: Zone of inhibition (ZoI) and relative percentage inhibition (RPI) of fractions of crude extracts of *P. longum* on various bacterial strains.

Sample/ Extract	Test Bacteria	ZoI (Inhibition zone) in mm±SE/% RPI (Relative percentage inhibition)				
		5 mg/mL	10 mg/mL	20 mg/mL	40 mg/mL	60 mg/mL
Root (DCM Fraction)	*Ab	9.0±1.73 (36.49%)	12.0±2.00 (48.66%)	15.0±1.00 (60.82%)	17.0±2.64 (68.93%)	20.0±0.57 (81.1%)
	*Bs	10.0±2.00 (35.29%)	12.0±2.08 (42.35%)	18.0±1.52 (63.53%)	20.0±1.00 (70.59%)	23.0±1.73 (81.18%)
	*Ec	14.0±1.00 (54.55%)	16.0±2.00 (62.35%)	20.0±2.08 (77.94%)	21.0±1.00 (81.83%)	23.0±0.57 (89.63%)
	*Pa	12.0±1.73 (43.38%)	17.0±0.57 (61.46%)	20.0±2.00 (72.3%)	22.0±1.00 (79.53%)	24.0±1.15 (86.76%)
	*Sa	20.0±2.08 (61.23%)	22.0±1.00 (67.36%)	25.0±1.15 (76.54%)	27.0±2.00 (82.66%)	30.0±0.57 (91.85%)
Root (Methanol Fraction)	*Ab	-	8.0±1.00 (32.44%)	11.0±2.00 (44.6%)	13.0±1.15 (52.71%)	15.0±0.57 (60.82%)
	*Bs	-	-	-	-	-
	*Ec	8.0±0.57 (31.17%)	10.0±2.64 (38.97%)	12.0±1.00 (46.76%)	16.0±2.00 (62.35%)	18.0±1.73 (70.14%)
	*Pa	-	8.0±1.52 (28.92%)	11.0±1.00 (39.76%)	16.0±1.73 (57.84%)	18.0±2.00 (65.07%)
	*Sa	-	-	-	-	-
Stem (DCM Fraction)	*Ab	-	-	-	-	-
	*Bs	10.0±2.64 (35.29%)	12.0±2.00 (42.35%)	18.0±1.00 (63.53%)	20.0±2.08 (70.59%)	21.0±1.73 (74.12%)
	*Ec	8.0±2.00 (31.17%)	10.0±0.57 (35.29%)	13.0±1.15 (50.66%)	15.0±1.00 (58.45%)	18.0±2.08 (70.14%)
	*Pa	-	9.0±0.57 (32.53%)	13.0±2.00 (46.99%)	18.0±1.00 (65.07%)	20.0±1.15 (72.3%)
	*Sa	-	-	-	-	-

Stem (Methanol Fraction)	**Ab*	-	-	-	-	-
	**Bs*	5.0±1.15 (17.64%)	8.0±2.00 (28.23%)	12.0±1.00 (42.35%)	17.0±0.57 (60.0%)	19.0±2.08 (67.06%)
	**Ec*	6.0±1.73 (23.38%)	8.0±2.00 (31.17%)	12.0±1.00 (46.76%)	17.0±2.64 (66.25%)	19.0±0.57 (74.04%)
	**Pa*	-	-	9.0±1.00 (32.53%)	15.0±2.00 (54.22%)	18.0±0.57 (65.97%)
	**Sa*	-	-	-	-	-
Leaf (DCM Fraction)	**Ab*	-	5.0±2.00 (20.27%)	12.0±2.08 (48.66%)	19.0±2.64 (77.04%)	21.0±1.00 (85.15%)
	**Bs*	-	-	-	-	-
	**Ec*	6.0±1.15 (23.38%)	8.0±1.00 (31.17%)	13.0±1.73 (50.66%)	14.0±2.00 (54.55%)	16.0±2.64 (62.35%)
	**Pa*	8.0±1.00 (28.92%)	9.0±2.00 (32.53%)	15.0±1.00 (54.22%)	17.0±0.57 (61.46%)	19.0±2.08 (68.69%)
	**Sa*	-	-	-	-	-
Leaf (Methanol Fraction)	**Ab*	-	-	-	-	-
	**Bs*	-	-	-	-	-
	**Ec*	5.0±1.73 (19.48%)	7.0±1.15 (27.27%)	12.0±1.00 (46.76%)	13.0±1.52 (50.66%)	15.0±1.00 (58.45%)
	**Pa*	-	-	-	-	-
	**Sa*	7.0±2.08 (25.20%)	9.0±2.64 (32.53%)	13.0±1.00 (46.99%)	18.0±2.00 (65.07%)	20.0±1.73 (61.23%)
Fruit (DCM Fraction)	**Ab*	7.0±2.00 (28.38%)	9.0±1.00 (36.49%)	12.0±1.15 (48.66%)	15.0±1.00 (60.82%)	18.0±1.73 (72.99%)
	**Bs*	10.0±1.00 (35.29%)	12.0±1.15 (42.35%)	16.0±2.00 (56.47%)	18.0±1.52 (63.53%)	20.0±1.73 (70.59%)
	**Ec*	10.0±1.00 (38.97%)	16.0±2.00 (62.35%)	20.0±0.57 (77.94%)	22.0±2.08 (85.73%)	23.0±1.15 (89.63%)
	**Pa*	12.0±1.15 (43.38%)	15.0±1.00 (54.22%)	17.0±0.57 (61.46%)	20.0±2.08 (72.3%)	22.0±2.00 (79.53%)
	**Sa*	13.0±1.00 (39.8%)	16.0±2.00 (48.98%)	20.0±1.00 (61.23%)	25.0±2.08 (76.54%)	27.0±1.73 (82.66%)
Fruit (Methanol Fraction)	**Ab*	-	-	-	-	-
	**Bs*	5.0±2.00 (17.64%)	10.0±1.00 (35.29%)	12.0±0.57 (42.35%)	18.0±1.15 (63.53%)	20.0±2.08 (70.59%)
	**Ec*	5.0±1.00 (19.48)	10.0±1.15 (38.97%)	12.0±1.00 (46.76%)	16.0±2.64 (62.35%)	20.0±1.73 (77.94%)
	**Pa*	-	-	-	-	-

	*Sa	14.0±0.57 (42.86%)	17.0±1.00 (52.05%)	20.0±2.00 (61.23%)	25.0±1.73 (76.54%)	27.0±2.64 (82.66%)
Ciprofloxacin (50 µg/mL)	For positive control: *Ab*= 22.66 mm, *Bs*=26.33 mm, *Ec*=24.66 mm, *Pa*=28.66, *Sa*=36.66 mm					
Absolute Methanol	For negative control: *Ab*= 0.0 mm, *Bs*=0.0 mm, *Ec*=0.0 mm, *Pa*=0.0, *Sa*=0.0 mm					

**Ab=Acinetobacter baumannii, *Ec=E. coli, *Pa=Pseudomonas aeruginosa, *Sa=Staphylococcus aureus*, **Bs=Bacillus subtilis*

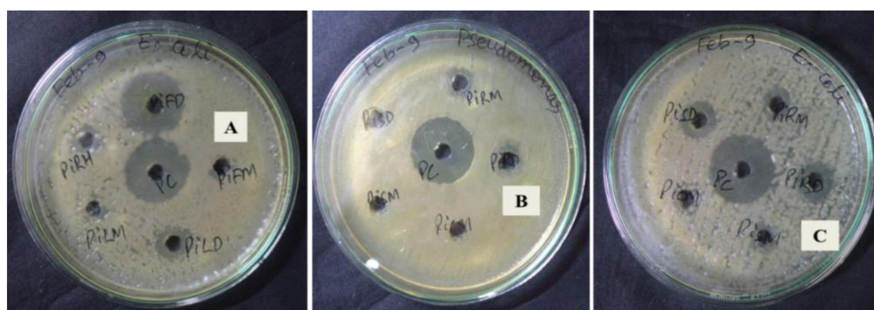


Figure 23: Zone of Inhibition by various fractions of *P. longum*:

(A) on *E. coli* at 20 mg/mL, (B) on *P. aeruginosa* at 5 mg/mL, (C) on *E. coli* at 10 mg/mL.

Note: (PiRM=Methanol fraction Root, PiRD=DCM fraction Root, PiSM=Methanol fraction Stem, PiSD=DCM fraction Stem, PiFD=DCM fraction Fruit, PiFM= Methanol fraction Fruit, PiLD= DCM fraction Leaf, PiLM= Methanol fraction Leaf, PiCM=Crude Methanol extract Callus, PC=Positive control).

This study found that crude extracts and DCM fractions of *P. longum* roots and fruits might be utilized for treating a variety of diseases and illnesses brought on by *P. aeruginosa, A. baumannii, E. coli, B. subtilis*, and *S. aureus*, since the extracts and fractions inhibited the bacterial growth *in vitro*. *A. baumannii* is a gram-negative, multidrug-resistant bacteria that causes wound infections, pneumonia, bacteremia, and urinary tract infections. Likewise, *E. coli* results in sepsis, infantile stomach flu, traveler's diarrhea, and gastrointestinal bleeding; *P. aeruginosa* results in pneumonia, infections in the blood, or other body parts after surgery; *S. aureus* results in skin infections and, in some cases, pneumonia, heart inflammation, and bone infection; and *B. subtilis* may ruin food and is regarded as an opportunist pathogen among people with weakened immune systems. However, before these extracts and fractions may be employed as natural medicines or antibacterial agents for diverse bacterial illnesses, *in vivo* testing is required to validate them.

4.2.3.2 Minimum inhibitory concentration and minimum bactericidal concentration

All MeOH crude extracts (excluding the crude callus extract) were not tested for MIC and MBC since they had narrower zones of inhibition and a lower relative percentage inhibition than their fractions on the investigated bacterial strains. Thus, only the methanolic crude extract of *in vitro*-grown callus and all fractions of wild-grown parts were tested for MBC and MIC on the three Gram-negative and two Gram-positive bacteria. The DCM fraction of root was found to have a minimal MIC (at 5.0 mg/mL) and MBC (at 8.35 mg/mL) against *S. aureus,* a gram-positive bacterium. It might be attributed to the increased amounts of antibacterial compounds in DCM compared to other fractions and crude extract of *in vitro*-grown callus (Fig. 24 and Table 24). However, pure compounds extracted from *P. longum* might have greater antibacterial activity. Lokhande *et al.* (2007) found that the isolated components (piperin) from the root extract of *P. longum* had higher antibacterial activity (MIC=12.5 mg/mL) against *Bacillus cereus* and *E. coli* than crude extracts. These values appeared to be greater than in the present research, which could be attributable to piperine purity, culture conditions, and the types of bacterial strains used in that study.

Table 23: MIC and MBC values of various fractions and crude callus extract in *P. longum.*

Plant parts	**Extract/ Fraction**	**Values of MIC/and MBC (mg/mL)**									
		Ab		***Bs***		***Ec***		***Pa***		***Sa***	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Root	DCM	25	27.65	15	18.55	10	12.45	10	13.25	5	8.35
	Methanol	40	42.65	-	-	35	37.55	30	31.55	-	-
Fruit	DCM	25	28.45	15	17.25	10	13.85	20	24.35	30	32.25
	Methanol	-	-	40	41.75	35	36.95	-	-	20	22.65
Stem	DCM	-	-	20	21.55	25	26.85	40	43.35	-	-
	Methanol	-	-	40	42.95	25	27.35	50	53.55	-	-
Leaf	DCM	30	32.55	-	-	20	22.15	25	26.25	15	16.65
	Methanol	-	-	-	-	20	23.35	-	-	40	42.25
Callus	Crude methanol	-	-	15	16.85	30	32.75	25	26.55	10	13.55

**Ab=Acinetobacter baumannii, *Ec=E. coli, *Pa=Pseudomonas aeruginosa, *Sa=Staphylococcus aureus*, **Bs=Bacillus subtilis*

Furthermore, the methanolic crude extract of *in vitro*-grown callus had the lowest minimum inhibitory concentration (10 mg/mL) and minimum bactericidal concentration (13.5 mg/mL) in *S. aureus* (Gram-positive), which could be attributed to the existence of substantial antibacterial compounds in the callus and the more susceptible nature of the bacterial strain. Adhikari *et al.* (2013) observed that compounds isolated from the *in vitro*-grown callus of *Withania somnifera* were more effective than those isolated from the wild-grown roots in inhibiting the growth of *Klebsiella pneumonia, Proteus vulgaris*, and *Pseudomonas aeruginosa*, whereas extract of wild grown roots was more effective on *Salmonella typhimurium, Bacillus subtilis, Escherichia coli*, and *Staphylococcus aureus.* As a result, the *in vitro*-grown callus of *P. longum* may include a variety of antibacterial compounds that require further investigation.

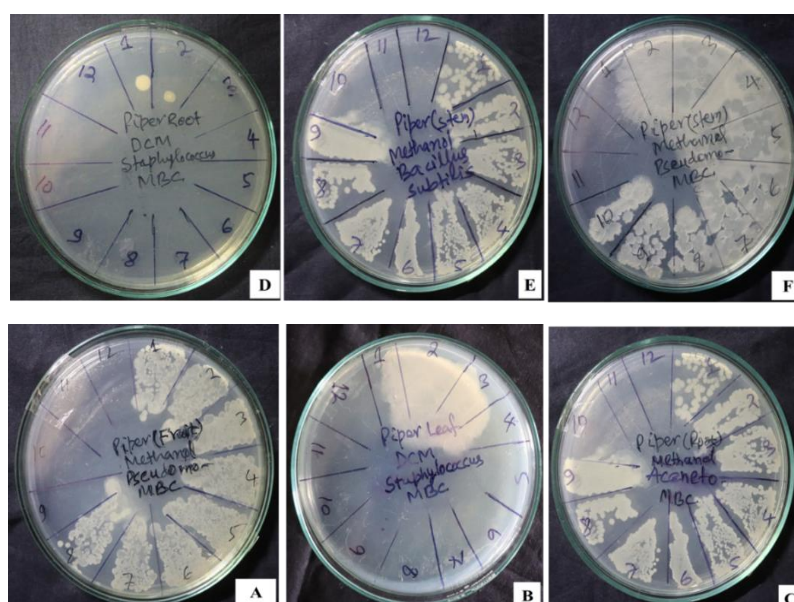


Figure 24: Minimum Bactericidal Concentration (MBC) of *P. longum*:

(A) on *P. aeruginosa* by methanol fraction of fruit, (B) on *S. aureus* by DCM fraction of leaf, (C) on *A. baumannii* by methanol fraction of root, (D) on *S. aureus* by DCM fraction of root, (E) on *B. subtilis* by methanol fraction of stem, (F) on *P. aeruginosa* by methanol fraction of stem.

[Note: Numbers 1-12 on MHA plates represent various concentrations of extracts starting from MIC].

The minimum bactericidal concentration (MBC) is an additional verification test for the MIC. The MIC and MBC values vary depending on the extraction solvent used, the plant part analyzed, and the microorganisms examined. Barua *et al.* (2014) found that chloroform, hexane, ethanol, and ethyl acetate extracts of *P. longum* seeds showed

MBC values of 20.23, 33.43, 36.23, and 64.09 mg/mL respectively to *Mycobacterium smegmatis*. Furthermore, the present investigation demonstrated that all *P. longum* extracts were bacteriostatic against the investigated bacterial strains, with MBC values greater than MIC values. Bactericidal agents kill 99.99% of bacteria, whereas bacteriostatic agents inhibit bacterial growth and reproduction but do not kill them completely.

4.2.4 Assessment of the *in vitro* antibacterial activity in *P. polyphylla*

The antibacterial property of crude extracts and fractions of *P. polyphylla* was assessed using five different bacterial strains (such as *P. aeruginosa, A. baumannii, E. coli, B. subtilis*, and *S. aureus*) concerning the zone of inhibition (ZoI), relative percentage of inhibition (RPI), minimum inhibitory concentration, and minimum bactericidal concentration as stated below.

4.2.4.1 Zone of inhibition (ZoI) and relative percentage inhibition (RPI)

Methanolic crude extracts of rhizome and callus prevented the growth of 60% of tested bacteria at five different concentrations (5, 10, 20, 40, and 60 mg/mL). Likewise, the DCM fraction of rhizome prevented the growth of all tested bacterial strains (100%), but the methanol fraction prevented the growth of 80% of the bacteria tested at five different concentrations (Fig. 25 and Table 25). A comparison was made to the antibiotic/drug ciprofloxacin (50 µg/mL). The DCM fraction of rhizome was more powerful than the methanol fraction and other crude extracts over the tested bacteria, possibly owing to the existence of more potent antibacterial molecules. The DCM fraction of the rhizome also prevented the multidrug-resistant *A. baumannii*, which causes pneumonia, bacteremia, urinary tract infection, and wound infection. DCM fraction of *Premna resinosa* demonstrated superior antibacterial properties towards Methicillin-resistant *Staphylococcus aureus*, with a minimum inhibitory concentration of 31.25 µg/mL (Njeru *et al*., 2015). According to experiments on antiprotozoal properties, DCM fractions of plants were shown to be more effective in ethnopharmacological research than other fractions and crude extracts (Ramadani *et al*., 2018).

Furthermore, the antibacterial activity of *in vitro*-raised callus was comparable to that of *in vivo*-grown rhizome, inhibiting the growth of one Gram +ve (*B. subtilis*) and two Gram -ve bacteria (*P. aeruginosa* and *E. coli*). The callus may have produced

antibacterial substances in MS media that stopped *B. subtilis, P. aeruginosa*, and *E. coli* from growing in MHA media, which might be used for sustaining wild-grown *P. polyphylla* rhizomes for long-term use. All of the extracts demonstrated a concentration-dependent zone of inhibition (ZoI) (Fig. 26 and 34) and relative percentage inhibition against the examined microorganisms. At 5 mg/mL, the DCM fraction of the rhizome demonstrated a relative percentage inhibition of 81.10%, which reached 97.32% at 60 mg/mL in *E. coli*. The lower intestines of Warm-blooded species typically include the rod-shaped *E. coli*, a gram-negative, facultatively anaerobic coliform bacterium. Some *E. coli* strains are capable of causing diarrhea, stomachaches, and a low-grade fever, and *P. polyphylla* rhizome and *in vitro*-raised callus may be utilized as a natural drug for *E. coli* infections. In the field of infectious disease, 82 (58%) of the 141 small compounds approved as antibacterial agents between 1981 and 2014 were derived from biological sources (Newman and Cragg, 2016). The maximum antibacterial property was found in *E. coli* where the inhibition zone was >31 mm, and the relative percentage inhibition of the methanol extracts of rhizome in *E. coli* and *S. aureus* was 95.58% at 5 mg/mL (Mayirnao and Bhat, 2017). The season in which rhizomes were harvested, the habitat and environment in which they grew, the solvents used for making the extract, the types of bacterial strains used, and other variables may have contributed to the differences between the results of this investigation and the prior study.

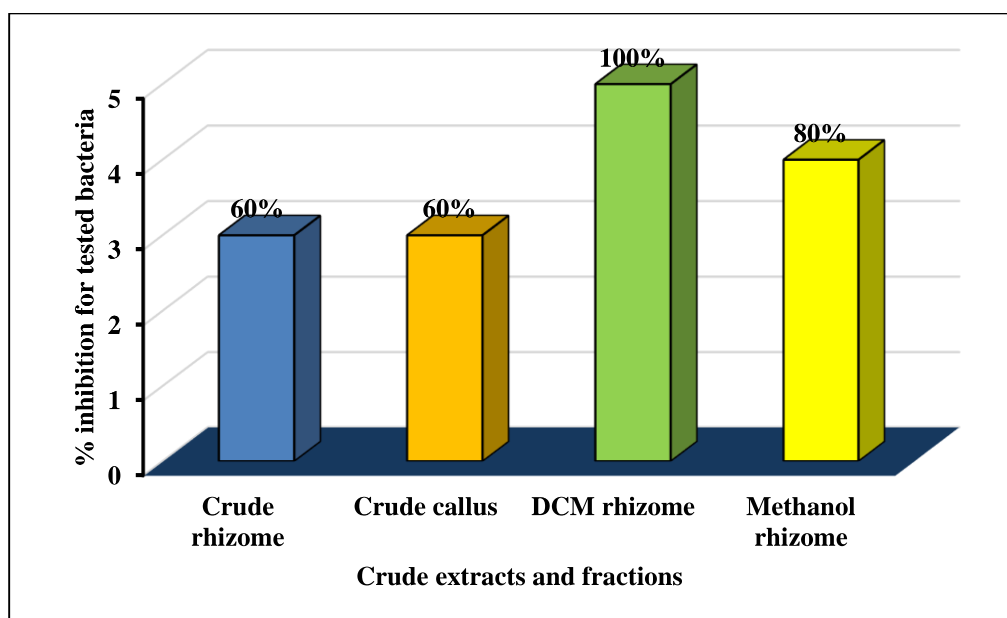


Figure 25: Percentage inhibition by crude extracts and fractions for five bacterial strains in *P. polyphylla*.

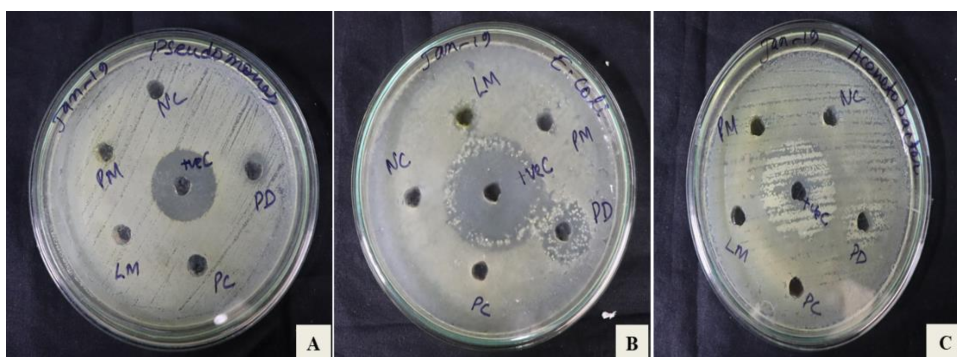


Figure 26: Zone of Inhibition (ZoI) shown by rhizome and callus of *P. polyphylla* at 10 mg/mL:

a) ZoI on *Pseudomonas aeruginosa*; b) ZoI on *E. coli*; c) ZoI on *Acinetobacter baumannii*.

Abbreviations: Methanol fraction (PM), Dichloromethane fraction (PD), Callus extract (PC), Positive control (+veC), and Negative control (NC).

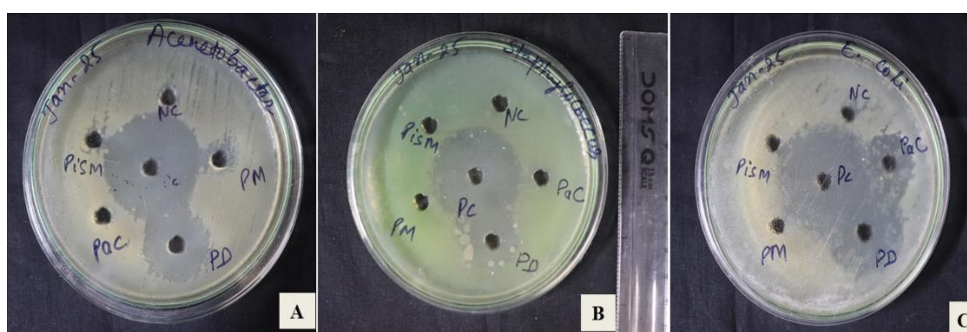


Figure 27: Zone of Inhibition (ZoI) shown by rhizome and callus of *P. polyphylla* at 60 mg/mL:

a) ZoI on *Acinetobacter baumannii*; b) ZoI on *Staphylococcus aureus*; c) ZoI on *E. coli*.

Abbreviations: Methanol fraction (PM), Dichloromethane fraction (PD), Callus extract (PaC), Positive control (PC), and Negative control (NC).

Table 24: Zone of inhibition (ZoI) and relative percentage inhibition (RPI) shown by rhizome and callus extracts *of P. polyphylla* on different bacteria.

Sample/ Extract	Test bacteria	Zone of inhibition (ZoI) in mm±SE/and Relative percentage inhibition				
		5 mg/mL	**10 mg/mL**	**20 mg/mL**	**40 mg/mL**	**60 mg/mL**
RCE	*Acinetobacter baumannii*	-	-	-	-	-
	Bacillus subtilis	-	-	-	-	-
	Escherichia coli	5.0±2.64 (20.27%)	7.0±2.00 (28.38%)	15.0±1.00 (60.82%)	18.0±2.00 (72.99%)	20.3±1.52 (81.10%)
	Pseudomonas aeruginosa	6.0±1.00 (23.38%)	8.33±1.52 (31.17%)	13.66±1.5 (54.55%)	17.0±3.00 (66.25%)	19.0±2.00 (74.04%)

	Staphylococcus aureus	7.33±2.08 (22.83%)	10.0±1.0 (32.61%)	15.0±2.64 (48.92%)	18.6±1.52 (61.96%)	21.0±2.00 (68.49%)
CCE	*Acinetobacter baumannii*	-	-	-	-	-
	Bacillus subtilis	4.66±1.52 (17.04%)	7.0±1.00 (23.86%)	15.0±2.64 (51.14%)	17.0±1.00 (57.96%)	18.0±2.64 (61.37%
	Escherichia coli	-	7.0±2.64 (28.38%)	13.0±2.00 (52.71%)	15.3±2.08 (60.82%)	19.0±3.00 (77.04%)
	Pseudomonas aeruginosa	6.0±1.00 (23.38%)	8.33±2.08 (31.17%)	15.6±2.08 (.55%)	18.0±2.00 (70.14%)	19.0±2.00 (74.04%)
	Staphylococcus aureus	-	-	-	-	-
DF	*Acinetobacter baumannii*	7.0±2.64 (29.58%)	9.66±1.52 (42.26%)	17.0±1.00 (71.85%)	19.0±2.64 (80.30%)	21.0±2.00 (88.75%)
	Bacillus subtilis	7.0±2.00 (23.86%)	15.0±1.00 (51.14%)	18.3±1.52 (61.37%)	20.0±3.00 (68.18%)	22.0±2.64 (75.0%)
	Escherichia coli	20.33±1.52 (81.10%)	21.0±2.00 (85.15%)	22.0±1.00 (89.21%)	22.6±1.2 (93.26%)	24.0±2.00 (97.32%)
	Pseudomonas aeruginosa	14.0±1.00 (54.55%)	19.0±3.00 (74.04%)	20.0±2.64 (77.94%)	22.0±2.00 (85.73%)	22.6±2.08 (89.63%)
	Staphylococcus aureus	18.66±2.08 (61.96%)	21.0±2.64 (68.49%)	22.0±2.00 (71.75%)	23.0±1.00 (75.01%)	25.0±1.00 (81.53%)
MF	*Acinetobacter baumannii*	-	-	-	-	-
	Bacillus subtilis	-	6.66±2.08 (23.86%)	14.0±3.00 (47.73%)	16.0±2.00 (54.55%)	19.0±1.00 (64.78%
	Escherichia coli	-	7.0±1.00 (28.38%)	15.3±2.08 (60.82%)	17.0±2.64 (68.93%)	19.0±2.00 (77.04%)
	Pseudomonas aeruginosa	8.0±2.64 (31.17%)	16.0±2.00 (62.35%)	18.0±1.00 (70.14%)	19.3±2.08 (74.04%)	21.0±3.00 (81.83%)
	Staphylococcus aureus	17.0±1.00 (54.44%)	20.33±1.5 (65.23%)	22.02.00 (71.75%)	23.0±1.00 (75.01%)	24.3±1.52 (78.27%)
Ciprofloxacin (50 µg/mL)	For positive control: *Acinetobacter baumannii*=23.66 mm, *Bacillus subtilis*=29.33 mm, *Escherichia coli*=24.66 mm, *Pseudomonas aeruginosa*=25.66, *Staphylococcus aureus*=30.66 mm					
Absolute Methanol	For negative control: *Acinetobacter baumannii*=0.0 mm, *Bacillus subtilis*=0.0 mm, *Escherichia coli*=0.0 mm, *Pseudomonas aeruginosa* =0.0, *Staphylococcus aureus*=0.0 mm					

Abbreviations: RCE=Rhizome Crude Extract, CCE=Callus Crude Extract, DF=Dichloromethane Fraction, MF= Methanol Fraction

4.2.4.2 Minimum bactericidal concentration (MBC) of callus and rhizome

All methanolic crude extracts (rhizome and callus) and fractions of rhizome were tested for MIC and MBC on two Gram +ve (*S. aureus* and *B. subtilis*) and three Gram -ve bacteria (*A. baumannii, E. coli*, and *P. aeruginosa*). The DCM fraction of rhizome had the lowest minimum inhibitory concentration at 5 mg/mL and minimum bactericidal concentration at 7.5 mg/mL on *E. coli* (Fig. 28 and Table 26).

Table 25: MIC and MBC values of various extracts of rhizome and callus in *P. polyphylla.*

Plant parts	Extract/ Fraction	Values of MIC/and MBC (mg/mL)									
		***Ab**		***Bs**		***Ec**		***Pa**		***Sa**	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Rhizome	Crude	-	-	-		40	42.25	25	28.25	30	33.25
	DCM	30	33.5	20	24.5	5	7.5	10	13.25	15	17.25
	Methano l	-	-	35	38.75	30	34.75	20	22.75	15	18.5
Callus	Crude	-	-	25	26.5	20	22.75	40	44.25	-	-

*Ab=*Acinetobacter baumannii,* *Bs=*Bacillus subtilis,* *Ec=*Escheriachia coli,* *Pa=*Pseudomonas aeruginosa,* and *Sa=*Staphylococcus aureus.*

Furthermore, the MeOH crude extract of *in vitro* callus inhibited the growth of *B. subtilis, E. coli*, and *P. aeruginosa*, with *B. subtilis* having the lowest MIC and MBC at 25 mg/mL and 26.5 mg/mL, respectively. Although the minimum inhibitory concentration and minimum bactericidal concentration of callus extract were greater than expected, this could be crucial for future researchers to investigate it for other useful bacteria. Elicitors/precursors in cell suspension culture might be utilized to raise the amount of antibacterial compound generated in calli in a shorter period, widening the use of callus in the pharmaceutical business. According to Adhikari *et al*. (2013) compounds obtained in the callus of *Withania somnifera* were more effective than those obtained in the natural root in preventing the growth of *P. aeruginosa, Proteus vulgaris*, and *Klebsiella pneumonia*, whereas compounds obtained in the natural root prevented the growth of *E. coli, S. aureus, B. subtilis*, and *Salmonella typhimurium*.

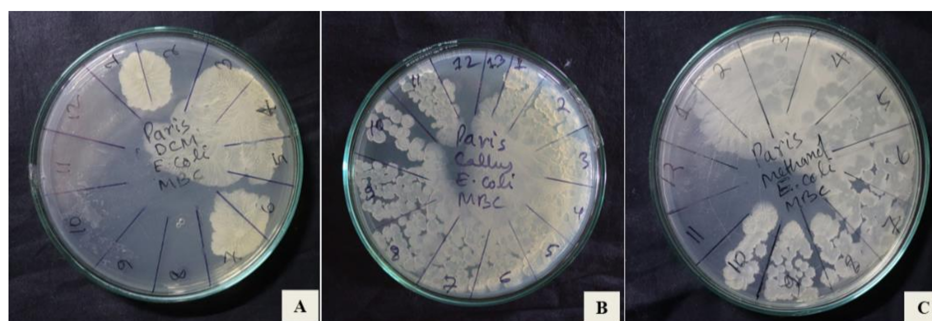


Figure 28: Minimum bactericidal concentration (MBC) shown by rhizome and callus extracts in *P. polyphylla***:**

a) MBC of Dichloromethane (DCM) fraction on *E. coli*; b) MBC of Callus extract on *E. coli*; c) MBC of Methanol fraction on *E. coli*.

Note: (1 to 13 numbers on Mueller Hinton Agar (MHA) petri plate represent various extract concentrations greater than the MIC, starting with the MIC of the particular extract on tested bacteria).

This investigation showed that the majority of the extracts and fractions performed as bacteriostatic agents against the tested bacterial strains with a lower MIC than the MBC value. Bacteriostatic drugs inhibit bacterial growth, while bactericidal agents kill bacteria (Bernatová *et al.,* 2003). There have been no reports of *P. polyphylla* callus exhibiting antibacterial activities when grown *in vitro*. However, prior studies on *P. polyphylla*, particularly *in vivo* parts, demonstrated antibacterial activity against a variety of bacterial strains. Total steroidal saponins of above-ground parts (TSSAPs) and total steroidal saponins of rhizomes (TSSRs) isolated from ethanol extract exhibited antibacterial properties on *E. coli* (Qin *et al.,* 2018). The MIC and MBC vary depending on the solvent used for extraction, the extraction technique, the plant part tested, soil or climate, post-harvest treatment or collection, and the microorganisms tested (Joshi *et al*., 2020). The principal ingredient accountable for the antibacterial efficacy is steroidal saponins, which are mainly included in the rhizomes (Qin *et al.,* 2012). A significant quantity of phenolics and flavonoids have been found in the plant species, which might contribute to its antibacterial properties (Mayirnao and Bhat, 2017)

4.2.5 Assessment of the cytotoxic activity in *P. longum*

The cytotoxic property of crude extracts and fractions of *P. longum* was determined using brine shrimp nauplii (*in vivo*) and various kinds of human carcinoma cells (*in vitro*), which are described below.

4.2.5.1 Assessment of the *in vivo* cytotoxic activity utilizing brine shrimp lethality bioassay in *P. longum*

The capacity to kill lab-cultured larvae (nauplii) makes the brine shrimp lethality bioassay a crucial instrument for the first-stage cytotoxicity testing of plant extracts. All the methanolic crude extracts of wild-grown parts and *in vitro*-grown callus, and most of the fractions, were found to be active against the brine shrimp larvae (Table 27). However, the methanol fractions of leaves and stems were found to be inactive against the nauplii. Greater LC_{50} denote reduced toxicity, whereas lesser LC_{50} denote greater toxicity. The most active crude extracts were those of roots with 50% lethal concentration (LC_{50}) of 156.78±5.49 μg/mL, followed by fruits (LC_{50}=180.59±14.17 μg/mL). Similarly, the most active fractions were those of root DCM (LC_{50}=71.87±5.58 μg/mL), followed by fruit DCM (LC_{50}=104.59±11.45 μg/mL), stem DCM (LC_{50}=126.98±4.66 μg/mL), and leaf DCM (LC_{50}=181.33±5.41 μg/mL). It was found that the lethality increased with extract concentration. The potassium dichromate ($K_2Cr_2O_7$), an inorganic chemical reagent that causes cancer in humans, was used as a positive control to compare the lethality of crude extract and its fractions. More cytotoxic activity was shown by DCM fractions of the root, fruit, and stem extracts on nauplii than by positive control or potassium dichromate (LC_{50}=134.34±15.66 μg/mL). Moreover, the DCM fraction of root was found to have the greatest cytotoxic effect on nauplii; this could be because it contains a higher concentration of cytotoxic compounds like piperine. In this study, an HRMS evaluation of the DCM fraction of the root showed the existence of piperine. Piperine exhibited a stronger cytotoxic activity in brine shrimp nauplii (LC_{50}: 2.8 μg/mL, Bazerra *et al.*, 2005). For bioactive compounds or pharmacologically active plant extracts, LC_{50} values generally fall between 10 and 1,000 μg/mL (Meyer *et al.*, 1982). However, the responsiveness of the shrimp species used, the experimental setup, and the kind of substance being evaluated are some of the variables that can affect the effective range of LC_{50} values in the brine shrimp assay. Padmaja *et al.* (2002) reported that the EtOH extracts of *P. longum* fruit had the highest toxicity on brine shrimp nauplii (LC_{50} of 6.9 μg/mL) compared to the other five plants, and the compound piperine had an LC_{50} of 2.4 μg/mL. Similarly, Shrestha (2018) also showed the toxicity of essential oil of *P. longum* fruit (LC_{50} of 251.1 μg/mL) on brine shrimp nauplii.

The cytotoxicity of *in vitro*-grown callus extract (LC_{50}=602.46±6.50 µg/mL) was comparable to those of *in vivo* parts of *P. longum*, which may be helpful for further research into the anticancer activity for the development of natural medicines for a variety of illnesses utilizing both callus and wild-grown parts of *P. longum*.

Table 26: Cytotoxic activity of crude extracts and fractions of *P. longum* on brine shrimp nauplii.

S.N.	Plant parts	Extracts/Fractions	50% lethal concentration (LC_{50}) µg/mL	Used concentrations (µg/mL)
1.	Leaf	Crude	365.48±4.75^{b}	156-1250 µg/mL
		DCM	181.33±5.41^{c}	78-625 µg/mL
		Methanol	1372.22±7.12^{a}	312-2500 µg/mL
2.	Stem	Crude	210.51±7.67^{b}	78-625 µg/mL
		DCM	126.98±4.66^{c}	78-625 µg/mL
		Methanol	1012.12±5.75^{a}	312-2500 µg/mL
3.	Root	Crude	156.78±5.49^{b}	62-500 µg/mL
		DCM	71.87±5.58^{c}	23-187 µg/mL
		Methanol	406.89±10.72^{a}	93-750 µg/mL
4.	Fruit	Crude	180.59±14.17^{b}	78-625 µg/mL
		DCM	104.59±11.45^{c}	23-167 µg/mL
		Methanol	463.58±11.05^{a}	156-1250 µg/mL
5.	Callus	Crude	602.46±6.50	156-1250 µg/mL
6.	Potassium control)	dichromate (Positive	(LC_{50}=134.34±15.66 µg/mL)	50-200 µg/mL

Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

4.2.5.2 Cytotoxic activity of *in vitro* callus and various wild-grown parts of *P. longum* in U-2 OS, HeLa, and normal cell lines by MTT assay

In vitro, all of the crude extracts and fractions showed anticancer activity by preventing the growth and division of HeLa (cervical carcinoma cells) and U-2 OS (bone carcinoma cells) cells. For determining cell metabolic activity and growth, the MTT assay is a popular colorimetric method. The yellow MTT tetrazolium salt is transformed into purple formazan crystals by mitochondrial enzymes in living cells, which forms the basis of the MTT test.

Cytotoxic activity of *in vitro* calli and wild-grown parts of *P. longum* in U-2 OS cell line

Table 28 reveals that the crude extracts of *in vivo* parts exhibited the highest anticancer activity with a higher inhibition percentage, followed by methanol and DCM fractions

in U-2 OS cell lines. The anticancer property of all the extracts was carried out comparison with the anticancer drug cisplatin (IC_{50}=28.43±1.94 µg/mL in U-2 OS cells and IC_{50}=24.56±0.775 µg/mL in HeLa cells). Among all the crude extracts, the crude extract of fruit exhibited the highest anticancer activity with 79.46% inhibition at 400 µg/mL, followed by the crude extract of root with 66.46% inhibition, and *in vitro*-grown callus with 56.30% inhibition, at 400 µg/mL on U-2 OS cells (Fig. 29). The least anticancer property was showed by the crude extracts of leaf with 46.33% inhibition at 400 µg/mL. Sawhney *et al*. (2011) reported that the MeOH crude extract was more potent in preventing the proliferation of HCC-827 cell lines than EtOAc and water extracts. Similarly, Benerjee *et al.* (2017) found that the hot methanolic extracts of *in vivo*-grown leaves of *P. longum* showed better cytotoxic activity in the leukemic carcinoma cells (K562) than *in vitro*-grown callus extracts. However, Sharma *et al.* (2014) observed that hexane extracts of *P. longum* fruit showed 90–92% toxicity to the majority of the tested cell lines, such as prostrate (DU-145), lung (A549), leukemia (THP-1), ovary (IGR-OVI-1), and breast (MCF-7) cancer cells.

Among all the fractions, the methanol fraction of fruit exhibited the highest anticancer activity with 65.46% inhibition at 400 µg/mL, followed by the methanol fraction of root with 53.50% inhibition at 400 µg/mL than DCM fractions. The reason for this could be that the concentration of anticancer compounds in crude extracts is higher than in fractions, and could be owing to the synergistic effects of several compounds present in crude extracts. Based on the particular plant, the kind of cancer being treated, and other variables, the effectiveness of crude extracts versus isolated fractions, however, could differ significantly.

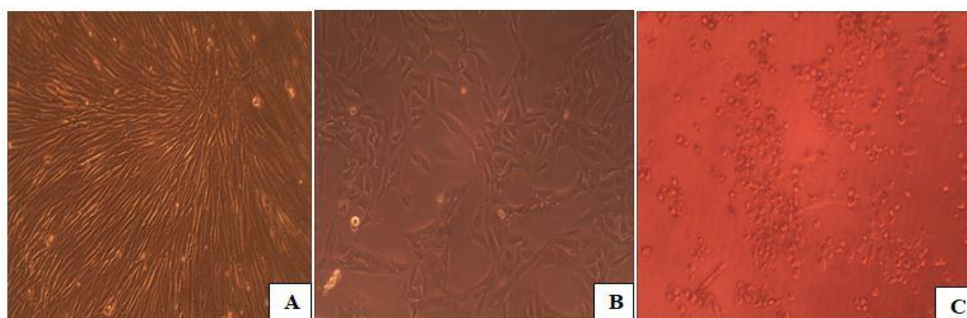


Figure 29: Microscopic images of cell lines at various stages in *P. longum*:
(A) Normal epithelial cells, (B) U-2 OS cells before the treatment of extract, (C) U-2 OS cells after the treatment of extracts**.**

Cytotoxic activity of *in vitro* calli and various wild-grown parts of *P. longum* in HeLa cell lines

The crude extracts of *in vivo* parts exhibited the highest anticancer activity (Table 28) with a higher inhibition percentage, followed by DCM and methanol fractions in HeLa cell lines. Among all the crude extracts, the crude extract of fruit exhibited the highest anticancer activity with 74.25% inhibition at 400 µg/mL, followed by the crude extract of root with 70.58% inhibition, and *in vitro*-grown callus (IC_{50}=513.28±12.49 µg/mL) with 36.84% inhibition at 400 µg/mL on HeLa cells. The least anticancer activity was exhibited by the crude extract of the leaf with 54.97% inhibition at 400 µg/mL. However, Ovadje *et al.* (2014) observed the crude ethanol extracts of fruits of *P. longum* had dose- and time-dependent effects on lowering the growth of carcinoma cells *in vitro*, comprising pancreatic (BxPC-3), colon (HCT116), melanoma cells, and ovarian cancer (OVCAR-3). Similarly, Sunila and Kuttan (2004) exhibited the anticancer property of ethanol extracts of *P. longum* fruits on Ehrlich ascites cancer (EAC) cells and Dalton's lymphoma ascites (DLA) cells at 500 and 250 µg/mL respectively.

Among all the fractions, the DCM fraction of fruit exhibited the highest anticancer activity with 54.65% inhibition at 400 µg/mL, followed by the DCM fraction of root with 46.19% inhibition at 400 µg/mL than methanol fractions (Fig. 30). The reason for this could be that the concentration of anticancer compounds in crude extracts is higher than in fractions, and could be owing to the synergistic impact of different compounds of crude extract.

In both carcinoma cells, the *in vitro*-grown callus exhibited anticancer properties; however, U-2 OS cell lines were more susceptible to the callus extracts than HeLa cells. It might be because the cell lines' responses to the treated extracts differ due to the various nature of carcinoma cells. Furthermore, as demonstrated by their higher IC_{50} values and lower percentage inhibition on normal epithelial cells, all of the crude extracts and fractions were comparatively less cytotoxic up to 400 µg/mL (Table 28). Higher concentrations of crude extracts and fractions, however, might be detrimental to normal epithelial cells.

Table 27: Anticancer activity of *in vitro* calli and wild-grown various parts of *P. longum* on U-2 OS (osteosarcoma cell line), HeLa, and Normal cell lines.

S.N.	Plant parts	Extracts/Fractions	IC_{50} values (μg/mL) in different human cell lines		
			U-2 OS cell line	HeLa cell line	Normal cell line
1.	Root	Crude	179.48±34.76[c]	290.44±7.32[c]	927.08±77.61[c]
		DCM	364.70±3.27[a]	406.88±14.36[b]	953.56±41.92[b]
		Methanol	311.16±1.91[b]	442.75±25.00[a]	1234.38±99.30[a]
2.	Stem	Crude	309.39±25.22[c]	357.02±9.47[c]	1127.04±130.81[c]
		DCM	498.52±17.92[a]	534.03±8.86[b]	1572.38±99.46[b]
		Methanol	467.62±22.54[b]	563.51±41.46[a]	1772.22±50.08[a]
3.	Fruit	Crude	146.55±2.31[c]	273.31±17.89[c]	884.87±73.49[b]
		DCM	320.14±5.92[a]	368.07±15.77[b]	1040.77±21.30[a]
		Methanol	210.59±3.02[b]	425.14±22.98[a]	1209.24±23.89[a]
4.	Leaf	Crude	404.16±21.53[c]	372.94±16.77[c]	1515.31±83.55[c]
		DCM	589.28±11.14[a]	689.26±5.26[b]	2316.76±19.80[b]
		Methanol	499.40±12.47[b]	731.96±17.02[a]	2768.02±19.01[a]
5.	Callus	Crude	272.73±7.28	513.28±12.49[c]	1267.35±67.39
6.	Positive control (Cisplatin)		28.43±1.94	24.56±0.775	-

Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

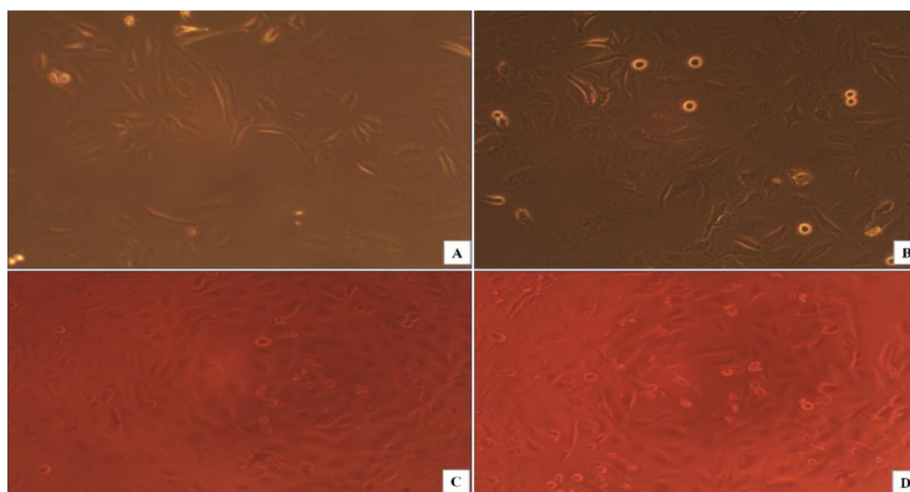


Figure 30: Microscopic Images of cell lines at various stages in *P. longum*:

(A and B) HeLa cells before treatment with extracts, (C and D) HeLa cells after treatment with extracts.

The findings of the anticancer activity assays and the brine shrimp lethality assays may probably show some correlation, but it's crucial to interpret each of them in light of the particular assay conditions and goals. Furthermore, additional research, such as *in vivo*

studies and clinical trials, is frequently required to confirm the possible safety and efficacy of substances found to have anticancer activity using *in vitro* assays. The primary objective of brine shrimp lethality assay is to evaluate acute toxicity, while anticancer activity assays evaluate a compound's capacity to particularly prevent cell growth or cause cell death in cancer cells. The results might not correspond if a substance acts differently on cancer cells than it does on brine shrimp.

4.2.6 Assessment of cytotoxic activity in *P. polyphylla*

The cytotoxic property of various crude extracts and fraction of *P. polyphylla* was determined using brine shrimp nauplii (*in vivo*) and various kinds of human carcinoma cells (*in vitro*), which are described below.

4.2.6.1 Assessment of the *in vivo* cytotoxic activity utilizing brine shrimp lethality bioassay in *P. polyphylla*

The effectiveness of phytochemicals contained in plant extracts is evaluated using the cheap and easy brine shrimp lethality assay. All the crude extracts (rhizome and callus) and DCM and methanol fractions (rhizome) of *P. polyphylla* were found to be cytotoxic on nauplii in this study because the $LC_{50}<1000$ µg/mL is referred to as cytotoxic (Meyer *et al.*, 1982) (Table 29).

Table 28: Cytotoxic activity of crude extracts and fractions of *P. polyphylla* on brine shrimp nauplii.

S.N.	Plant parts	Extracts/Fractions	50% lethal concentration (LC_{50}: µg/mL	Used concentrations (µg/mL)
1	Rhizome	Crude	304.16±60.57[c]	100-1000 µg/mL
		DCM	201.78±70.97[c]	100-1000 µg/mL
		Methanol	457.00±49.53[b]	100-1000 µg/mL
2	Callus	Crude	587.76±52.23[a]	100-1000 µg/mL
3	Potassium dichromate (Positive control)		(LC_{50}=134.34±15.66 µg/mL).	50-200 µg/mL

Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

It was found that the rhizome crude extract exhibited greater cytotoxicity on nauplii in comparison to the callus crude extract. Similarly, the DCM fraction of rhizome was found to be the most cytotoxic compared to the methanol fraction and other crude extracts, presumably because it contained a greater concentration of cytotoxic

compounds. The cytotoxic capacity of crude extract and fractions was compared with a positive control potassium dichromate, which had an LC_{50} of 134.34±15.66 µg/mL. Though the crude extract of callus showed a relatively weaker cytotoxic activity on nauplii, it could be used as a possible cytotoxic substance instead of wild-grown parts of *P. polyphylla*. To the best of our knowledge, no studies on the toxicity of various extracts of *P. polyphylla* on brine shrimp nauplii have been reported.

This is a quick and thorough test for bioactive substances, whether they are synthetic or natural. It uses a large number of organisms for statistical validation, doesn't need any devices, and only needs a small amount of sample (2–20 mg or less) (Sarah *et al.*, 2017). Several researchers have found that brine shrimp (*Artemia salina*) to screen for lethality can be effective for different compounds found in different biological activities. This assay is very helpful for determining the potential toxicity of different plant extracts, even though it does not give enough information about the method of toxic action. This technique yields preliminary screening results that are corroborated by a more focused bioassay.

4.2.6.2 Cytotoxic activity of *in vitro* callus and rhizome of *P. polyphylla* in HeLa, MCF-7, and normal epithelial cell lines

Methanolic crude extract of wild-grown rhizome, *in vitro*-raised calli from leaf, and *in vivo*-grown rhizome fractions were evaluated for anticancer properties. In comparison to the synthetic anticancer agent cisplatin, all crude extracts and fractions in HeLa (cervical carcinoma cell) and MCF-7 (breast carcinoma cell) cells, except for crude extract of callus in MCF-7 cells, had considerable cytotoxic activity (Table 30). The DCM fraction showed higher cytotoxic activity on HeLa cell lines, and had an IC_{50} of 235.94±0.720 µg/mL and a dose-dependent suppression of cell growth, reaching over 62% inhibition at 400 µg/mL after 48 hours. On the other hand, the methanol fraction had a higher cytotoxic activity on MCF-7 cell lines, and had an IC_{50} of 211.36±0.570 µg/mL and a considerable dose-dependent decrease in cell growth, reaching >68% inhibition at 400 µg/mL within 48 hours of treatment (Fig. 31). It suggests that distinct chemical compounds, present in varying amounts in the DCM and methanol fractions, could have had varied impacts on HeLa and MCF-7 cells. The polarity of the solvents during the extraction process may also affect biological activity. The crude extracts and fractions were not inhibiting normal human epithelial cells (IC_{50} values greater than those of HeLa and MCF-7 cell lines), indicating they were not hazardous to normal

cells at the concentration of 50-400 μg/mL (Table 30). On normal cell lines, the callus extract was less hazardous than the rhizome extract, DCM fraction, and methanol fraction. Higher doses of all the extracts and fractions may harm normal cell lines.

Furthermore, the crude extract of callus showed a lower cytotoxic activity on HeLa cells than the crude extract of rhizome, with an IC_{50} of 548.49±0.580 μg/mL and a considerable dose-dependent decrease in cell growth, achieving >40% inhibition at 400 μg/mL after 48 hours (Fig. 31). In contrast, the crude extract of callus demonstrated no cytotoxicity in MCF-7 carcinoma cells. It might be because the features of the HeLa and MCF-7 carcinoma cells are different, and the callus contains fewer toxic compounds than the rhizome. Although various research has been published on the cytotoxicity of *P. polyphylla* rhizome and other parts, this is the first effort to examine the cytotoxicity of *in vitro*-raised callus. In HeLa cells, the ethanolic crude extract of the leaf was more potent than that of the callus (Jafarain *et al.,* 2014). Because of variations in cytotoxic effects between wild and *in vitro* samples, samples produced from *in vivo*-raised plant material had more inhibitory action (Pant *et al*., 2021).

The present research found that the DCM and methanol fractions were more cytotoxic than crude methanol extracts on HeLa and MCF-7 carcinoma cells. The EtOAc fractions of *Pseudocedrela kotschyi* stem bark were more cytotoxic to HeLa cells and RD cells than the methanol crude extract (Elufioye *et al.,* 2017). However, an isolated compound may have higher cytotoxicity than the crude extract and fractions. Saponin isolated from ethyl acetate fraction demonstrated greater cytotoxicity than fraction and standard drugs (Elufioye *et al.,* 2017).

Table 29: Cytotoxicity of rhizome and callus extract with their IC_{50} value in HeLa, MCF-7, and Normal Epithelial cell lines in *P. polyphylla*.

Plant parts	**Extracts/Fractions**	**IC_{50} (μg/mL) values for different cell lines**		
		HeLa Cells	**MCF-7 Cells**	**Normal Epithelial Cells**
Rhizome	Crude Extract	473.39±0.47^{b}	448.87±0.67^{a}	1107.05±16^{b}
	DCM Fraction	235.94±0.72^{d}	296.70±0.83^{b}	991.67±4.4^{c}
	Methanol Fraction	296.48±0.81^{c}	211.36±0.57^{c}	880.79±14.25^{d}
Callus	Crude Extract	548.49±0.58^{a}	-	1191.12±6.91^{a}
Positive control (Cisplatin)		24.56±0.77	26.43±0.530	-

Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

Previous studies on *P. polyphylla* found that isolated pure compounds were more cytotoxic than crude extracts on HeLa and MCF-7 cells (Lee *et al*., 2005; Zhang *et al.,* 2014; Qin *et al*., 2018; Lepcha *et al*., 2019), which could be attributed to the purity of the targeted compounds. This study found a positive correlation between free radical scavenging, antibacterial, and cytotoxic activities in callus and rhizome crude extracts and rhizome fractions (except the methanol fraction in MCF-7 cells) of *P. polyphylla*. Extracts with stronger antioxidant activity, such as the DCM fraction, exhibited greater antibacterial and cytotoxic effects, and vice versa. Similar chemical compounds could be accountable for all three types of bioactivities.

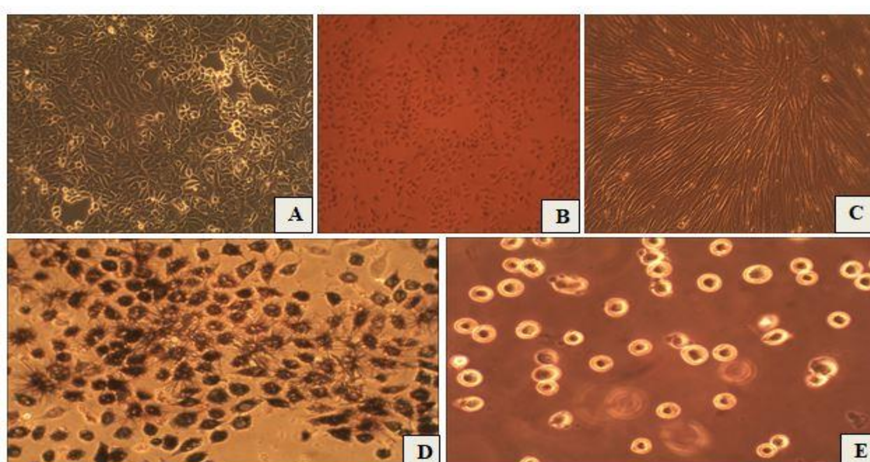


Figure 31: Microscopic images of cell lines at various stages in *P. polyphylla*:
a) MCF-7 cells; b) HeLa confluent cells; c) Normal human epithelial cells; d) Formazan formation; e) Trypsinized cells (Cells cultured in EMEM media in a 5% CO_2 incubator at 37°C).

Figure 32: Microscopic images of cell lines after treatment of extracts in *P. polyphylla*:
a) HeLa cells; b) MCF-7 cells; c) Normal epithelial cells.

4.2.7 Assessment of the *in vitro* antidiabetic activity in *P. longum*

Crude extracts of *in vitro* callus and various *in vivo* parts such as roots, stems, leaves, and fruits, and fractions of various *in vivo* parts were tested for antidiabetic activity *in vitro* with alpha-amylase and alpha-glucosidase enzymes, as detailed below.

4.2.7.1 *In vitro* alpha-amylase and alpha-glucosidase inhibition assay

Alpha-amylase breaks down complex carbs into oligosaccharides or disaccharides, while α-glucosidase breaks down disaccharides into simple glucose molecules, increasing postprandial blood glucose levels. Inhibiting the enzymes α-amylase and α-glucosidase reduces the breakdown of dietary polysaccharides into simple saccharides, lowering postprandial hyperglycemia (Kumar *et al.,* 2011). The roots had the greatest alpha-amylase and alpha-glucosidase inhibition among the studied crude methanolic extracts, with IC_{50} of 1066.60±56.186 and 1255.90±2.764 µg/mL, respectively, followed by callus with IC_{50} of 1165.15±15.637 and 1304.76±12.431 µg/mL, respectively, when contrasted with the antidiabetic drug acarbose (IC_{50}=125.73±13.54 µg/mL) (Table 31). Acarbose is a type 2 diabetes drug that helps control blood sugar levels. Among the tested fractions of wild-grown parts, the DCM fraction of root had the highest alpha-amylase and alpha-glucosidase inhibition, with IC_{50} of 365.21±31.021 and 489.07±27.966 µg/mL, respectively. The DCM fraction of the leaf had IC_{50} of 831.14±18.140 and 922.37±21.347 µg/mL, respectively (Table 31). The methanol fractions of crude extracts of wild-grown parts exhibited lower α-glucosidase inhibitory activity than the DCM fractions. DCM fractions might contain more α-glucosidase inhibitory chemical compounds compared to other fractions and methanolic crude extracts. According to studies on olive mill plant waste (Mwakalukwa *et al.,* 2020), *Clerodendrum volubile* (Erukainure *et al.,* 2018), and *Syzygium cumini* leaf (Franco *et al.,* 2020) DCM has a greater anti-diabetic effect than other fractions. This study found that the DCM fraction had significant antioxidant and antidiabetic characteristics, possibly due to its higher TPC and TFC than the other fractions. The antioxidant properties of phenolic substances such as flavonoids have been found to drastically decrease *in vitro* diabetes via methylation and acetylation of hydroxyl groups (Sarian *et al*., 2017).

Table 30: Alpha-amylase and Alpha-glucosidase inhibition activity by various extracts and fractions in *P. longum.*

S.N.	Plant parts	Extract/Fractions	α-amylase inhibition (IC_{50}: (µg/mL)	α-glucosidase inhibition (IC_{50}: (µg/mL)
1.	Root	Crude	1066.60±56.186[b]	1255.90±2.764[b]
		DCM	365.21±31.021[c]	489.07±27.966[c]
		Methanol	3434.53±57.853[a]	4248.05±35.637[a]
2.	Leaf	Crude	1514.18±70.127[b]	1360.20±13.948[b]
		DCM	831.14±18.140[c]	922.37±21.347[c]

		Methanol	3348.48±149.183[a]	3223.11±13.419[a]
3.	Fruit	Crude	1123.05±13.724[b]	1467.17±43.418[b]
		DCM	921.38±59.881[c]	1058.14±4.45[c]
		Methanol	3199.02±134.046[a]	4331.27±51.216[a]
4.	Stem	Crude	1855.68±38.085[b]	1731.85±20.989[b]
		DCM	1138.77±35.339[c]	1380.85±15.696[c]
		Methanol	2534.98±73.510[a]	2324.01±57.118[a]
5.	Callus	Crude	1165.15±15.637	1304.76±12.431
6.	Acarbose (Positive control)		118.23±27.321	125.73±13.54

Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

Moreover, crude extracts and methanol fractions of leaf and stem showed reduced α-amylase inhibition but stronger α-glucosidase inhibition (Table 31). Alqahtani *et al.* (2019) found variations in the inhibitory ability of α-amylase and α-glucosidase in the same samples, including n-hexane fraction, crude extract, n-butanol fraction, and chloroform fraction. Alpha-amylase inhibition was greater in crude extract, n-butanol, and chloroform fractions than α-glucosidase inhibition, whereas α-glucosidase inhibition was higher in n-hexane than in α-amylase. Kyon *et al.* (2008) found reduced or no α-amylase inhibition in the same samples (four types of teas, red and white wines), but higher α-glucosidase inhibition. Yilmazer-Musa *et al.* (2012) found that tea extracts and catechin 3-gallates were effective at inhibiting α-glucosidase but not α-amylase. Saini and Gangwar (2022) reported that an ethyl acetate extract of an endophytic fungal strain derived from *Aegle marmelos* considerably inhibited α-glucosidase but only moderately to weakly inhibited α-amylase. It might be a consequence of the existence of different bioactive compounds in the same samples, resulting in two distinct types of enzyme inhibition. Different inhibitors may exhibit various modes of activity and selectivity for enzymes (Wang *et al*., 2022). As a result, the degree of inhibition varies depending on the inhibitor's efficacy and the enzyme in issue.

In vitro-grown callus extracts had higher alpha-amylase and alpha-glucosidase inhibition as compared to leaf and stem crude extracts (Table 7). It might be because *in vitro*-grown callus has been shown to generate biologically active secondary metabolites such as piperin from *Piper longum* (Siddique *et al*., 2019) and *Piper nigrum* (Ahmad *et al*., 2014), terpenoid compounds from *Piper retrofractum* (Faramayuda *et*

al., 2021), flavonoid, terpenoid, octadecanoic acid from *Piper betle* (Junairiah *et al*., 2019). Similarly, catechin was obtained from the callus of *Camellia sinensis* (Sutini *et al*., 2020). Callus cultures yield a variety of compounds, including tropane alkaloids, reserpine, flavonoids, α-tocopherol, ajmaline, serpentine, stilbene, scopolamine, paclitaxel, resveratrol, and anthocyanins (Efferth, 2019). Significant contributions could be made to the development and extraction of important chemical compounds from *in vitro*-grown callus as a potential natural drug for a variety of diseases and illnesses, as well as the *ex-situ* management of wild plants in their natural environments.

Some researchers carried out *in vivo* studies utilizing animal models (rats) on the fruit (Kumar *et al.,* 2011), root (Nabi *et al*., 2012; Nabi *et al.,* 2013), and oil (Kumar *et al*., 2013) of *P. longum* and found potential antidiabetic properties. However, this could be the first study comparing the antidiabetic activity of different parts of *P. longum* including roots, stems, fruits, and leaves, *in vitro*. This study found that root extracts have considerably stronger antidiabetic activity *in vitro* than leaf, fruit, and stem extracts, particularly the dichloromethane fraction.

4.2.8 Assessment of the *in vitro* antidiabetic activity in *P. polyphylla*

Crude extracts of rhizome and callus and fractions of rhizomes were tested for antidiabetic activity *in vitro* with alpha-amylase and alpha-glucosidase enzymes, as detailed below.

4.2.8.1 *In vitro* alpha-amylase and alpha-glucosidase inhibition assay

The *in vitro* inhibition of alpha-amylase and α-glucosidase was assessed in crude extracts of rhizome and callus, as well as rhizome fractions. Compared to callus crude extract and rhizome fractions, the rhizome crude extract exhibited the highest inhibition of α-glucosidase (IC_{50}=51.40±8.62 μg/mL) and α-amylase (IC_{50}=95.45±8.71 μg/mL) (Table 32). It might be because the mixture of compounds in the crude extract works synergistically to increase the inhibitory effects on the alpha-glucosidase and alpha-amylase enzymes. Some of these synergistic interactions may be lost when fractions are isolated, which would lower the total inhibitory activity. Similarly, reduced inhibitory activity may arise from the loss of some small but powerful components during fractional isolation.

Table 31: Alpha-amylase and alpha-glucosidase inhibition by rhizome and callus in *P. polyphylla.*

S.N.	Plant parts	Crude extract/Fraction	α-amylase inhibition (IC_{50}: µg/mL)	α-glucosidase inhibition (IC_{50}: µg/mL)
1.	Rhizome	Crude extract	95.45 ± 8.71^{d}	51.40 ± 8.62^{d}
		Methanol fraction	390.60 ± 29.04^{b}	186.24 ± 5.71^{c}
		DCM fraction	156.02 ± 11.66^{c}	397.97 ± 23.33^{b}
2.	Callus	Crude extract	564.64 ± 12.67^{a}	536.10 ± 44.83^{a}
3.	Acarbose (positive control)		118.23±27.321	125.73±13.54

Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

Similarly, the DCM fraction showed higher α-amylase inhibition (IC_{50}=156.02±11.66 µg/mL) than the methanol fraction, whereas the methanol fraction showed higher α-glucosidase inhibition (IC_{50}=186.24±5.71 µg/mL) than DCM fraction. It could be because of the differential solubility of compounds, which means that the methanol fraction and the DCM fraction probably contain different sets of compounds. Callus crude extract, however, had the least α-amylase (IC_{50}=564.64±12.67 µg/mL) and α-glucosidase inhibition (IC_{50}=536.10±44.83 µg/mL). The absence of significant concentrations of inhibitors targeting alpha-amylase and alpha-glucosidase enzymes, extraction efficiency, and the presence of interacting or non-inhibitory compounds may be the cause of the lower alpha-amylase and alpha-glucosidase inhibition observed in the callus crude extract when compared to rhizome extracts and their fractions. The alpha-amylase and alpha-glucosidase inhibition of all crude extracts and fractions were compared with the antidiabetic drug acarbose.

A few studies on the antidiabetic properties of *P. polyphylla* have been conducted. The α-amylase and α-glycosidase enzymes were shown to be inhibited by the diosgenin-enriched rhizome extracts of *P. polyphylla* extract. When streptozotocin (STZ)-induced diabetic rats were given rhizome extract orally every day for 28 days, their hyperglycemic state was reduced, and their liver and kidney tissues' antioxidant levels were enhanced (Kshetrimayum *et al*., 2023).

Furthermore, there isn't much research on the anti-diabetic effects of *in vitro*-grown callus of *P. polyphylla*. However, Rajan *et al.* (2022) found that *Veronia anthelmintica* leaf and callus extracts inhibited alpha-amylase and alpha-glucosidase enzymes significantly. The EtOAc extract of calli and methanolic extracts of leaves

demonstrated the greatest inhibition of α-amylase, while the EtOAc extract of both leaf and calli demonstrated noteworthy inhibition of α-glucosidase enzyme.

4.3 Assessment of the secondary metabolites production in callus suspension culture by using phenylalanine and salicylic acid in *Piper longum* and *Paris polyphylla*

Secondary metabolites produced from callus suspension culture using salicylic acid as elicitor and phenylalanine as precursor were assessed for antioxidant activity, TPC, and TFC.

4.3.1 Assessment of antioxidant activity, total phenolic contents (TPC), and total flavonoid contents (TFC) in suspension culture of *P. longum*

For suspension culture, the most effective media for producing friable callus and proliferation from compact callus, produced in MS + 2,4-D + 10% CW, was MS + 0.5 mg/L TDZ + 1.5 mg/L NAA in *P. longum.* The combined use of TDZ and NAA provides a hormonal environment or synergistic effect that promotes the proliferation of undifferentiated cells over structured tissue development. The increased auxin-to-cytokinin ratio (1.5 mg/L NAA to 0.5 mg/L TDZ) may favor the production of loose, friable calluses rather than compact ones. This callus (about 5 g) was cultured in semi-solid and liquid media for 15 and 30 days in MS medium added with 0.5 mg/L TDZ and 1.5 mg/L NAA, and that were designated as control. The callus, on the other hand, was cultured in liquid media with MS + 0.5 mg/L TDZ + 1.5 mg/L NAA + 15 mg/L salicylic acid, MS + 0.5 mg/L TDZ + 1.5 mg/L NAA + 30 mg/L salicylic acid, MS + 0.5 mg/L TDZ + 1.5 mg/L NAA + 50 mg/L phenylalanine, and MS + 0.5 mg/L TDZ + 1.5 mg/L NAA + 100 mg/L phenylalanine that were designated as treatment. Salicylic acid was used at lower concentrations than phenylalanine to efficiently activate defense-related pathways and increase the production of metabolites while causing no stress or damage to cells. Phenylalanine was used at higher concentrations than salicylic acid to ensure adequate substrate availability for secondary metabolite biosynthesis, resulting in the maximum amount of product.

After the calli were harvested, the percentage increase in callus biomass was computed and the methanolic crude extracts of all the controls and treatments were prepared for the determination of antioxidant activity, TPC, and TFC (Fig. 33).

Table 32: Percentage increase in biomass of callus in callus suspension culture of *P. longum.*

Treatments	% Increase in callus biomass	
	After 15 days	After 30 days
MS+TN (SSM)	15.49±3.01[e]	49.45±1.73[f]
MS+TN (LM)	17.48±1.53[e]	68.91±2.23[e]
MS+TN+SA (15)/LM	83.29±2.25[b]	113.20±5.77[b]
MS+TN+SA (30)/LM	100.47±2.43[a]	123.31±3.45[a]
MS+TN+PHE (50)/LM	22.89±1.32[d]	91.05±5.42[d]
MS+TN+PHE (100)/LM	56.82±3.17[c]	102.54±3.38[c]

MS, Murashige and Skoog; TN, TDZ and NAA; SSM, Semi-solid media; LM, Liquid media; SA (15), Salicylic acid 15 mg/L; SA (30), Salicylic acid 30 mg/L; PHE (50), Phenylalanine 50 mg/L; PHE (100), Phenylalanine 100 mg/L. Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

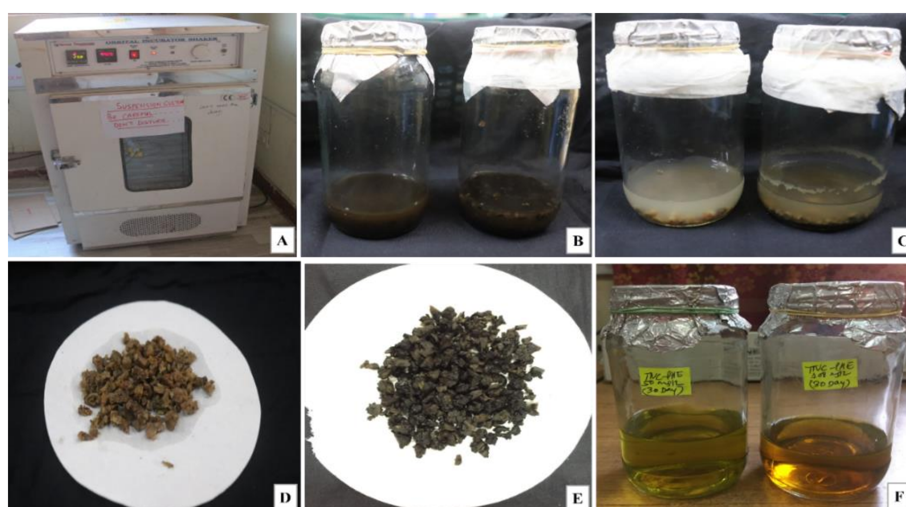


Figure 33: Callus suspension culture in *P. longum*:

(A) Culture jars kept inside the orbital incubator shaker, (B) Callus in liquid MS media with 15 mg/L and 30 mg/L salicylic acid, (C) Callus in liquid MS medium with 50 mg/L and 100 mg/L phenylalanine, (D) Harvested callus treated with 50 mg/L phenylalanine, (E) Harvested callus treated with 30 mg/L salicylic acid, (F) Liquid extracts prepared from the dry powdered callus in 90% methanol.

This study showed that the control as well as the treatment with salicylic acid and phenylalanine showed an increase in callus biomass after the 15 and 30 days of culture (Table 33). In the case of control, the increase in callus biomass (%) was higher in liquid media than in semi-solid media after the 15 and 30 days of culture. Compared with a semi-solid medium, a liquid medium provides a more homogeneous and favorable environment for callus proliferation, with higher nutrient availability, gas exchange, and fewer physical restrictions, resulting in increased biomass production.

Linh *et al.* (2021) found that callus biomass increased 67-fold after 20 days in MS medium enriched with 2,4-D and KN in a suspension culture of *Catharanthus roseus*. Similarly, following 15 and 30 days of culture, salicylic acid treatment as elicitor resulted in a greater rise in callus biomass (%) than phenylalanine treatment as a precursor. Salicylic acid is a type of plant growth regulator which contributes to a variety of physiological processes, including enhanced biomass production via cell proliferation. Al-Khayri and Naik (2020) reported that adding 50 mg/L salicylic acid to the MS medium-supplemented cell culture of *Phoenix dactylifera* enhanced biomass in terms of packed cell volume (PCV). However, Rattan *et al.* (2022) found that adding 1 mM of phenylalanine significantly increased biomass production (both fresh and dry weight) in *Rhodiola imbricata* suspension cultures. Furthermore, Chavan *et al.* (2021) reported that after four weeks of culture in MS media added with jasmonic acid in the cell suspension culture of *Salacia chinensis*, the biomass of callus rose five times.

Following callus harvesting, the amount of TPC, TFC, and antioxidant activity was measured to determine the metabolite production in suspension culture in the methanolic crude extract of callus. In the case of controls, antioxidant activity (263.32±43.27 µg/mL), total phenolic contents (53.64±3.07 mg of GAE/g dry wt.), and total flavonoid contents (8.93±0.43 mg of QE/g dry wt.) were higher in the liquid/suspension culture than in semi-solid media after the 15 days and the 30 days of culture (Table 33). These values were higher in the treatment of 30 days than in the 15 days of culture. The synthesis of secondary bioactive compounds may be higher in callus suspension cultures than in semi-solid cultures for several reasons. These include the fact that suspension cultures provide a more homogenous distribution of nutrients and growth factors, which can encourage more consistent cell growth and metabolite production, and that callus suspension cultures have a greater surface area subjected to the culture medium than semi-solid cultures, possibly leading to higher metabolite production. Metabolite production is higher at 30 days of suspension culture than at 15 days because the cells in suspension culture may have experienced substantial proliferation and accumulation of biomass in 30 days, and by thirty days, the cells might have reached the optimum state for metabolite synthesis.

Table 33: Antioxidant, total phenolic content (TPC), and total flavonoid content (TFC) in suspension culture of *P. longum*.

Treatments	Antioxidant activity (IC_{50}: µg/mL)		Total phenolic contents (mg of GAE/g extract wt)		Total flavonoid contents (mg of QE/g extract wt)	
	After 15 days	After 30 days	After 15 days	After 30 days	After 15 days	After 30 days
MS+TN (SSM)	444.78±30.75[a]	341.11±19.60[a]	12.50±0.47[e]	13.32±0.66[e]	3.38±0.25[d]	4.13±0.32[a]
MS+TN (LM)	335.51±8.84[b]	263.32±43.27[b]	15.51±1.55[e]	53.64±3.07[b]	3.98±0.03[cd]	8.93±0.43[b]
MS+BN+SA (15)	173.46±19.69[c]	242.11±18.98[b]	35.01±1.17[d]	31.96±1.21[d]	4.29±0.07[cd]	3.98±0.27[a]
MS+TN+SA (30)	142.81±8.70[c]	180.46±2.54[c]	52.79±3.72[a]	47.44±1.54[c]	10.06±0.9[b]	4.81±0.39[bc]
MS+TN+PHE(50)	164.44±10.34[c]	90.43±6.30[d]	40.33±2.40[c]	79.38±3.29[a]	6.91±0.42[c]	7.21±0.21[bc]
MS+TN+PHE (100)	92.01±10.37[d]	22.44±1.96[e]	44.64±0.40[b]	83.08±1.08[a]	17.78±0.3[a]	18.26±0.11[a]

Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

In comparison to the control treatment using semisolid and liquid media, the salicylic acid (elicitor) and phenylalanine (precursor) treatments showed greater antioxidant activity and higher values of TPC and TFC (Table 34). Salicylic acid is a phenolic compound composed of a variety of antioxidant component that improves plant immunological responses to abiotic and biotic stresses but also increases antioxidant activity, as well as secondary metabolite production such as total phenolic and flavonoid content, N_2-containing compound, and sulfur-containing compounds (Khan *et al.*, 2022; Singh, 2023). Similarly, increasing the concentration and duration of phenylalanine treatment led to greater metabolite production with higher antioxidant activity, and higher TPC and TFC values. However, treatment with 100 mg/L phenylalanine exhibited the highest free radical scavenging activity (IC_{50} of 92.01±10.37 µg/mL at 15 days of treatment and IC_{50} of 22.44±1.96 µg/mL at 30 days of treatment) and the highest values of TPC (44.64±0.40 mg of GAE/g of dry wt. at 15 days of treatment and 83.08±1.08 mg of GAE/g of dry wt. at 30 days of treatment) and TFC (17.78±0.37 mg of QE/g of dry wt. at 15 days of treatment and 18.26±0.11 mg of QE/g of dry wt. at 30 days of treatment). Precursor feeding is an established plan for enhancing the industrial synthesis of bioactive chemicals in plant cell culture systems (Rattan *et al*., 2022). As a result, phenylalanine serves as an important precursor for the phenylpropanoid synthesis pathway, influencing cell proliferation, antioxidant activity, and secondary bioactive compound production including phenolic compounds in cell suspension cultures. Rattan *et al.* (2022) exhibited that treating *Rhodiola imbricata*

callus suspension cultures with phenylalanine led to the maximum synthesis of cell biomass and phenylpropanoid, which in turn increased the production of TPC and TFC. On the other hand, phenylalanine functions as a heterocyclic amino acid precursor, leading to salicylic acid production (Khan *et al.*, 2015) that might lead to increased metabolite production and higher antioxidant activity, TPC and TFC. Moreover, the production of metabolites with greater antioxidant activity and higher values of TPC and TFC was found by increasing the concentration of salicylic acid treatment; however, metabolite production with lower antioxidant activity and lower values of TPC and TFC was found by increasing the duration of salicylic acid treatment from 15-30 days. It suggests that prolonged salicylic acid treatment in suspension culture is ineffective for enhancing antioxidant activity, TPC, and TFC in *P. longum*. However, Rodrguez-Sanchez *et al.* (2020) found that the addition of 100 mM salicylic acid in the suspension culture of *Piper cumanense* was successful in increasing certain metabolites, such as (Z)-9 octadecenamide (8.8%), phenol, and 5-hydroxymethylfurfural (6.3%). Similarly, Al-Khayri and Naik (2020) showed increased TPC and free radical scavenging activity in the MS media-supplemented cell culture with 50 mg/L Salicylic acid. Maqbul *et al.* (2023) found that antioxidant compounds, TPC, and TFC increased in MS medium enriched with salicylic acid from shoot-derived callus suspension culture when compared to the control treatment in *Aerva sanguinolenta*.

4.3.2 Assessment of antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC) in suspension culture of *P. polyphylla*

The callus was initially induced in MS medium added with 2,4-D, KN, and 10% coconut water from *P. polyphylla* leaf explants. The best media for callus proliferation was MS medium fortified with 1.0 mg/L BAP and 1.5 mg/L NAA for suspension culture. The suspension culture was performed using these calli (about 5 g) for 15 and 30 days in semi-solid and liquid media with MS media containing 1.0 mg/L BAP and 1.5 mg/L NAA as a control. On the other hand, the callus was cultured for 15 and 30 days in liquid media with MS + 1.0 mg/L TDZ + 1.5 mg/L NAA + 15 mg/L salicylic acid; MS + 1.0 mg/L BAP + 1.5 mg/L NAA + 30 mg/L salicylic acid; MS + 1.0 mg/L BAP + 1.5 mg/L NAA + 50 mg/L phenylalanine; and MS + 1.0 mg/L BAP + 1.5 mg/L NAA + 100 mg/L phenylalanine. Salicylic acid was used at lower concentrations than phenylalanine to efficiently activate defense-related pathways and increase the

production of metabolites while causing no stress or damage to cells. Phenylalanine was used at higher concentrations than salicylic acid to ensure adequate substrate availability for secondary metabolite biosynthesis, resulting in the maximum amount of product.

After the calli were harvested, the callus biomass percentage increase was computed and the methanolic crude extracts of all the treatments were prepared for the determination of antioxidant activity, TPC, and TFC (Fig. 34).

Table 34: Percentage increase in biomass of callus in suspension culture of *P. polyphylla***.**

Treatments	% Increase in callus biomass	
	After 15 days	**After 30 days**
MS+BN (SSM)	9.80±3.42[e]	44.91±7.89[d]
MS+BN (LM)	29.88±2.12[d]	66.77±9.35[c]
MS+BN+SA (15)/LM	58.61±5.24[c]	87.86±6.56[b]
MS+BN+SA (30)/LM	90.59±2.83[a]	152.28±2.73[a]
MS+BN+PHE (50)/LM	34.93±2.38[d]	96.56±5.47[b]
MS+BN+PHE (100)/LM	80.72±4.49[b]	145.79±10.44[a]

Abbreviations: MS, Murashige and Skoog; BN, BAP and NAA; SSM, Semi-solid media; LM, Liquid media; SA(15), Salicylic acid 15 mg/L; SA(30), Salicylic acid 30 mg/L; PHE(50), Phenylalanine 50 mg/L; PHE(100), Phenylalanine 100 mg/L. Duncan's Test (α=0.05) indicated no significant difference between means in columns with the same letter.

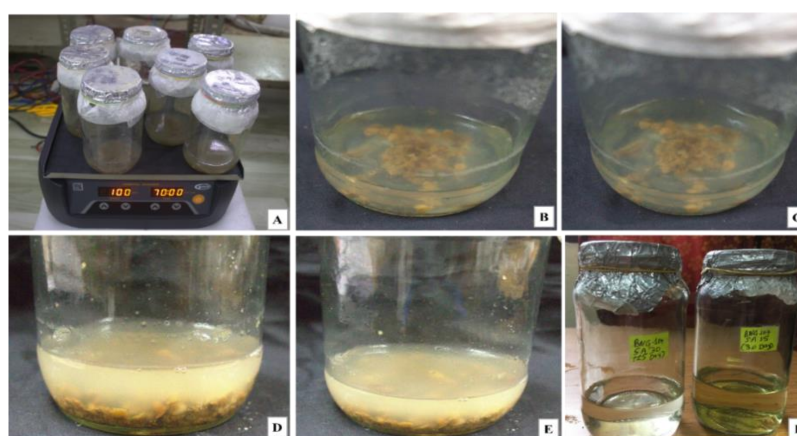


Figure 34: Callus suspension culture in *P. polyphylla*:

(A) Culture jars in orbital incubator shaker, (B) Calli in liquid MS medium with 15 mg/L salicylic acid, (C) Calli in liquid MS medium with 30 mg/L salicylic acid, (D) Calli in liquid MS medium with 50 mg/L phenylalanine, (E) Calli in liquid MS medium with 100 mg/L phenylalanine, (F) Liquid extracts prepared from the dry powdered callus in 90% methanol.

This study showed that the control treatment with semisolid and liquid media as well as the treatment with salicylic acid and phenylalanine showed an increase in callus biomass after the 15 and 30 days of culture (Table 35). In the case of control, callus biomass increase (%) was higher in liquid media than in semi-solid media after the 15 and 30 days of culture. Moreover, in the case of treatment with salicylic acid and phenylalanine, the callus biomass increase (%) was higher in salicylic acid treatment than in phenylalanine treatment after 15 and 30 days of culture. Salicylic acid (SA) is a type of plant growth regulator, which contributes to several physiological functions, one of which is increased biomass production through cell proliferation. Similarly, appropriate phenylalanine concentrations may result in enhanced cell mass and total yield (Rattan *et al.*, 2022). However, Rawat *et al.* (2023) found that the medium with 0.5 mM TDZ showed the highest callus biomass (2.98 g fresh wt.) in the suspension culture of *P. polyphylla*.

Following callus harvesting, the amount of TPC, TFC, and antioxidant activity were measured to determine the metabolite production in suspension culture from the methanolic crude extract of callus. In the case of controls, antioxidant activity, TPC, and TFC were higher in the liquid/suspension culture than in semi-solid media after the 15 days and the 30 days of culture (Table 36). These values were higher in the culture of 30 days than in the 15 days of culture. The synthesis of secondary bioactive compounds may be higher in callus suspension cultures than in semi-solid cultures for several reasons. These include the fact that suspension cultures provide a more homogenous distribution of nutrients and growth factors, which can encourage more consistent cell growth and metabolite production, and that callus suspension cultures have a greater surface area subjected to the culture medium than semi-solid cultures, possibly leading to higher metabolite production.

Table 35: Antioxidant, total phenolic contents (TPC), and total flavonoid contents (TFC) in suspension culture of *P. polyphylla*.

Treatments	**Antioxidant activity (IC_{50}: µg/mL)**		**Total phenolic contents (mg of GAE/g dry wt.)**		**Total flavonoid contents (mg of QE/g dry wt.)**	
	After 15 days	**After 30 days**	**After 15 days**	**After 30 days**	**After 15 days**	**After 30 days**
MS+BN (SSM)	767.45±53.18^{a}	94.11±19.34^{a}	11.74±1.26^{d}	12.14±0.03^{c}	2.33±0.49^{a}	3.10±0.12^{a}
MS+BN (LM)	165.63±5.82^{e}	102.52±1.29^{e}	23.90±1.01^{ab}	42.20±0.78^{a}	4.51±0.46^{a}	8.66±1.19^{a}

MS+BN+SA (15)/LM	598.09±44.96^{b}	230.07±7.47^{c}	18.31±0.78^{c}	24.05±2.27^{abc}	2.83±0.09^{a}	3.24±1.72^{a}
MS+BN+SA (30)/LM	322.98±10.89^{c}	96.48±31.43^{b}	17.37±1.19^{c}	21.55±0.56^{bc}	3.75±0.23^{a}	4.64±0.25^{a}
MS+BN+PHE (50)/LM	261.35±6.75^{d}	258.18±5.26^{c}	22.44±0.40^{bc}	24.49±1.92^{abc}	4.11±0.18^{a}	4.46±0.08^{a}
MS+BN+PHE (100)/LM	251.25±42.97^{d}	153.85±4.45^{d}	27.79±1.99^{a}	37.62±1.06^{ab}	5.28±0.30^{a}	7.78±0.47^{a}

Abbreviations: MS, Murashige and Skoog; BN, BAP and NAA; SSM, Semi-solid media; LM, Liquid media; SA(15), Salicylic acid 15 mg/L; SA(30), Salicylic acid 30 mg/L; PHE(50), Phenylalanine 50 mg/L; PHE(100), Phenylalanine 100 mg/L.

In comparison to the treatment with salicylic acid (elicitor) and phenylalanine (precursor), the control treatment using liquid media exhibited the highest free radical scavenging activity (with IC_{50} of 165.63±5.82 µg/mL at 15 days of culture and IC_{50} of 102.52±1.29 µg/mL at 30 days of treatment) as well as higher values of total phenolic content (23.90±1.01 mg of GAE/g dry wt. at 15 days of treatment and 42.20±0.78 mg of GAE/g dry wt. at 30 days of treatment) and total flavonoid contents (4.51±0.46 mg of QE/g dry wt. at 15 days of treatment and 8.66±1.19 mg of QE/g dry wt. at 30 days of culture treatment) (Table 36). Rawat *et al.* (2018) found that the medium with 1.0 mM TDZ and 50 mM methyl jasmonate had the best antioxidant activity in the callus suspension culture of *P. polyphylla*. Salicylic acid and phenylalanine can either boost or modify metabolite synthesis in suspension cultures, based on the culture environment and concentrations utilized (Li *et al.*, 2021). Therefore, control treatment with liquid media was effective for producing metabolites with increased antioxidant activity, TPC, and TFC in *P. polyphylla*. The control treatment of liquid MS media was fortified with 1.0 mg/L BAP + 1.5 mg/L NAA, which served as an elicitor for the increased metabolite with higher antioxidant activity, TPC, and TFC. On the other hand, the control treatment with semisolid media had the lowest antioxidant activity and TPC and TFC compared to the control treatment with liquid media and the treatment with salicylic acid and phenylalanine. Semisolid callus culture may produce fewer metabolites due to limited nutrient diffusion, oxygen availability, and uneven cell distribution (Jamil *et al.*, 2018). However, increasing the concentration and duration of phenylalanine and salicylic acid treatments led to greater metabolite production with higher antioxidant activity, and higher TPC and TFC values. Treatment with 100 mg/L phenylalanine showed higher antioxidant activity (IC_{50} of 251.25±42.97

µg/mL at 15 days of treatment and IC_{50} of 153.85±4.45 µg/mL at 30 days of treatment) and higher values of TPC (27.79±1.99 mg of GAE/g of dry wt. at 15 days of treatment and 37.62±1.06 mg of GAE/g of dry wt. at 30 days of treatment) and TFC (5.28±0.30 mg of QE/g of dry wt. at 15 days of treatment and 7.78±0.47mg of QE/g of dry wt. at 30 days of treatment) than treatment with salicylic acid. Phenylalanine functions as a heterocyclic amino acid precursor, leading to salicylic acid production (Khan *et al.*, 2015) which might lead to increased metabolite production and higher antioxidant activity, TPC, and TFC. However, Sharifi *et al.* (2019) found that a callus suspension of *Ruta graveolens* treated with salicylic acid (10 mg/mL) increased its total flavonoid content by up to 3.14 times, total phenolic content by 18.33-fold and its antioxidant activity by up to 3.55-fold as compared to the control.

4.4 Screening of phytoconstituents and assessment of putative compounds in crude extracts of *in vitro*-raised callus and *in vivo* parts of *Piper longum* and *Paris polyphylla* by LC-HRMS analysis

Phytochemicals present in the crude extracts of *in vitro*-raised callus and wild-grown parts were assessed using standard qualitative methods, and the potential secondary metabolites responsible for different bioactivities were identified using the LC-HRMS analysis.

4.4.1 Screening of phytoconstituents in crude extracts of *P. longum* and *P. polyphylla*

Screening of phytoconstituents is the process of analyzing plant secondary metabolites to identify the existence of different classes of active chemical compounds. Qualitative screening of phytoconstituents in plant extracts is an initial analytical approach for determining the presence or absence of various chemical compounds found in plant material. This screening assists researchers or analysts in understanding the chemical makeup of the plant extract and offers information about its possible pharmaceutical or biological properties. Moreover, phytochemical screening not only reveals the different components of plant extracts and which one dominates over the others, but it also aids in the discovery of bioactive compounds that may be utilized as medicinal and dietary supplements (Mukherjee *et al.*, 2017). In this study, crude extracts of *in vivo* parts and *in vitro* callus of *P. longum* and *P. polyphylla* were qualitatively analyzed using

standard techniques. Table 37 displays the results, showing positive test outcomes with a plus (+) sign and negative test outcomes with a minus (-) sign.

Table 36: Screening of phytoconstituents in crude extracts of wild-grown parts and *in vitro* callus of *P. longum* and *P. polyphylla*.

Phytoconstituents	Performed test	*Piper longum*					*Paris polyphylla*	
		Rt	Ft	Sm	Lf	Cs_1	Rm	Cs_2
Alkaloid	Meyer's test	+	+	+	+	+	-	-
	Wagner's test	+	+	+	+	+	-	-
	Dragendorff's test	+	+	+	+	+	-	-
Tannins	Ferric chloride test	-	-	+	+	-	-	-
	Gelatin test	-	-	+	+	-	-	-
Glycosides	Borntrager's test	-	+	+	+	+	+	+
	Keller-Kiliani test	-	+	+	+	-	+	+
Flavonoids	Ferric chloride test	+	+	-	+	-	+	+
	Alkaline reagent test	+	+	+	+	+	+	+
	Shinoda test	+	+	+	+	+	+	+
Terpenoids	Liebermann-Burchardtest	+	+	+	+	-	+	-
Phenols	Ferric chloride test	-	+	-	+	+	+	-
Steroids	Salkowski test	+	+	-	-	-	+	+
Saponin	Frothing test	+	+	+	+	-	+	+
Carbohydrates	Fehling's test	-	+	+	+	+	+	+
	Benedict's test	-	+	+	+	+	+	+

Abbreviations: Rt, Root; Ft, Fruit; Sm, Stem; Lf, Leaf; Cs_1, Callus of *P. longum*; Rm, Rhizome; Cs_2, Callus of *P. polyphylla*; '+' represents presence, and '-' represents absence.

Various *in vivo* parts and *in vitro* callus of *P. longum* showed common phytoconstituents including alkaloids, flavonoids, terpenoids, saponins, glycosides, and carbohydrates, however, steroids were not detected in the stem, leaf, and callus. Similarly, tannins were not detected in the roots, fruits, and callus; glycosides were absent in the roots; terpenoids were absent in the callus; phenols were absent in the roots and stem. The phytoconstituents of wild-grown parts of *P. longum* described in this investigation were supported by previous findings (Zaveri *et al.*, 2010; Saraf and Saraf, 2014; Sultana *et al*., 2019; Luca *et al.*, 2021). Callus cells can build up different types of bioactive molecules, such as glycosides, terpenoids, flavonoids, sterols, and

phenolic compounds. These compounds may differ based on the plant type and the environment in whereby the callus develops (Rattan *et al.*, 2021).

Moreover, *In vivo* rhizome and *in vitro* callus of *P. polyphylla* showed common phytoconstituents including flavonoids, terpenoids, saponins, glycosides, phenol, steroids, and carbohydrates, however, alkaloids and tannins were not detected in all the *in vivo* rhizome and *in vitro* callus. Similarly, terpenoids and phenols were not detected in the callus. The phytoconstituents of *in vivo* rhizome of *P. polyphylla* described in this investigation were endorsed by previous findings (Chen *et al.,* 1995; Zhou and Yang, 2003; Wang *et al*., 2005; Xiao *et al.,* 2009; Kang *et al*., 2012; Li *et al*., 2013; Thapa *et al*., 2022). The existence or lack of phytoconstituents in plant extracts could be determined by a combination of intrinsic (plant species, plant parts, plant age, and growth stage) and extrinsic factors, including cultivation practice, harvesting and post-harvest handling, environmental conditions, and extraction method. Furthermore, the absence of chemical compounds in certain extracts could be the result of compounds disintegrating in the heat generated during the rotary evaporator which is the method of extract concentration.

4.4.2 Assessment of putative plant metabolites in crude extracts of *in vitro*-raised callus and *in vivo* parts of *P. longum* by LC-HRMS analysis

Crude extracts of *in vitro*-grown callus and DCM fraction of wild-grown roots of *P. longum* were examined using LC-HRMS to identify the bioactive compounds. The DCM fraction of root extract was chosen to find possible compounds since it showed higher bioactivities *in vitro* than other parts, but the crude extract of callus was chosen to compare its bioactive compounds to that of *in vivo*-grown roots. The compounds that have been identified are presented in Tables 40 and 41, along with information about their ion intensity, precursor mass and exact mass (PEPMass), molecular formula, mass-to-charge errors in parts per million (ppm), retention time (Rt) in minutes, GNPS spectral ID, and ESI positive ion mode measurements. The compounds were identified using the measured mass spectrum, and the results were validated by comparing them to existing literature. There were 27 compounds found from both the root and the callus, with 24 compounds found in the root and 19 in the callus. GNPS databases exhibited 17 compounds, whereas Sirius databases showed 10 compounds. Not all compounds are found in GNPS databases for a variety of reasons, including a lack of complete data

coverage (GNPS does not include every compound ever studied), delays in scientific community submission-driven content, private or confidential data, and limitations in compound detection and characterization. The common adduct ions generated during measurement were $[M+H]^+$, $[M+Na]^+$, $[M]^+$, and $[M+NH_4]^+$.

Present work showed that roots contained more bioactive compounds than callus, the majority of which were alkaloid and polyphenolic compounds. The higher number of compounds detected in wild-grown plant roots compared to *in vitro*-raised callus may be due to the complex interaction of environmental (sunlight, soil composition, temperature, humidity, and stress), and developmental, genetic, and microbial factors in wild-grown plants. The most important alkaloids found in both root and callus were piperine (m/z 285.13), piplartine (m/z 317.12), piperlonguminine (m/z 273.13), piperanine (m/z 287.15), etc (Table 38 and 39, and Fig. 35 and 36). According to Zaveri *et al.* (2010), *P. longum* has a considerable number of alkaloids in its fruits and roots, with piperine accounting for 3-5% of the dry weight. It was found that callus was capable of synthesizing key bioactive compounds in MS media fortified with PGRs, which could explain why callus exhibited antioxidant, antibacterial, cytotoxic, and antidiabetic activities *in vitro*, as demonstrated by wild-grown parts of *P. longum*. However, the type of compounds produced by the callus and their quantities in the callus may be influenced by the culture environment, variation in genomes, the age of the callus, the use of PGRs in culture, and other factors. The major compound piperine and its derived compounds have various types of biological activities, including anticancer activity, antioxidant activity, antiinflammatory activity, antidiabetic activity, antimycobacterial activity, insecticidal activity, cardioprotective, hepatoprotective, and neuroprotective properties (Qu *et al*., 2015; Haq *et al*., 2021). Similarly, piplartine (piperlongumine) has numerous documented medicinal properties, including cytotoxic, anticancer, antimetastatic, antidepressant, antidiabetic, antibacterial, antifungal, leishmanicidal, trypanocidal, and inflammatory effects (Bezerra *et al*., 2013; Henrique *et al*., 2020).

Moreover, some glycosides, polyphenolic compounds, and phenolic acid derivatives were also found in the callus extract of *P. longum*, such as campyloside A, lanceolin B, and 5-0-Feruloylnigrumin respectively. Campyloside A isolated from *Campylospermum glaucum* roots had antibacterial activity against Gram-positive cocci, including *Enterococcus hirae* ATCC 9790, *Enterococcus* sp. P054,

Staphylococcus aureus ATCC 25923, *S. aureus* U271, and *S. saprophyticus* (Abouem à Zintchem *et al.*, 2008). According to Sore *et al*. (2020) lanceolin B showed an antimalarial activity, which was a strong suppressor of *Plasmodium's* initial sporogonic phases.

This study also found that there were 16 compounds common to both roots and callus, however, their ion concentration was higher in roots than in callus (Table 38). It might be due to the differences in tissue-specific metabolism, environmental and nutritional variables, as well as age and differentiation factors. Roots and calli have distinct metabolic activity. The root, as a specialized tissue, may naturally develop and accumulate larger quantities of some secondary metabolites than callus tissue, which is more undifferentiated and unspecialized. Similarly, 8 compounds were found only in the roots, while 3 compounds were found only in the callus.

The above discussion can help to elucidate that the antioxidant, antibacterial, anticancer, and antidiabetic properties of various parts and callus of *P. longum* may be related to the synergistic effects of all these compounds. However, further research on *in vivo* study and isolation of pure compounds is necessary to validate all these bioactivities.

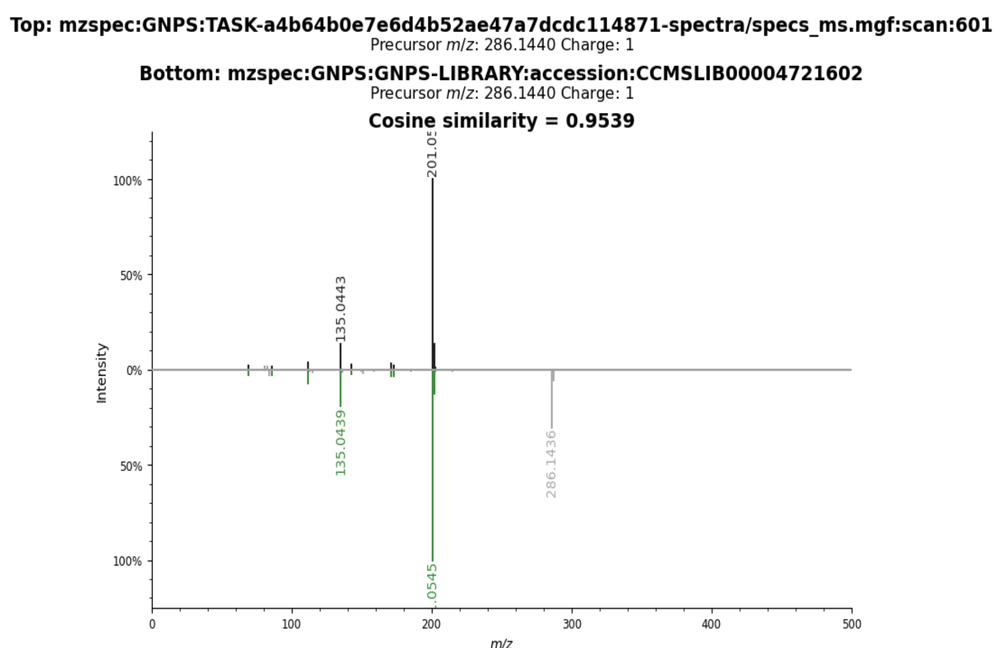


Figure 35: Individual spectrum of piperine with precursor m/z 286.14 Da. [Mirror-match universal spectrum match (USI)]

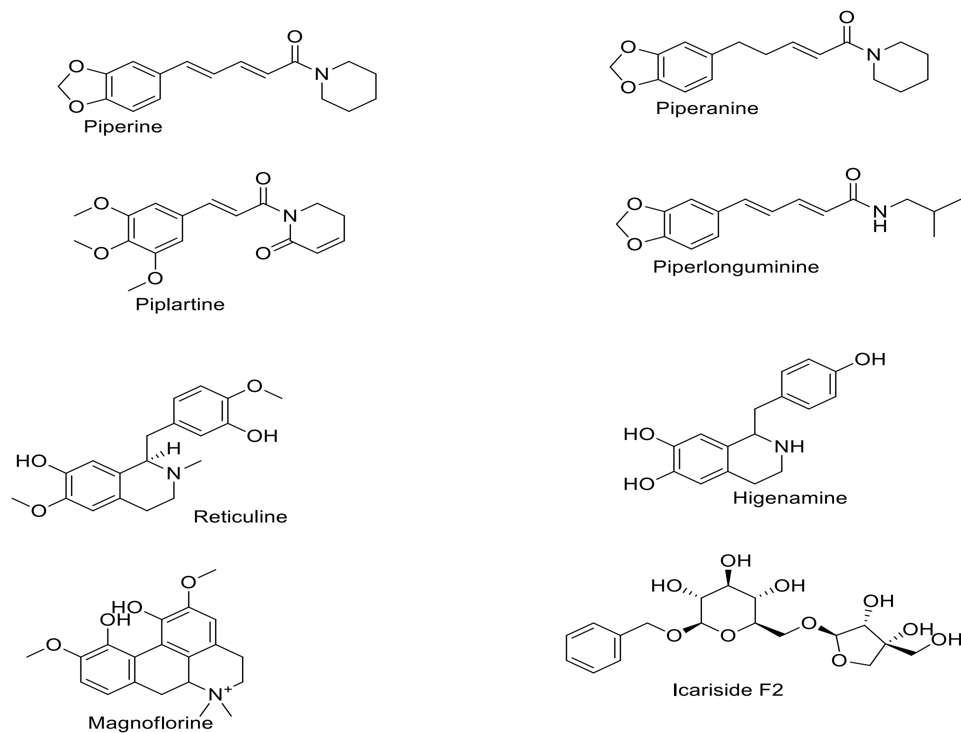


Figure 36: Molecular structure of some bioactive compounds identified in *P. longum* by LC-HRMS analysis.

Table 37: List of compounds detected by HRMS analysis using GNPS databases in roots and callus of *Piper longum*.

Adduct	Compound name	Ion intensity		Ion source	Ion mode	Mass diff	Molecular formula	MzError ppm	Precursor mass	PEPMass	RT min	GNPS spectral ID
		Root	Callus									
$[M+H]^+$	Piperlonguminine	139259.7	92558.01	ESI	+ve	0.00036	$C_{16}H_{19}NO_3$	1.33583	274.14	273.1364	27.58	CCMSLIB00000080181
$[M+Na]^+$	2-beta-D-Glucopyranosyloxy-4-methoxy-cis-cinnamic acid	139259.7	791608.4	ESI	+ve	0.00036	$C_{16}H_{20}O_9$	0.966001	379.1	356.1109	13.16	CCMSLIB00004698822

$[M+H]^+$	$[M+H]^+$	$[M+NH_4]^+$	$[M+H]^+$	$[M]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
Piplartine	Piperanine	Icariside F2	Diosgenin	Magnoflorine	Laurotetanine	Isoboldine	Higenamine	Feruloyltyra mine	Boldine	Asimilobine	Machiline
139259.7	139259.7	139259.7	139259.7	139259.7	139259.7	139259.7	139259.7	139259.7	139259.7	139259.7	139259.7
4736	2437	0	1047	3562212	67481.13	161193.1	489268.5	405288.9	1027	2832	832597.1
ESI	ESI	ESI	ESI	ESI	ESI	ESI	ESI	ESI	ESI	ESI	ESI
+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
0.000397	0.000336	0.000885	0.00064	0	0.00018	0.00039	0.00811	0.00024	0.00473	0.00415	0.00033
$C_{17}H_{19}NO_5$	$C_{17}H_{21}NO_3$	$C_{18}H_{26}O_{10}$	$C_{27}H_{42}O_3$	$C_{20}H_{24}NO_4$	$C_{19}H_{21}NO_4$	$C_{19}H_{21}NO_4$	$C_{16}H_{17}NO_3$	$C_{18}H_{19}NO_4$	$C_{19}H_{21}NO_4$	$C_{17}C_{17}NO_2$	$C_{17}H_{19}NO_3$
1.24705	1.16496	2.10623	1.54307	0	0.557986	1.20897	2.8312	0.777174	1.4148	1.4791	1.17316
318.134	288.159	420.187	415.32	342.17	328.15	328.15	272.12	314.13	328.15	268.13	286.14
317.1262	287.1521	402.153	414.3131	342.1628	327.1469	327.1471	271.1208	313.1316	327.1474	267.1259	285.1364
24.76	27.22	12.74	26.26	13.50	14.39	13.28	11.29	18.71	13.93	14.93	12.80
CCMSLIB00 004720840	CCMSLIB00 004714483	CCMSLIB00 000854306	CCMSLIB00 005769950	CCMSLIB00 010127350	CCMSLIB000 05436056	CCMSLIB00 005436060	CCMSLIB00 006516737	CCMSLIB00 004692249	CCMSLIB00 006439036	CCMSLIB00 005436078	CCMSLIB00 005724962

$[M+H]^+$	Reticuline	139259.7	269923.8	ESI	+ve	0.000427	$C_{19}H_{23}NO_4$	1.29402	330.17	329.1632	13.58	CCMSLIB00005436048
$[M+H]^+$	S-Isocorydine (+)	139259.7	189664.1	ESI	+ve	0.000366	$C_{20}H_{23}NO_4$	1.07026	342.17	341.1631	14.73	CCMSLIB00004694379
$[M+H]^+$	Piperine	808027.9	88910.85	ESI	+ve	3.05E-05	$C_{17}H_{19}NO_3$	3.05E-05	286.144	285.1362	27.44	CCMSLIB00004721602

Table 38: List of compounds detected by HRMS analysis using Sirius 5.6.3 platform in roots and callus of *Piper longum.*

Precursor mass (Da)	**Molecular mass (Da)**	**Extracts**	**Peak**	**Retention time (min)**	**Adduct**	**Collision energy**	**Predicted compound Sirius**	**Molecular formula**	**Databases**	**CSI: Finger ID Score**
220.1181	219.23	Root	48	10.1 min	$[M+H]^+$	25.0 eV	DL-Pantothenic acid (100%)	$C_9H_{17}NO_5$	PubChem	-7.300
239.0911	238.24	Root	77	25.5 min	$[M+H]^+$	20.4 eV	3,4,5-trimethoxyl cinnamic acid (100%)	$C_{12}H_{14}O_5$	PubChem	-2.282
241.1073	240.25	Root	54	19.1 min	$[M+H]^+$	25.5 eV	3(3,4,5)Trimethoxyphenyl)propionic acid (99.53%)	$C_{12}H_{16}O_5$	PubChem	-14.952
288.1233	287.31	Root	52	24.1 min	$[M+H]^+$	26.7 eV	3'-Demethoxypiplartine (74.19%)	$C_{16}H_{17}NO_4$	Pubchem	-46.056

452.1560	451.4	Callus	55	12.0 min	$[M+H]^+$	33.1 eV	5-O-Feruloylnigrumin (60.68%)	$C_{21}H_{25}NO_{10}$	Pubchem	-248.908
452.1560	451.4	Callus	55	12.0 min	$[M+H]^+$	33.1 eV	Lanceolin B (56.40%)	$C_{21}H_{25}NO_{10}$	Pubchem	-213.319
452.1560	451.4	Callus	55	12.0 min	$[M+H]^+$	33.1 eV	Campyloside A (55.50%)	$C_{21}H_{25}NO_{10}$	Pubchem	-238.606
320.1492	319.4	Root	60	24.2 min	$[M+H]^+$	27.8 eV	8,9-Dihydropiplartine (91.17%)	$C_{17}H_{21}NO_5$	Pubchem	-40.986
302.1754	301.4	Root	57	28.3 min	$[M+H]^+$	27.1 eV	7-Benzo[1,3]dioxol-5-yl-hepta-2,4-dienoic acid isobutyl-amide (99.97%)	$C_{17}H_{21}NO_5$	PubChem	-44.187
384.2556	383.5	Root	148	35.3 min	$[M+H]^+$	30.5 eV	Guineesine (100%)	$C_{24}H_{33}NO_3$	PubChem	-16.075

4.4.3 Assessment of putative plant metabolites in crude extracts of *in vitro*-raised callus and *in vivo* rhizome of *P. polyphylla* by LC-HRMS analysis

Crude extracts of *in vitro*-grown callus and DCM fraction of wild-grown rhizome of *P. polyphylla* were examined using LC-HRMS to identify the bioactive compounds. The bioactive compounds of the DCM fraction of rhizome were analyzed and compared to those of callus produced *in vitro*. The compounds that have been identified are presented in Tables 42 and 43, along with information about their ion intensity, parent mass and exact mass (PEPMass), molecular formula, mass-to-charge errors in parts per million (ppm), retention time (Rt) in minutes, GNPS spectral ID, and ESI positive ion

mode measurements. The compounds were identified using the measured mass spectrum, and the results were validated by comparing them to existing literature. There were 31 compounds found from both the rhizome and the callus, with 24 compounds from the rhizome and 18 from the callus. GNPS databases revealed 19 compounds, while Sirius databases revealed 12 compounds. The common adduct ions generated during measurement were $[M+H]^+$, $[M+Na]^+$, and $[M+H-H_2O]^+$.

This study also showed that rhizomes contained more bioactive compounds than callus, the majority of which were steroidal saponin and polyphenolic compounds. The higher number of compounds detected in wild-grown rhizome compared to *in vitro* generated callus may be due to the complex interaction of environmental (sunlight, soil composition, temperature, humidity, and stress), developmental, genetic, and microbial factors in wild-grown plants. *P. polyphylla* contains steroidal saponins, flavonoid glycosides, sterols, triterpenoid saponins, and polysaccharides (Thapa *et al.,* 2022). This study identified a few steroidal saponins and ecdysteroids from the rhizome and callus that were previously reported from the *P. polyphylla* rhizome, including Paris saponin V (m/z 723.43) (Xiao *et al.*, 2009), polyphyllin VI (m/z 739.427) (Lin *et al.*, 2015; Liu *et al.*, 2020), diosgenin (m/z 414.31) (Wang *et al.*, 2010), pinnatasterone (m/z 480.6) and 20-hydroxyecdyson (m/z 480.6) (Kang *et al.*, 2012). However, polyphenolic, glycosides, and oxylipins were also identified from the rhizome and callus. Important polyphenols identified from the rhizome and callus were chlorogenic acid (m/z 354.09), feruloylputrescin (m/z 313.13), caffeoylputrescin (m/z 250.13), gaultherin (m/z 446.14), etc. (Fig. 37 and 38).

Polyphyllin VI is a steroidal saponin that shows anticancer properties on human lung cancer cell lines (Lin *et al*., 2015) and brain carcinoma cell lines (Liu *et al*., 2020). Paris saponin V can be utilized as an antioxidant, antimicrobial, and anticancer agent (Negi *et al.*, 2014). Diosgenin is also a steroidal saponin that has free radical scavenging and inflammation reducing effects and can be used to treat blood and cerebral disorders, allergies, diabetes and obesity, symptoms of menopause, and skin aging; it may additionally protect against cardiovascular disease and act as an anticancer agent (Manivannan *et al*., 2913; Jesus *et al*., 2016). Similarly, 20-Hydroxyecdysone is a naturally occurring ecdysteroid hormone that regulates cell division, growth, and apoptosis (cell death) (Nelson and Baehrecke, 2014). Pinnatasterone, an ecdysteroid derived from *Vitex pinnata*, has limited action against *Musca domestica* larvae

(Suksamrarn and Sommechai, 1993). Furthermore, chlorogenic acid has medicinal properties such as inflammation reducer, free radical scavenger, antibacterial, antitumor, liver and kidney protection, neurological protection, and glucose and cholesterol control (Dai *et al.*, 2024). It was identified from a callus extract of *P. polyphylla*. Similarly, gaultherin, methyl salicylate glycoside, was also identified from a callus extract. It functions as an effective anti-inflammatory drug, with medium antioxidant and antibacterial action *in vitro* and considerable insecticide and larvicidal potential (Michel and Olszewska, 2024). It was found that callus was capable of synthesizing key bioactive compounds in MS media fortified with PGRs, which could explain why callus exhibited antioxidant, antibacterial, cytotoxic, and antidiabetic activities *in vitro*, as demonstrated by wild-grown parts of *P. polyphylla*.

This study also found that there were 11 compounds common to both rhizome and callus, however, their ion concentration was higher in the rhizome than in callus (Table 40). It might be due to the differences in tissue-specific metabolism, environmental and nutritional variables, as well as age and differentiation factors. Rhizomes and calli have distinct metabolic activity. The rhizome, as a specialized tissue, may naturally develop and accumulate larger quantities of some secondary metabolites than callus tissue, which is more undifferentiated and unspecialized. Similarly, 13 compounds were found only in the rhizome, while 7 compounds were found only in the callus.

The above discussion can help to elucidate that the antioxidant, antibacterial, anticancer, and antidiabetic properties of *P. polyphylla* rhizome and callus may be related to the synergistic effects of all these compounds. However, further research on *in vivo* study and isolation of pure compounds is necessary to validate all these bioactivities.

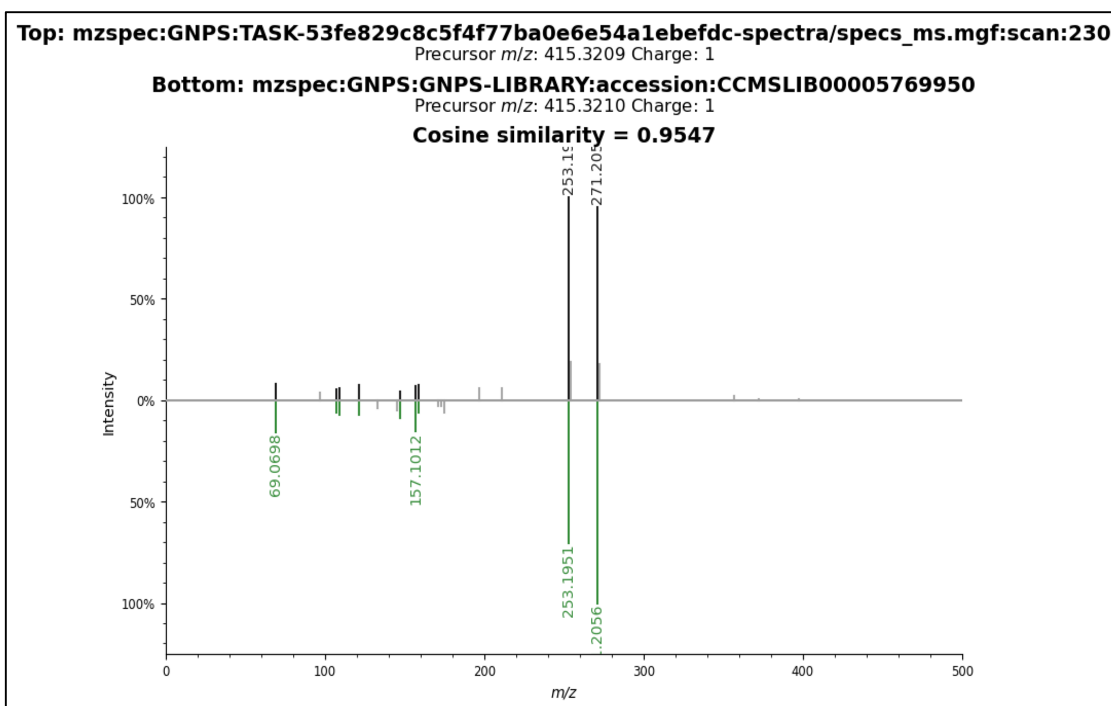


Figure 37: Individual spectrum of diosgenin with precursor m/z 415.32 Da**.** [Mirror match-universal spectrum identifier (USI)]

Table 39: List of compounds detected by HRMS analysis using GNPS databases in rhizome and callus of *Paris polyphylla.*

Adduct	Compound name	Ion intensity		Ion source	Ion mode	Mass diff.	Molecular formula	MzError ppm	Precursor mass	PEPMass	RT min	GNPS spectral ID
		Rhizome	Callus									
$[M+H]^+$	Chlorogenic acid	0	802150.4	LC-ESI	+ve	0.00030	$C_{16}H_{18}O_9$	0.8594	355.10	354.095	12.53	CCMSLIB00010152856
$[M+H]^+$	Feruloyltyramine	0	181086.5	nan	+ve	0.00027	$C_{18}H_{19}NO_4$	0.8743	314.13	313.131	18.72	CCMSLIB00004692249
$[M+H]^+$	Diosgenin	2802676.7	1619	ESI	+ve	0.00012	$C_{27}H_{42}O_3$	0.2939	415.32	414.313	25.71	CCMSLIB00005769950
$[M+H]^+$	Succinoadenosine	119474.55	2515	LC-ESI	+ve	0.00473	$C_{14}H_{17}N_5O_8$	1.3144	384.115	383.108	9.71	CCMSLIB00005467722

$[M+H-H_2O]^+$	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-H_2O]^+$	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$
Coronaric acid	Vernolic Acid	Beta-Gentiobiose	Feruloyl putrescine	Phytosphingosine	1-Palmitoyl-sn-glycero-3-phosphocholine	Linoleoyl ethanolamide	13-Keto-9Z,11E-octadecadienoic acid	9s,13r-12-Oxophytodienoic Acid	Tetradecyldiethanolamine	Gaultherin	Caffeoyl putrescin
2230677.7	1149161.6	0	4142	357718.31	159288.63	2063867.8	577230.69	1998946	77453.48	0	4458
90559.38	0	237836.9	4813913	4066	7749	1225	1890	0	357341.78	1582804	3048873
ESI	ESI	ESI	ESI	ESI	ESI	ESI	ESI	LC-ESI	ESI	LC-ESI	ESI
+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
0.000275	0.002258	0.006378	0.000458	0.000519	0.000183	0.000183	0.000122	0.000519	0.000336	3.05E-05	0.00114
$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$	$C_{12}H_{22}O_{11}$	$C_{14}H_{20}N_2O_3$	$C_{18}H_{39}NO_3$	$C_{24}H_{50}NO_7P$	$C_{20}H_{37}NO_2$	$C_{18}H_{30}O_3$	$C_{18}H_{28}O_3$	$C_{18}H_{39}NO_2$	$C_{19}H_{26}O_{12}$	$C_{13}H_{18}N_2O_3$
0.98362	1.59757	1.6187	1.7264	1.62991	0.368911	0.564635	0.440344	1.88517	1.11045	0.0650	1.5568
279.232	297.242	325.113	265.155	318.301	496.34	324.29	277.216	275.201	302.305	469.13	251.13
296.4	296.2348	342.30	264.1474	317.2932	495.3329	323.2826	294.22	292.204	301.2981	446.142	250.131
31.26	33.89	11.93	11.07	23.77	29.09	34.917	29.544	22.04	24.27	13.30	9.32
CCMSLIB00003134722	CCMSLIB00003135278	CCMSLIB00003137457	CCMSLIB00005748443	CCMSLIB00003139655	CCMSLIB00003136336	CCMSLIB00003138141	CCMSLIB00003137023	CCMSLIB00000855852	CCMSLIB00005765229	CCMSLIB00000855202	CCMSLIB00005742467

$[M+H-H_2O]^+$	9,10-Dihydroxy-12Z-octadecenoic acid	171611.95	1938	ESI	+ve	0.000275	$C_{18}H_{34}O_4$	0.924022	297.242	314.246	27.13	CCMSLIB00003135128
$[M+H]^+$	Paris Saponin V	121487.7	0	ESI	+ve	-	$C_{39}H_{62}O_{12}$	-	723.431	722.9	27.72	-
$[M+H]^+$	Polyphyllin VI	203103.3	0	ESI	+ve	-	$C_{39}H_{62}O_{13}$	-	739.427	738.9	15.95	-

Table 40: List of compounds detected by HRMS analysis using Sirius 5.6.3 platform in rhizome and callus of *Paris polyphylla.*

Precursor mass (Da)	Molecular mass (Da)	Extract	Peak	Retention time	Adduct	Collision energy	Predicted compound (Sirius)	Molecular formula	Databases	CSI: Finger ID Score
481.3171	480.6	Rhizome	93	14.2 min	$[M+H]^+$	34.3 eV	20-Hydroxyecdysone (Similarity: 99.99%)	$C_{27}H_{44}O_7$	PubChem	-3.787
855.4750	855.02	Rhizome	66	26.4 min	$[M+H]^+$	42.1 eV	Chonglou saponin I (Similarity: 99.57%)	$C_{44}H_{70}O_{16}$	PubChem	-29.304

315.2531	285.2424	464.3590	274.2743	315.25331	313.2371
314.5	284.4	463.6	252.26	314.5	330.5
Rhizome	Rhizome	Rhizome	Rhizome	Rhizome	Rhizome
135	64	63	57	8	8
27.5	36.3 min	22.4 min	21.7 min	27.6 min	23.2 min
$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M-H_2O+H]^+$
27.6 eV	26.6 eV	33.6 eV	26.4 eV	27.6 eV	27.5 eV
12,13-Dihydroxy-9-octadecenoic acid (Similarity: 100%)	7-Ketoheptadecanic acid(Similarity: 100%)	D-Glucosyldihydrosphingosine (Similarity: 99.25%)	3-beta-D-Glucopyranosyloxy-2-butanol(Similarity: 100%)	9,12-Dihydroxy-10-octadecenoic acid (Similarity: 100%)	9,12,13-Todea (Similarity: 100%)
$C_{18}H_{34}O_4$	$C_{17}H_{32}O_3$	$C_{24}H_{49}NO_7$	$C_{16}H_{35}NO_2$	$C_{18}H_{34}O_4$	$C_{18}H_{34}O_5$
Pubmed	Pubchem	PubChem	PubChem	Pubmed	Pubmed
-19.610	-40.670	-15.00	-18.224	-16.717	-29.882

344.1495	284.1284	251.1387	481.3163
343.4	283.32	250.29	480.6
Callus	Callus	Callus	Rhizome
18	30	61	188
19.0	18.9	10.2	14.4
$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
28.8 eV	26.6 eV	25.8 eV	34.3 eV
N-trans-Feruloylmethoxytyramine (Similarity: 9.94%)	Cinnamamide, p-hydroxy-N-(p-hydroxyphenethyl)-(Similarity: 88.50%)	N-(4-aminobutyl)-3-(3,4-dihydroxyphenyl)prop-2-enamide(Similarity: 99.90%)	Pinnatasterone(Similarity: 99.99%)
$C_{19}H_{21}NO_5$	$C_{17}H_{17}NO_3$	$C_{13}H_{18}N_2O_3$	$C_{27}H_{44}O_7$
Pubmed	Pubmed	Pubmed	Pubmed
-32.756	-5.243	-7.064	-5.225

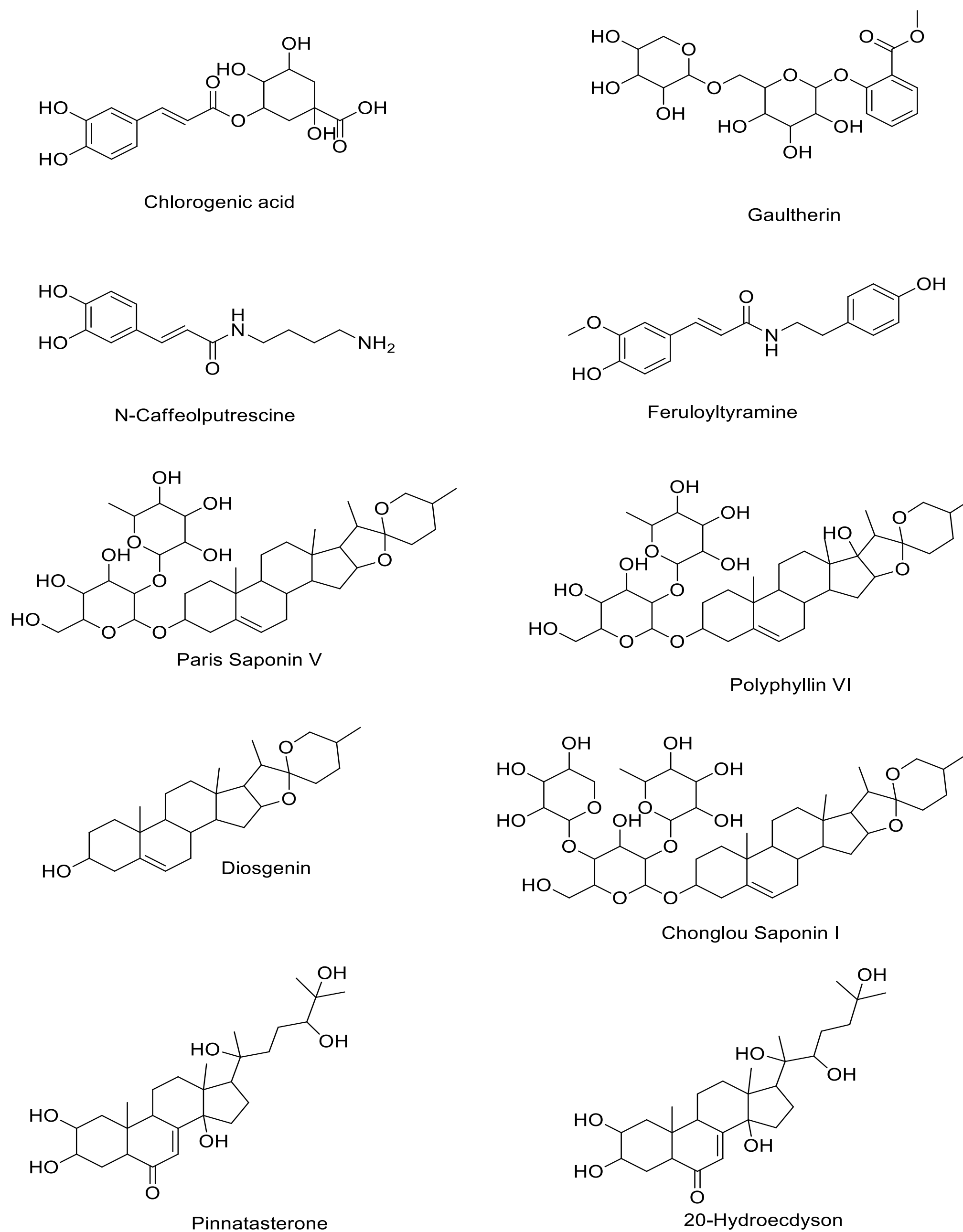


Figure 38: Molecular structure of some compounds identified in *P. polyphylla* by LC-HRMS analysis.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The whole study concludes that the valuable plant resources *Piper longum* and *Paris polyphylla* can be established *in vitro* utilizing nodal and leaf explants in MS medium for *ex-situ* conservation, as their populations are declining in their natural habitat. Regeneration of plants from nodal explants in *P. longum* via direct organogenesis is difficult due to frequent contamination by endogenous systemic bacteria and metabolite release, which causes media browning. However, regeneration of plants from leaf explants via indirect organogenesis, such as shoot buds differentiation or somatic embryogenesis, is safer for culture management and can produce a larger number of plants. Similarly, plant regeneration in *P. polyphylla* from shoot apex and rhizome explants is difficult due to the slow response of explants to nutrient media. Therefore, plant regeneration from leaf explants via indirect organogenesis is beneficial for the generation of a higher number of plants in a shorter duration. Furthermore, although the *in vitro*-raised callus of *P. longum* and *P. polyphylla* exhibited comparable antioxidant, antibacterial, anticancer, and antidiabetic activities, *in vivo* parts showed higher antioxidant, antibacterial, anticancer, and antidiabetic properties due to the existence of higher number and more important bioactive compounds. However, *in vitro*-raised callus of *P. longum* and *P. polyphylla* exhibited higher antioxidant activity, TPC, and TFC in suspension culture in MS media treated with salicylic acid and phenylalanine, as well as in their absence. Thus, metabolite enhancement can be done using salicylic acid and phenylalanine in a callus suspension culture. Similarly, a greater number of known metabolites have been identified in *in vivo* parts than in *in vitro*-grown callus of both plants, which could be attributed to the growth of *in vivo* plants under more harsh environmental conditions. Moreover, *in vitro*-grown callus can be utilized to regenerate plants as well as produce valuable bioactive compounds, which will aid in the conservation of wild-grown plants in natural habitats. This study concludes with the following points.

1) *Piper longum*

- Induction of callus and embryogenic/nodular callus with embryos was effective in 2,4-D and KN in leaf explants, while NAA was effective in inducing callus

and embryogenic/nodular callus with embryos from nodal explants. This is the first report of somatic embryogenesis in *P. longum*.

- TDZ favors both direct and indirect organogenesis over BAP and KN and also favors embryo differentiation and conversion over full-strength MS in the absence of PGRs.
- IBA was more effective for rooting during direct and indirect organogenesis from leaf callus, but NAA was effective during indirect organogenesis from nodal callus.
- Based on the significant *in vitro* activities and higher number of compounds (alkaloids and polyphenols), the DCM fraction of roots can be utilized as a source of antioxidants, antibacterials, antidiabetics, and cytotoxics for brine shrimp nauplii. DCM fractions of *in vivo* parts were more active than methanol fractions and crude extracts. However, crude fruit extract was more active against U2-OS and HeLa cell lines, indicating that it could be utilized as a source of natural medicine for bone and cervical cancers.
- Methanolic crude extract of callus could be used as an antioxidant, antibacterial, cytotoxic, and antidiabetic agent. Because it exhibited comparable bioactive compounds and significant bioactivity *in vitro*.
- Phenylalanine can be utilized for the enhancement of metabolite synthesis and production in suspension culture.

2) *Paris polyphylla*

- Callus can be induced with a combination of 2,4-D and KN from leaf explants, however, callus differentiation into mini-rhizomes and roots occurs at greater KN concentrations.
- Based on the significant *in vitro* bioactivities and higher number of compounds (steroidal saponin and polyphenols), the DCM fraction of rhizome can be used as an antioxidant, antibacterial, cytotoxic for brine shrimp nauplii, and an anticancer agent against HeLa cell lines. The methanol fraction of rhizome can be utilized as an anticancer agent against MCF-7 cells. However, crude methanol rhizome extracts may be utilized as a source of anti-diabetic drugs.

- Methanolic crude extract of callus could be used as an antioxidant, antibacterial, cytotoxic, and antidiabetic agent. Because it exhibited comparable bioactive compounds and bioactivities *in vitro*.
- MS media enriched with BAP and NAA can be used for the enhancement of metabolite production in suspension culture.

5.2 Recommendations

- A detailed investigation of the acclimatization of *in vitro*-raised plants in various substrates is required for the successful propagation of *P. longum* and *P. polyphylla* in natural habitats.
- As the government of Nepal has prioritized *P. longum* and *P. polyphylla* for research and development as well as agrotechnology development, there should be a collaborative effort between the government and researchers to conserve these plants *ex-situ* by micropropagation and to analyze bioactive compounds in detail.
- Further works such as alternative and advanced assays for antioxidant (e.g., ABTS, HRSA assays), anticancer (e.g. Annexin V/Propidium iodine staining, Western blot analysis), and antidiabetic (e.g., RLAR inhibition) activities are required to confirm bioactivities and validate tested assays.
- To confirm the bioactivities of *in vitro*-raised callus with alternative PGR combinations (other than those used in this study) and suspension culture using precursor and elicitors, as well as to identify and characterize their bioactive compounds, further research is needed.
- Bioactivity analysis and compound identification in callus that was grown for more than 20 weeks is necessary.
- Isolation and characterization of bioactive compounds produced/synthesized in callus suspension culture using NMR is necessary.

CHAPTER 6

6. SUMMARY

Piper longum L. (Piperaceae) is a valuable tropical and subtropical medicinal plant used for the treatment of coughs, colds, bronchitis, insomnia, and anti-diabetes. Similarly, *Paris polyphylla* Sm. (Melanthiaceae) is an endangered medicinal plant that grows from at altitudes of 1800 to 3300 meters and is used to treat cuts, burns, boils, vermifuge, stomachaches, and various types of cancer. However, unsustainable utilization, illegal collection and export, habitat destruction, and climate change are threatening its survival in its natural habitats. As a result, their population in natural habitats is declining. In addition, the Nepalese government has prioritized *P. longum* and *P. polyphylla* for research and development, as well as agrotechnology for the development of agrotechnologies. Therefore, this research aims to regenerate plants *in vitro* from different explants and compare the bioactivity and bioactive compounds of tissue culture-grown callus with wild-grown parts of *P. longum* and *P. polyphylla.*

The *in vitro* culture was performed using the standard technique in MS medium supplemented with dichlorophenoxyacetic acid (2,4-D), kinetin (KN), naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), thidiazuron (TDZ), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA) and 10% coconut water. Similarly, antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, total phenolic content (TPC) by Folin-Ciocalteu reagent, and total flavonoid content (TFC) by $AlCl_3$-complex assay, antibacterial activity by agar well diffusion assay, anticancer activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, and antidiabetic activity by alpha-glucosidase and alpha-amylase inhibition assays. Callus suspension culture was prepared in an MS medium containing salicylic acid (elicitor) and phenylalanine (precursor). The putative compounds of the extracts were analyzed using LC-HRMS, Metaboscape, GNPS databases, and Sirius databases.

P. longum was micropropagated *in vitro* using node and leaf explants. The most efficient medium for direct organogenesis by regeneration of multiple shoots (5.33±1.15) from nodal explants was MS + 1.0 mg/L TDZ. The highest number of roots (7.0±1.0) was regenerated in MS + 1.0 mg/L IBA from *in vitro* shoots. The plant was also regenerated by indirect organogenesis using leaf and node explants, and by the

process of somatic embryogenesis. Calli were induced from leaf explants in MS media enriched with 2,4-D alone or together with 2.4-D and KN, whereas they were induced from nodal explants in MS mediua containing only NAA. The most effective medium for multiple shoot differentiation was MS + 0.25 mg/L TDZ (25.33±1.52) for leaf callus and MS + 3.0 mg/L NAA (18.33±1.50) for nodal callus. Subsequently, roots were regenerated from *in vitro* shoots using MS + 0.5 mg/L IBA (8.33±1.52) from the leaf callus and MS + 2.0 mg/L NAA (10.66±1.52) from the nodal callus. In addition, the best media for inducing a maximum number of embryogenic/nodular callus with somatic embryos (SEs) were MS + 3.5 mg/L 2,4-D (16.66±2.08 SEs), MS + 1.5 mg/L 2,4-D + 1.0 mg/L KN (28.33±3.05 SEs) for leaf callus, and MS + 1.0 mg/L NAA (12.66±2.51 SEs) for nodal callus. Nodular calli with SEs differentiated into plantlets/seedlings both in MS + 0.5-2.5 mg/L TDZ and in full-strength MS media without PGRs. Similarly, *P. polyphylla* was also micropropagated *in vitro* using leaf explants, where the best media for callus formation and mini-rhizomes with root differentiation were MS + 0.25 mg/L 2,4-D + 0.5 mg/L KN and MS + 2.5 mg/L KN respectively.

Regarding the analysis of the bioactivity of crude extracts and fractions of *P. longum*, the DCM fraction of the root showed the highest antioxidant activity (IC_{50}: 134.81±1.16 µg/mL), TPC, and TFC compared to other extracts of wild-grown parts and *in vitro* callus. It showed 100% growth inhibition against five bacterial strains, including three Gram-negative (*P. aeruginosa, E. coli*, and *A. baumannii*) and two Gram-positive (*S. aureus* and *B. subtilis*), with the lowest MIC at 5.0 mg/mL and MBC at 8.35 mg/mL on *S. aureus*. It was more cytotoxic to brine shrimp nauplii (LC_{50}: 156.78±5.49 µg/mL), while the crude extract of the fruit showed higher anticancer activity (IC_{50}: 146.55±2.31 µg/mL) on U-2 OS and HeLa cell lines (IC_{50}: 273.31±17.89 µg/mL). Similarly, the DCM fraction of the root exhibited the strongest inhibition of α-amylase (IC_{50}: 365.21±31.021 µg/mL) and α-glucosidase (IC_{50}: 489.07±27.966 µg/mL) compared to other extracts of wild parts and *in vitro* callus. In addition, the callus suspension culture of *P. longum* revealed that MS medium treated with salicylic acid increased callus biomass compared to other treatments, while MS medium treated with 100 mg/L phenylalanine resulted in maximum antioxidant activity (IC_{50}: 22.44±1.96 µg/mL), TPC, and TFC.

Also, when analyzing the bioactivity of the crude extract and fractions of *P. polyphylla*, the DCM fraction of the rhizome showed the highest antioxidant activity (IC_{50}: 197.06±0.635 µg/mL), TPC, and TFC compared to other extracts of the wild-grown rhizome and *in vitro* callus. It also showed 100% growth inhibition against five bacterial strains, including *P. aeruginosa, E. coli, A. baumannii, S. aureus,* and *B. subtilis*, with the lowest MIC at 5.0 mg/mL, and MBC at 7.5 mg/mL for *E. coli*. It was more cytotoxic to brine shrimp nauplii (LC_{50}: 201.78±70.97 µg/mL). It also showed higher anticancer activity (IC_{50}: 235.94±0.72 µg/mL) on HeLa cell lines, but methanol fraction on MCF-7 cell lines (IC_{50}: 211.36±0.57 µg/mL). The crude extract of the rhizome inhibited α-amylase (IC_{50}: 95.45±8.71 µg/mL) and α-glucosidase (IC_{50}: 51.40±8.62 µg/mL) more effectively than other extracts from the rhizome and *in vitro* callus. In addition, the callus suspension culture of *P. polyphylla* revealed that MS media treated with salicylic acid increased callus biomass more than other treatments, but liquid MS media enriched with BAP and NAA resulted in the highest antioxidant activity (IC_{50}: 102.52±1.29 µg/mL), TPC, and TFC than MS media treated with phenylalanine or salicylic acid.

LC-HRMS analysis of the DCM fraction of the root and crude extract of the *in vitro*-grown callus of *P. longum* revealed a total of 27 compounds (mainly alkaloids and polyphenols), with 16 compounds present in both the roots and the callus. Similarly, the DCM fraction of the rhizome and the crude extract of the *in vitro*-grown callus of *P. polyphylla* had a total of 31 compounds (mainly steroidal saponins and polyphenols), with 11 compounds present in both the rhizome and the callus. The protocols developed for the regeneration of plants either by direct organogenesis or indirect organogenesis could be useful for the *ex-situ* management and conservation of these medicinal plants. In addition, the roots of *P. longum* and the rhizomes of *P. polyphylla* can be used for the preparation of natural medicines, especially for antibacterial, anticancer, and antidiabetic diseases, and *in vitro* callus could be a valuable source of antibacterial, anticancer, and antidiabetic agents after increasing metabolite production by suspension culture. However, further studies are needed to isolate pure compounds and test them *in vivo* required to validate bioactivity.

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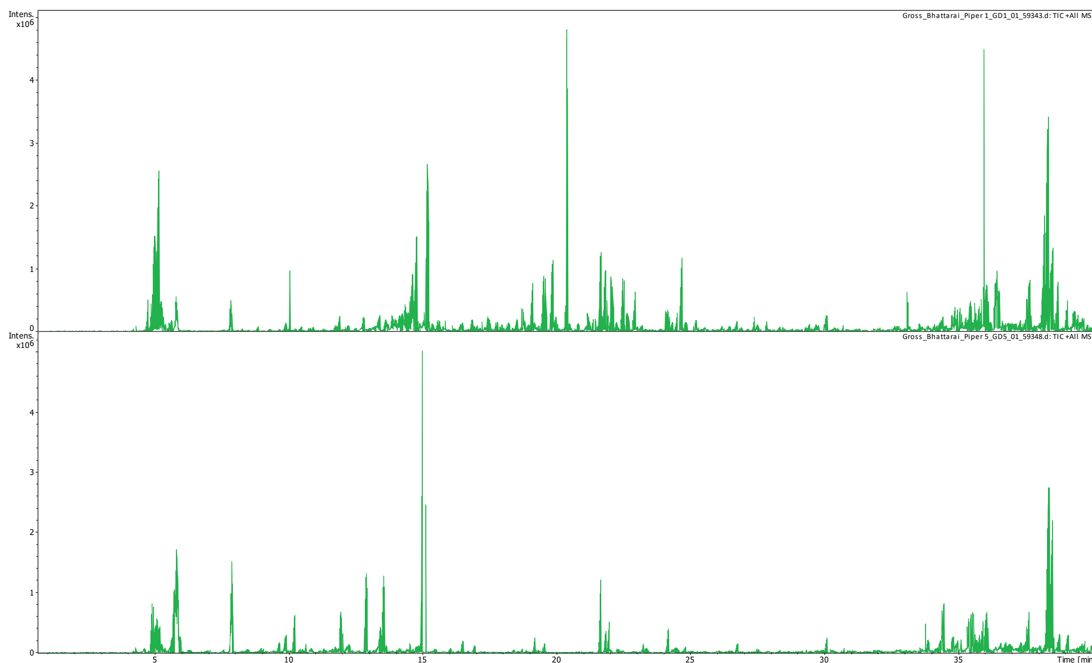
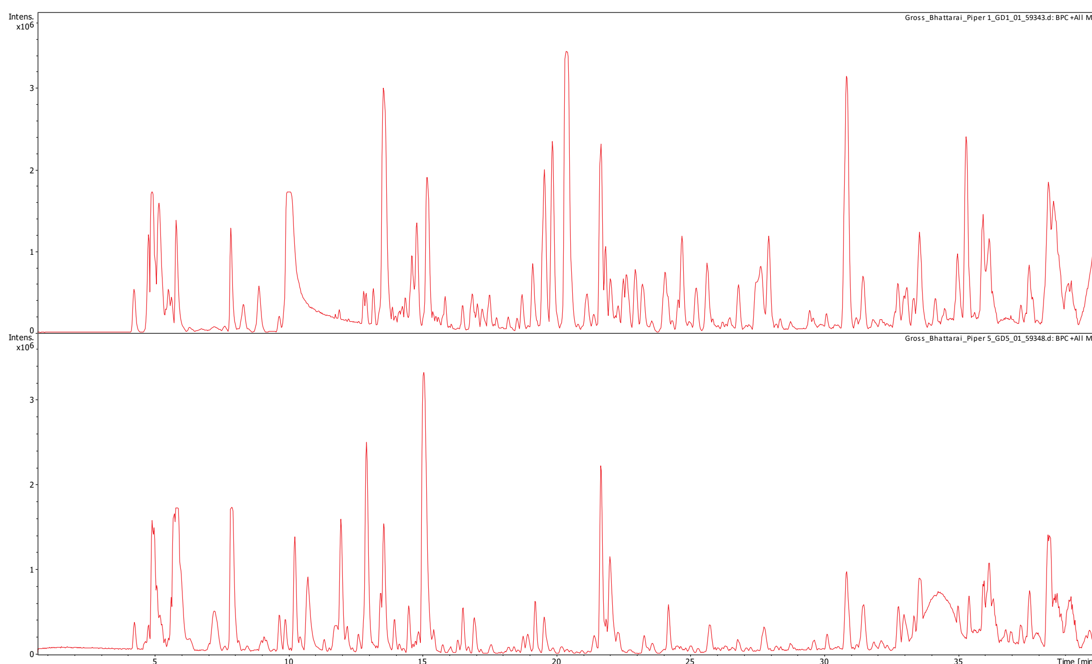
APPENDICES

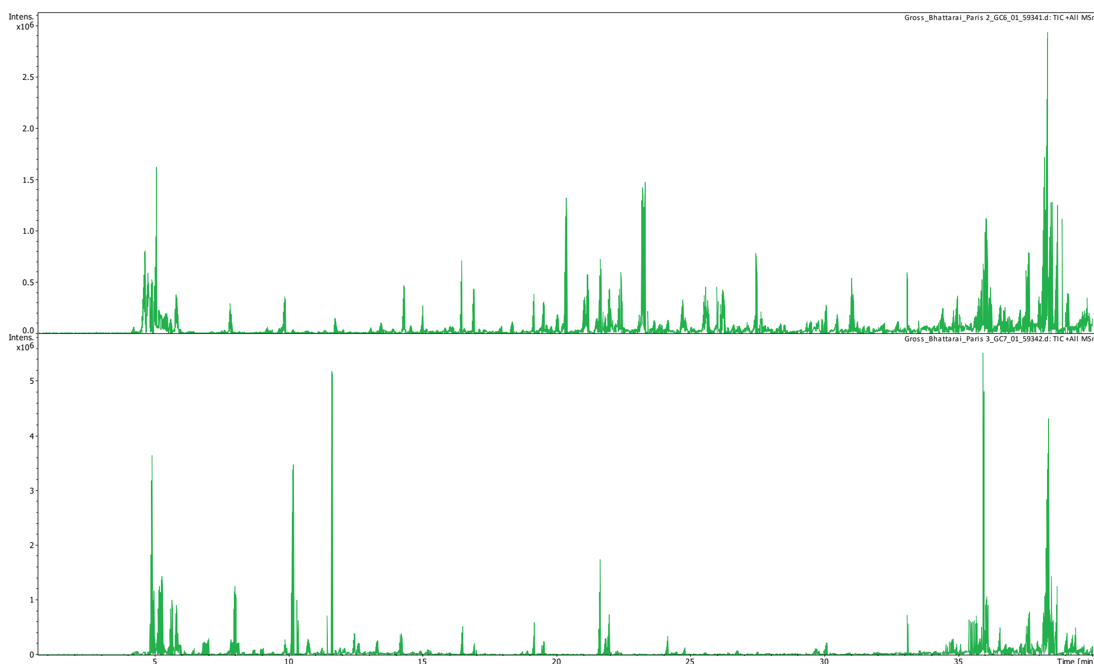
Appendix A: Chromatograms, molecular structures, and fragmentation (HRMS analysis)



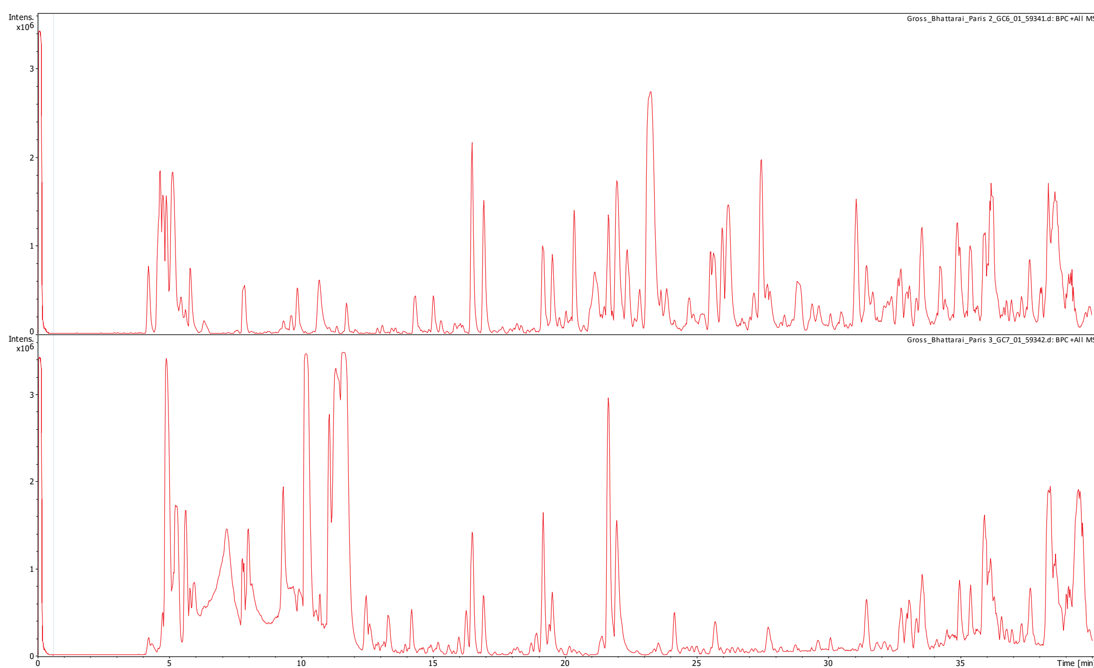
Total Ion Chromatogram (TIC): DCM fraction of root and methanol crude extract of callus of *Piper longum*



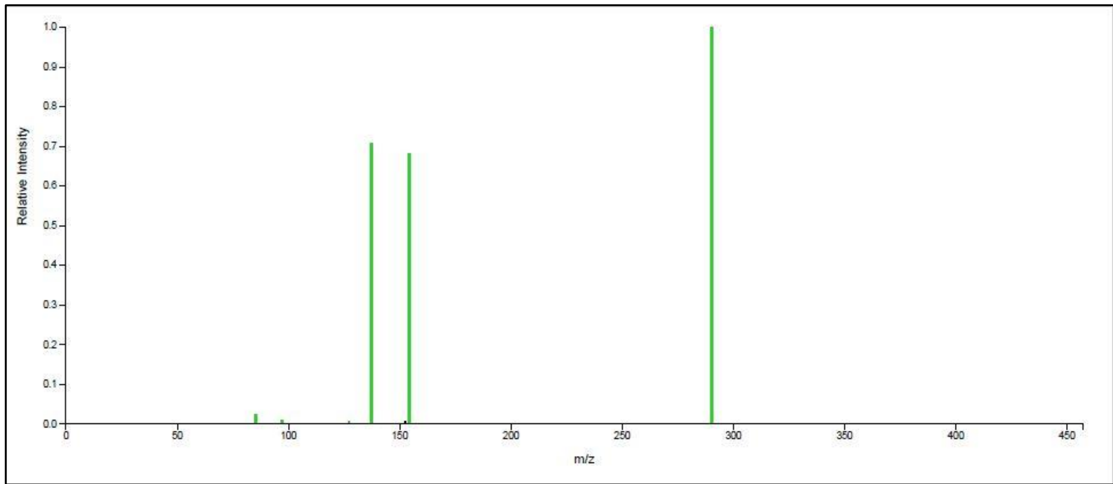
Base Peak Chromatogram (BPC): DCM fraction of root and methanol crude extract of callus of *Piper longum*



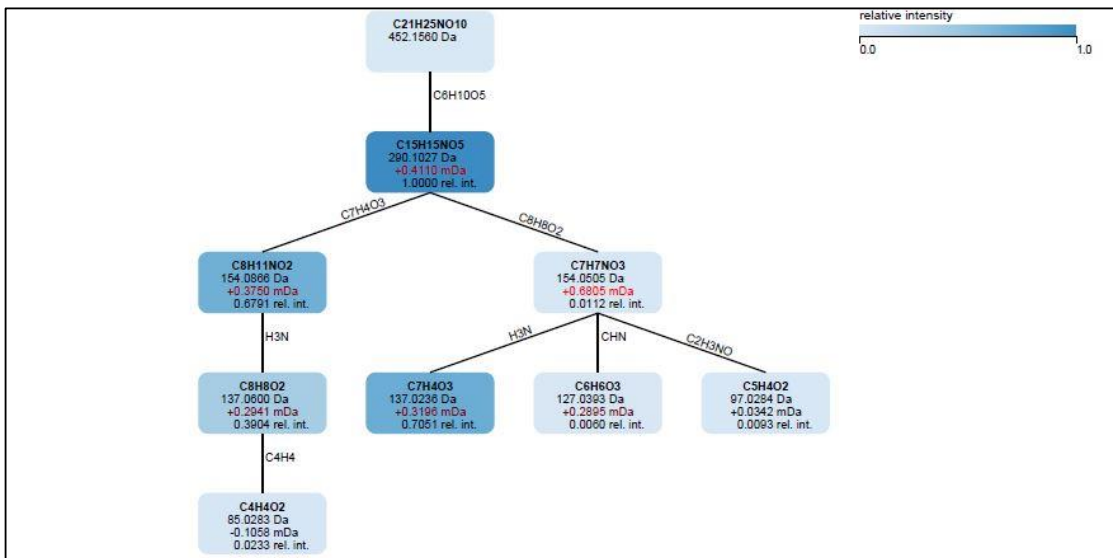
Total Ion Chromatogram (TIC): DCM fraction of rhizome and methanol crude extract of callus of *Paris polyphylla*



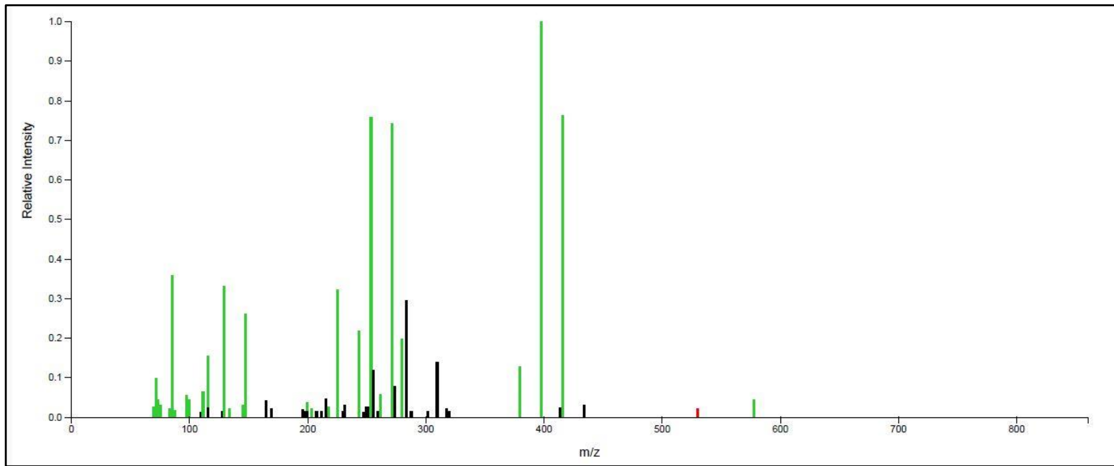
Base Peak Chromatogram (BPC): DCM fraction of rhizome and methanol crude extract of callus of *Paris polyphylla*



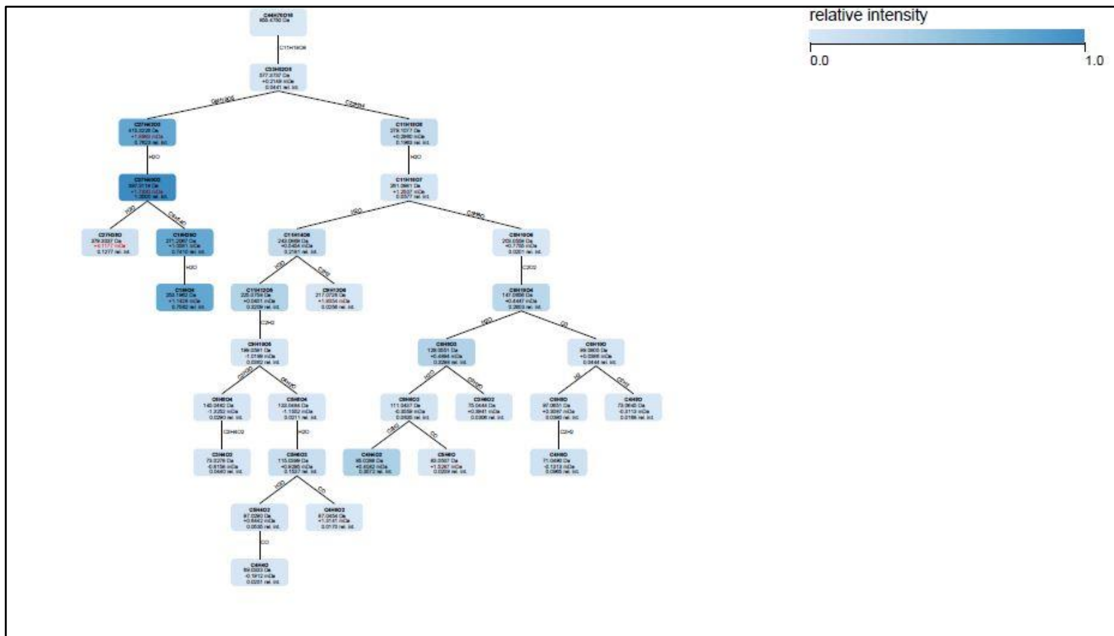
Spectrum Campyloside A isolated from Sirius databases precursor mass m/z 452.15 Da (*Piper longum*)



Fragmentation pattern from Sirius databases precursor mass m/z 452.15 Da (*Piper longum*)



Spectrum Chonglou saponin I isolated from Sirius databases precursor mass m/z 855 Da (*Paris polyphylla*)



Fragmentation pattern Chonglou saponin I from Sirius databases precursor mass m/z 855 Da (*Paris polyphylla*)

Appendix B: Scientific Publications and Conference/Seminar/Workshop Participation

Appendix B1: List of published research articles

(1) **Thapa, C.B.**, Bhattarai, H.D., Pant, K.K., Joshi, P.R., Chaudhary, T.L., and Pant, B. (2023). Antioxidant, antibacterial, and cytotoxic effect of *in vitro* callus and *in vivo* rhizome of *Paris polyphylla* Sm. *Process Biochemistry*, 124: 33–43. https://doi.org/10.1016/j.procbio.2022.11.005.

(2) **Thapa, C. B**., Bhattarai, H. D., Pant, K. K., & Pant, B. (2024). Comparative Antioxidant, Antibacterial, and Antidiabetic Activities of *in vitro*-grown Callus and Wild-grown Various Parts of *Piper longum* L. *Phytomedicine Plus*, 4: 100586. https://doi.org/10.1016/j.phyplu.2024.100586.

(3) **Thapa, C. B.**, Pant, K. K., Bhattarai, H. D., Ghimire, M., Sah, A. & Pant, B. (2024). *In Vitro* Propagation and Evaluation of Genetic Homogeneity Using RAPD, ISSR, and SCoT Markers in *Piper longum* L. *South African Journal of Botany*, **172**, 609-618. https://doi.org/10.1016/j.sajb.2024.07.066

(4) **Thapa, C. B**., Pant, K. K., Bhattarai, H. D., & Pant, B. (2023). *In Vitro* Induction and Proliferation of Callus in *Piper longum* L. through Leaf Culture. *Nepal Journal of Science and Technology,* **21**(1): 13-22. https://doi.org/10.3126/njst.v21i1.49892

(5) **Thapa, C. B**., Bhattarai, A., Pant, K. K., Bhattarai, H. D., Pant, B. (2023). Evaluation of antioxidant, antidiabetic, and cytotoxic activities of *Lilium nepalense* D. DON. *Journal of Institute of Science and Technology,* 28(2): 63-70. https://doi.org/10.3126/jist.v28i2.61174

(6) **Thapa, C. B.,** Pant, K. K., Bhattarai, H. D., Thapa, M., & Pant, B. (2024). Induction, Proliferation and Differentiation of Callus in *Paris polyphylla* Sm. through Leaf Culture. *Journal of Nepal Biotechnology Association*, 5 (1): 8-15

(7) **Thapa, C. B.**, Bhattarai, H. D., Pant, K. K., & Pant, B. (2024). Comparative Antioxidant, Antibacterial, and Antidiabetic Activities of *in Vitro*-Grown Callus and Wild-Grown Various Parts of *Piper longum* L. *Phytomedicine Plus*, 4 (100586): 1-13, https://doi.org/10.1016/j.phyplu.2024.100586

(8) Thapa, C. B, Paudel, M. R, Bhattarai, H. D, Pant, K. K, Devkota, H. P, Adhikari, Y. P., & Pant, B. (2022). Bioactive secondary metabolites in *Paris polyphylla* Sm. and their biological activities: A review. *Heliyon*, **8**(2): e08982. http://dx.doi.org/ 10.1016/j.heliyon.2022.e08982.

(9) Thapa, C. B., Pant, K. K., Bhattarai, H. D., Thapa, M., & Pant, B. (2024). Somatic Embryogenesis and Plant Regeneration through Callus Cultures of *Piper longum* L. *Banko Janari*, 31(2): 16-28. https://doi.org/10.3126/banko.v34i2.62729.

Appendix B2: Participation in Conference/Seminar/Workshop

(1) **"8th Graduate Conference 2022: Himalayan Knowledge Conclave**" held on April 4-5, 2022, organized by Mid-West University in Birendranagar, Surkhet, Nepal.

(2) **"International Conference on Biodiversity and Bioprospecting"** held on June 22-24, 2022, organized by the Department of Plant Resources (DPR), Ministry of Forest and Environment, Government of Nepal in Kathmandu, Nepal.

(3) **"9th National Conference on Science and Technology"** held on June 26-28, 2022, organized by the Nepal Academy of Science and Technology (NAST) in Kathmandu, Nepal.

(4) **"International Scientific Conference Kathmandu Humboldt-Kolleg 2022"** held on October 16-19, 2022, organized by Humboldt Club Nepal with Alexander VON Humboldt Foundation, Germany in Kathmandu, Nepal.

(5) **"4th International Conference on Biotechnology"** held on March 17-19, 2023, organized by Nepal Biotechnology Association (NBA) in Kathmandu, Nepal.

(6) **"Ph. D. Festival 2080"** held on October 9-10, 2023, organized by the Institute of Science and Technology, Tribhuvan University, Kathmandu, Nepal.

(7) **"International Collaborative Seminar 2023"** held on October 18, 2023, organized by Professor Emeritus Acram Taji, Australia.

(8) **"8th International Botanical Conference 2023"** held on 23-24 February 2024, organized by Bangladesh Botanical Society, Dhaka University.

(9) **"Workshop on Predatory Academic Practices in South Asia"** held virtually on 25-26 March 2022, organized by the National Young Academy of Nepal (NaYAN).

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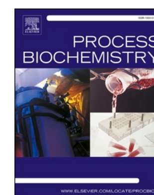

Antioxidant, antibacterial, and cytotoxic effect of *in vitro* callus and *in vivo* rhizome of *Paris polyphylla* Sm

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A R T I C L E I N F O

Keywords:
Agar Well diffusion
Bactericidal
Cytotoxicity
IC_{50}, MTT assay

A B S T R A C T

This study aims to evaluate the antioxidant, antibacterial, and cytotoxicity activity of *in vitro*-raised callus and *in vivo*-grown rhizome of the vulnerable medicinal plant *Paris polyphylla*. The antioxidant activity was assessed by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, the antibacterial activity by agar-well diffusion assay, and cytotoxicity by MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay. The highest antioxidant activity was found in the dichloromethane (DCM) fraction of rhizome (IC_{50} = 197.06 ± 0.635 μg/mL) followed by a crude extract of rhizome (IC_{50} = 339.60 ± 0.680 μg/mL). The DCM fractions showed a minimum inhibitory concentration of 5 mg/mL and a minimum bactericidal concentration of 7.5 mg/mL against *E. coli.* Similarly, the methanol fraction had the lowest IC_{50} of 211.36 ± 0.570 μg/mL against MCF-7 cells and the DCM fraction 235.94 ± 0.720 μg/mL against HeLa cells, both of which showed no deleterious effects on normal epithelial cells. Additionally, the *in vitro* callus' antioxidant (IC_{50} = 650.54 ± 0.515 μg/mL), antibacterial (inhibited 60 % of tested bacteria), and cytotoxic activities (IC_{50} = 548.49 ± 0.580 μg/mL on HeLa cells) were comparable to those of the rhizome, and were reported for the first time in *P. polyphylla*. These results showed that *P. polyphylla* rhizome and *in vitro*-raised callus could be a rich source of natural antioxidants, antimicrobials, and anticancer for therapeutic usage.

1. Introduction

Paris polyphylla, also called Satuwa in Nepali, is mostly found in South Asian countries between 1800 and 3500 m above sea level, where it is a vulnerable medicinal plant because of a declining population and challenges to its natural habitats [1–3]. In traditional medicine, rhizomes are used in cuts, wounds, blisters, burns, headache, fever, anthelmintic, scabies, rashes, or itching, diarrhea, nose, lung, and throat problems, liver cancer, febrifuge, analgesic, antiphlogistic, antitussive and depurative, breast cancer, fractures, convulsions, and strains; antidote, alexipharmic, demulcent, hemostatic, homeopathy [1,4,5]. Furthermore, it is used in traditional Chinese medicine (TCM), and Chinese patent medicines including "Jidesheng Sheyaopian," "Yunnan Baiyao," and Gong Xue Ning (GXN) capsule [6–8]. Steroidal saponin is the major component of *P. polyphylla,* which includes Paris saponin I, II, VI, VII, H, polyphyllin D, diosgenin, pennogenin, dioscin, and polyphyllin VII [9–13].

Medicinal Plants have long been known to have anticancer effects. Drugs to treat testicular and small-cell lung cancer were eventually developed as a result of the isolation of podophyllotoxin and lignans from the *Podophyllum peltatum* [14]. Several undesirable side effects might occasionally happen when receiving chemotherapy. Natural remedies, such as the usage of cancer therapy materials made from plants, may lessen unfavorable side effects. Numerous investigations have been made on the cytotoxic effects of *P. polyphylla* rhizome on various human cancer cell lines from China, India, and other nations [15]. Experimental evidence strongly suggests that oxidative stress plays a role in carcinogenesis [16,17]. Oxidative stress occurs when free radicals produced by the oxidation process surpass antioxidants. Reactive oxygen species (ROS) production that is increased within cells has the potential to be directly genotoxic or indirectly carcinogenic, for example, through altering signaling pathways that lead to altered gene

Abbreviations: IC50, Half-maximal inhibitory concentration.

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expression [18]. Moreover, ROS can deteriorate proteins, lipids, and DNA, causing essential genes to mutate and turn healthy cells into cancerous ones [19,20]. Antioxidants have been used to prevent or reduce the formation or multiplication of cancer cells by reducing the excessive production of intracellular ROS [21]. Many plants have been discovered to exhibit high antioxidant activity (ROS-scavenging action), which has been linked to antineoplastic activity against cancer cells and hence could be employed as therapeutic and preventative agents [22, 23].

Bioactive compounds produced *in vivo* plants have been thoroughly investigated in terms of their biological activity; however, callus cultured *in vitro* may contain useful compounds that are effective on a variety of bacteria, parasites, and anticancer agents, and further phytochemical research is required. The synthesis of therapeutically relevant secondary metabolites from callus cultures has shown economic promise [24,25]. There is some research on the *in vitro* cytotoxic, antibacterial, and antioxidant effects of callus extracts. Comparative studies of the *in vitro* antioxidant, antibacterial, and cytotoxic activities of callus and wild plant extracts were conducted in *Crotalaria retusa* [26], *Curculigo latifolia* [27], and *Ampelopsis grossedentata* [28].

Some natural products are now available on the medicinal market as free radical scavengers and are used to fight reactive oxygen species (ROS), which are known to cause a variety of malignancies in humans [29]. Numerous cancers, including leukemia, osteosarcoma, gastric cancer, breast cancer, lung cancer, liver cancer, and ovarian cancer, are being treated with substances derived from plants. Furthermore, one of the most pressing issues confronting today's age is the failure of chemotherapeutics and antibiotic resistance, which has led to the testing of various medicinal plants for antimicrobial characteristics [30]. Medicinal plants are evaluated for antibacterial potential since they are thought to be a rich source of antimicrobial compounds [31]. The antioxidant, antibacterial, and cytotoxic properties of plant extracts might be positively correlated with each other. Alkaloids, polyphenols, flavonoids, tannins, saponins, and steroids are secondary metabolites of plant extracts that are responsible for antioxidant activities [32] as well as potential antibacterial [33] and cytotoxic [34] effects on bacterial cells and tumor cells. Therefore, plant extracts with significant antioxidant properties may be employed as herbal medicines to prevent various malignancies and bacterial infections. This paper aims to investigate and compare the antioxidant, antibacterial, and anticancer properties of *in vitro* grown callus and *in vivo* rhizome of *P. polyphylla*. Several bioactive secondary metabolites, particularly those with anticancer, antibacterial, and antioxidant properties, have been consistently documented in *in vivo* rhizome of *P. polyphylla*. It is the first report to document the antioxidant, antibacterial, and anticancer properties of *in vitro*-produced *P. polyphylla* callus.

2. Materials and method

2.1. Plant material collection

P. polyphylla was gathered at an elevation of 2100 m above sea level in November 2021 in the Baglung district of western Nepal. The plant specimen was identified by tallying with the Tribhuvan University Central Herbarium (TUCH) specimen, and the voucher specimen was deposited in the TUCH in Kathmandu, Nepal (Voucher No. C15). A few plants were grown in pots in the garden of the Central Department of Botany, TU for callus induction from the leaf.

2.2. Chemical reagents

All the chemicals and reagents utilized for *in vitro* antioxidant and MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assays were purchased from the local dealer of ThermoFisher scientific company, India. All of the chemicals and reagents used in the experiment were of high-grade analytical quality. The media used for antibacterial assays such as NA (Nutrient Agar), NB (Nutrient Broth), MHA (Mueller Hinton Agar), and hormones (viz. 2,4-D, KN, NAA) used for callus induction were purchased from the local dealer of HiMedia, India. Ciprofloxacin was purchased from Lomus Pharmaceuticals, Kathmandu, Nepal.

2.3. Bacterial strains and cell lines

The bacterial strains selected for this research were ATCC strains received from the National Public Health Lab, Kathmandu Nepal. *Acinetobacter baumannii* (ATCC 19606), *E. coli* (ATCC MA 35218), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC baa977), and *Bacillus subtilis* (ATCC 6051-U) were used for antibacterial activity. The HeLa cell lines, MCF-7 cell lines, and normal epithelial cell lines were obtained from the Shikhar Biotech Pvt. Ltd, Nepal.

2.4. Callus induction and its biomass production

Leaves were surface sterilized with 70 % ethanol for 30 s, decanted ethanol, and added 0.1 % mercuric chloride was for 3–4 min pursued by three thorough washes with sterile distilled water to eliminate remnants of mercuric chloride. Then, explants (0.5 cm^2) were inoculated on full MS, half MS, and quarter MS [35] with or without supplemented hormones (2,4-D and KN). The culture tubes (20 mm × 150 mm) containing explants were transferred to the culture room and exposed to artificial fluorescent light (3000–4000 lux) with a 14–16 h photoperiod at 25 ± 2 °C. The callus, produced after 12 weeks of culture in MS media, was proliferated for another 8 weeks in the same condition to obtain significant biomass of callus.

2.5. Extract preparation

2.5.1. Rhizome extract preparation

Rhizomes were dried in the shade and ground into powder using an electric grinder (Electron). For 72 h, 350 g of powder was macerated in 500 mL of 90 % methanol and the mixture was filtered with the use of filter paper. This method was repeated three times for complete extraction. A Rotary evaporator (EYELA) was used to concentrate the crude extract under reduced pressure, and the extract was collected in vials and stored at 4° C in the refrigerator for further chemical analysis.

2.5.2. Callus extract preparation

In a mortar and pestle, 25 g of dry callus was crushed with 50 mL absolute methanol. The mixture was macerated with 150 mL methanol. After 72 h, the mixture solution was filtered using filter paper and the supernatant was evaporated in a Rotary evaporator under diminished pressure. For further chemical analysis, the crude extract was collected in vials and stored at 4° C in the refrigerator.

2.5.3. Fractionation of crude extracts

Based on a preliminary test, the crude extract of *in vivo* rhizome was found to be more active than the *in vitro*-grown callus extract, thus only the crude extract of rhizome was fractionated using the liquid-liquid partition method based on polarity with a variety of nonpolar to polar solvents, including n-hexane, dichloromethane (DCM), and absolute methanol. The crude extract (65 g) was first dissolved in 100 mL distilled water, filtered using filter paper, and then combined in a 1:2 ratio with hexane in a separating funnel. The hexane and the aqueous layers were separated by shaking the mixture solution in a separating funnel and the hexane layer was removed to obtain the hexane fraction. The aqueous layer was then mixed in a separating funnel with DCM in a 1:2 ratio, agitated thoroughly, and the aqueous layer and the DCM layer were separated. The DCM layer was separated with filter paper to obtain the DCM fraction. The aqueous layer was evaporated in a rotary evaporator under diminished pressure to obtain an aqueous extract, which was then dissolved in absolute methanol. The methanol fraction was obtained by

filtering it with filter paper. All the fractions were concentrated under reduced pressure in a rotary evaporator separately to obtain the hexane, DCM, and methanol extracts. These extracts were weighed, kept in vials, and reserved at 4 °C for further use. Moreover, the hexane fraction was disregarded because initial tests showed it to be less efficient than other fractions.

2.6. Evaluation of antioxidants, total phenolic contents, and total flavonoid contents

2.6.1. Antioxidant or free radical scavenging activity (DPPH assay)

The antioxidant activity of the extracts was accessed by applying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method [36] with slight modification. In a 96-well plate, 50 μL of each sample (1 mg/mL) was combined with 150 μL of DPPH (0.1 mM of DPPH in methanol). The plate was left in the dark for 30 min. For positive control, various concentrations of ascorbic acid were made ready from the stock solution of ascorbic acid (1 mg/mL) and methanol was used as a negative control. The absorbance was measured at 517 nm by using a 96-well microplate reader (Azure Biosystem). Using the formula below, the percentage of the DPPH free radical scavenging activity was determined.

$$\% \text{ Inhibition} = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100$$

Where $Abs_{Control}$ = Absorbance of control reaction (DPPH + Methanol).

Abs_{sample} = Absorbance in the presence of sample (DPPH+Sample).

The IC_{50} values were estimated by applying the inhibition curve by graphing extract concentration against the correspondent scavenging effect.

2.6.2. Total phenolic content (TPC)

The amount of total phenolic content in the extract was evaluated using a spectrophotometer with Folin-Ciocalteau reagent (FCR) involving gallic acid as standard following Zhang et al. [37] with slight modification. In a 96-well plate, 100 μL of Folin-reagent Ciocalteu's (1:10) was added to 20 μL of extract (1 mg/mL). After 6 min, 80 μL of sodium carbonate (1 M) solution was added. The absorbance was determined at 765 nm following a 15-min incubation period in the dark. Using a standard curve of gallic acid (10 μg/mL to 60 μg/mL), the concentration of total phenolic content was reported as mg of gallic acid equivalents per g of dried extract (mg of GAE/g of E). All measurements were carried out in three replicates.

2.6.3. Total flavonoid contents (TFC)

An aluminium chloride complex-forming assay was applied to estimate the total flavonoid contents of the extracts following Chang et al. [38] with some changes. Quercetin (1 mg/mL) was dissolved in pure methanol and was used as standard. The entire assay mixture, included 20 μL of standard quercetin (10–80 μg/mL) or plant extract (1 mg/mL), 60 μL of ethanol, 110 μL of distilled water, 5 μL of 10 % AlCl3, and 5 μL of potassium acetate (1 M), was incubated in the dark for 30 min before the absorbance was measured at 415 nm. The amount of total flavonoid content was manifested as milligrams of quercetin equivalent per gram of dry weight (mg of QE/g) of the extract using the quercetin standard curve.

2.7. Evaluation of antibacterial activity

Antibacterial activity was estimated by the agar-well diffusion method [39,40] with slight modification. Plant extracts were dissolved in absolute methanol. All the bacterial strains were smeared on nutrient agar plates and incubated at 37 °C for 24 h to revive the bacteria as well as to obtain a pure culture. Then 2–3 similar colonies of bacteria were transferred in normal saline and approximately diluted to 0.5 McFarland standard turbidity to obtain a cell suspension of 1.5×10^8 CFU/mL. Single bacterial strains from the diluted bacterial suspensions were implanted on Mueller Hinton Agar plates with a sterile cotton swab. Wells with a diameter of 6 mm was constructed with a sterile cork borer. Each well was filled with 35 μL of extracts. The plates were incubated at 37° C for 24 h. The antibacterial activity was determined by calculating the zone of inhibition (ZOI) in mm (diameter) around the point of application of each sample solution (5, 10, 20, 40, and 60 mg/mL). The broad-spectrum antibiotic ciprofloxacin (50 μg/mL) was used as positive control and absolute methanol as a negative control. Three replicates of the test samples were used to calculate the average for the inhibition zone.

2.7.1. Relative percentage inhibition

By comparing the zones of inhibition of positive and negative controls, the relative percentage inhibition of bacterial strains against the test extract was obtained [41].

$$\text{Relative percentage inhibition of test extract} = \frac{(A\text{-}B)}{(C\text{-}B)} \times 100$$

Where, A=Total zone of inhibition of the plant extract.

B= Total zone of inhibition of the negative control.

C= Total zone of inhibition of the positive control.

2.7.2. Minimum inhibitory concentration (MIC) assay

Only extracts that showed an inhibitory zone with selected bacteria were used to evaluate MIC and minimum bactericidal concentration (MBC) values. The micro-broth dilution approach was used to obtain the minimum inhibitory concentrations (MICs) of various extracts [42,43]. The extracts were serially diluted (1.0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, and 60 mg/mL) in absolute methanol. Each dilution (1 mL) was added to 1 mL of sterile nutrient broth along with 0.05 mL of the tested bacterial strain, and the mixture was then incubated at 37 °C for 24 h. The negative control was 0.05 mL of plant extracts of each measured concentration in 2 mL of nutrient broth, while the positive control was 0.05 mL of bacterial suspension in 2 mL of nutrient broth. The lowest extract concentration (mg/mL) at which bacteria could not proliferate in nutrient broth was used to determine the MIC visually. In addition, the sample solution's concentration was gradually increased in a small range starting at the MIC, treated with a bacterial suspension of nutrient broth, and incubated at 37 °C for 24 h. By smearing each treated sample solution with bacterial suspension in Mueller Hinton Agar media and incubating for 24 h at 37 °C, the MBC of each extract was determined. The lowest concentration of an antibacterial agent (extracts) required to kill a certain bacterium is known as the minimum bactericidal concentration. At MBC the bacteria cannot grow in MHA plates.

2.8. Evaluation of cytotoxic activity of extracts (MTT Assay)

The cytotoxic action of extracts was assessed using the standard MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric test with some modification in 96-well flat-bottomed microtiter plates (Corning) [44,45]. Human cervical cancer cells (HeLa), breast cancer cells (MCF-7), and normal epithelial cells (dermal and fibroblast in origin) were grown in T25 flasks in Eagle's minimum essential medium (EMEM) fortified with 10 % fetal bovine serum (FBS), 1 % streptomycin, and 1 % L-glutamine and maintained in a 5 % CO_2 incubator at 37 °C. In 100 μL of media, cells were cultured in 96-well plates (2×10^4 cells/well) and incubated at 37 °C for 24 h in a 5 % CO_2 incubator. The cells were treated with 100 μL of plant extracts (50, 100, 200, and 400 μg/mL) diluted in 0.1 % DMSO (dimethyl sulfoxide, Merck, 99.9 %) for 48 h after attachment and cell confluence. After 48 h of incubation, the supernatant was replaced with 125 μL of medium containing 25 μL of MTT in each well. A purple formazan crystal of living cells was formed after 4 h of incubation. To dissolve the formazan crystals, 150 μL of 0.1 % DMSO was added to the plates, which were then incubated for another 15 min at room temperature. A microplate reader was used to measure

Fig. 1. *P. polyphylla*: a) Habit of the plant; b) In vivo-grown rhizome; c) In vitro-grown callus.

Table 1
In vitro callus induction and proliferation from leaf explant in *P. polyphylla*.

	Strength of MS media	Plant growth hormones (mg/L)	Dry callus (g) ± SD
1.	MS	–	–
2.	½ MS	–	–
3.	¼ MS	–	–
4.	MS	0.25 2,4-D + 0.25 KN + 10 % CW	0.492 ± 0.113
5.	MS	0.5 2,4-D + 0.25 KN + 10 % CW	1.312 ± 0.229
6.	MS	1.0 2,4-D + 0.0.25 KN + 10 % CW	1.045 ± 0.187
7.	MS	1.5 2,4-D + 0.25 KN + 10 % CW	0.579 ± 0.082
8.	MS	2.0 2,4-D + 0.25 KN + 10 % CW	0.240 ± 0.021

Culture condition: fluorescent light with 14–16 h photoperiod, 25 ± 2 °C temperature, and three replicates for each combination (at 12 weeks of culture).

the absorbance of the samples at a wavelength of 595 nm. As a positive control, the anticancer drug cisplatin was used. The following formula was used to compute the percentage of cytotoxic activity:

$$\% \quad \textbf{Cytotoxic} \quad \textbf{Activity} = \frac{\textbf{Abs}_1 - \textbf{Abs}_2}{\textbf{Abs}_1} \times \textbf{100}$$

Where, Abs_1 is the absorbance of cells in the absence of plant extracts and Abs_2 is the absorbance of cells in the presence of all components, including plant extracts.

2.9. Statistical analysis

For *in vitro* callus induction and proliferation, the average values with ± standard deviation (SD) of a week's measurements were computed. The concentration of each plant extract necessary to suppress DPPH activity by 50 % (IC_{50}) was calculated using linear regression of the percentage of radical scavenging against concentration in Microsoft Excel 2010 (Microsoft Corporation, WA, USA). Results from experiments in triplicate using antibacterial assays including ZOI, MIC, and MBC were expressed as a percentage. Furthermore, a dose-response curve was plotted for each extract and a linear regression equation was utilized to evaluate the cytotoxic capacities of extracts (IC_{50} μg/mL) in Microsoft Excel 2010.

3. Results and discussion

3.1. Callus induction and proliferation from P. polyphylla Leaf

After 12 weeks of culture, callus was induced from the leaf explants in MS media fortified with 2,4-D, KN, and 10 % coconut water, but not in full MS, half MS, and quarter MS without additional hormones.

The callus biomass was increased from 0.25 to 0.5 mg/L 2,4-D at a constant concentration of KN (0.25 mg/L) in the full strength of MS media, and then further decreased to 2.0 mg/L concentration of 2,4-D. A large callus (dry wt 1.312 ± 0.229 g) was induced in MS media fortified with 0.5 mg/L 2,4-D + 0.25 mg/L KN and 10 % coconut water than other treatments (Fig. 1C and Table 1). It was proliferated for 8 weeks under the same conditions producing a significant amount of callus biomass, which was then used to make the methanolic crude extract.

3.2. Antioxidant activity of callus and rhizome by DPPH method

The methanolic crude extracts of rhizome showed a higher free radical scavenging capacity, with an IC_{50} of 339.60 ± 0.680 μg/mL than the crude extract of callus with an IC_{50} of 650.54 ± 0.515 μg/mL (Table 2). Moreover, the DCM fraction of rhizome showed a higher free radical scavenging capacity, with an IC_{50} of 197.06 ± 0.635 μg/mL than the methanol fraction, with an IC_{50} of 317.97 ± 0.730 μg/mL in comparison to standard ascorbic acid (IC_{50} = 7.40 ± 0.875 μg/mL) (Table 2). DCM is an organic solvent for many chemical and industrial processes due to its volatility and ability to dissolve a wide variety of non-polar and polar organic compounds, establishing both London type interactions and dipole-dipole interactions [46]. The synergistic effect of both polar and non-polar compounds may be the cause of the high antioxidant and bioactivity in the DCM fraction. The DCM fraction of *Apium graveolens* had a higher antioxidant activity than ethyl acetate and butanol fractions, and mother liquor [47]. This study also showed that the capacity of fractions to scavenge free radicals is higher than that of their methanolic crude extracts. The activity could be linked to the

Table 2
DPPH radical scavenging activity, total phenol and total flavonoid content of the rhizome and callus extracts of *P. polyphylla*.

Crude Extract /Fraction	Antioxidant activity (IC_{50}: μg/mL)	Total phenol content (TPC)	Total flavonoid content (TFC)
RCE	339.60 ± 0.680	13.07 ± 0.23	28.38 ± 0.71
CCE	650.54 ± 0.515	6.62 ± 0.23	25.27 ± 0.37
DF	197.06 ± 0.635	26.40 ± 0.61	43.72 ± 0.61
MF	317.97 ± 0.730	16.81 ± 0.84	30.20 ± 0.81
Ascorbic acid (Positive control)	7.40 ± 0.875	–	–

Abbreviations: RCE=Rhizome Crude Extract, CCE=Callus Crude Extract, DF=DCM Fraction, and MF=Methanol Fraction.

overall amount of phenols, a family of chemicals known to have antioxidant properties. Ethyl acetate fraction, dichloromethane fraction, and n-butanol fraction were all more active than crude extract of stem bark of *Tabernaemontana catharinensis* [48]. It could be due to impurities/contaminants such as carbohydrates, lipids, or a mixture of other non-polar compounds in the crude extract. In addition, DCM fractions had a better capability for scavenging free radicals than methanol fractions, which could be attributed to the buildup of more potent antioxidant compounds such as phenolic and flavonoids (Table 2). With an increase in extract concentration from 25 to 400 μg/mL, percentage of DPPH radical scavenging effect increased.

Though, the methanolic crude extract of rhizome had stronger antioxidant activity (IC_{50} = 339.60 ± 0.680 μg/mL) than the methanolic crude extracts of callus (IC_{50} =650.54 ± 0.515), *in vitro*-raised callus have been reported to synthesize and accumulate antioxidants like phenolic compounds. It's observed that the callus regenerated from the *P. polyphylla* leaf had produced antioxidants in the culture medium. Bergenin (trihydroxybenzoic acid glycoside), an antioxidant compound, was isolated by Shrestha and Pant [49] from an *in vitro*-produced callus of a *Bergenia ciliata* leaf. In *Habenaria edgeworthii*, callus cultured on 1/2 MS increased antioxidant activity and considerable phenolic content was seen in MS medium that had 3.0 μM BA added to it [50]. Methanol extracts of dark-grown and light-grown callus reduced the stable DPPH free radicals in *Inula crithmoides* [51]. Utilizing the *in vitro*-raised callus from *P. polyphylla* leaves as an antioxidant and natural drug for the treatment of illnesses might aid in the long-term conservation of the wild plant species.

According to previous studies, the ability and strength of crude rhizome extracts to scavenge free radicals varied in various extracts, such as methanol extract [41,52], ethanol extract [53] in *P. polyphylla.* The results of this study were also different from those of the previous study could be due to the effect of solvent purity, extraction with polar or non-polar solvents, geographical location and habitat of the plant, and other environmental conditions. Pure molecules isolated from various crude extracts and fractions, on the other hand, can have considerable antioxidant activity.

DPPH is a stable nitrogen-centered free radical, violet in color, containing an odd electron in its structure. Due to the stability in the

Table 3
Zone of inhibition (ZOI) and relative percentage inhibition shown by rhizome and callus extracts *of P. polyphylla* on different bacteria.

Sample/ Extract	Test bacteria	Zone of inhibition (ZOI) in mm±SD/and Relative percentage inhibition (Excluding diameter of the well)				
		5 mg/mL	10 mg/mL	20 mg/mL	40 mg/mL	60 mg/mL
RCE	*Acinetobacter baumannii*	–	–	–	–	–
	Bacillus subtilis	–	–	–	–	–
	Escherichia coli	5.0 ± 2.64 (20.27 %)	7.0 ± 2.00 (28.38 %)	15.0 ± 1.00 (60.82 %)	18.0 ± 2.00 (72.99 %)	20.3 ± 1.52 (81.10 %)
	Pseudomonas aeruginosa	6.0 ± 1.00 (23.38 %)	8.33 ± 1.52 (31.17 %)	13.66 ± 1.5 (54.55 %)	17.0 ± 3.00 (66.25 %)	19.0 ± 2.00 (74.04 %)
	Staphylococcus aureus	7.33 ± 2.08 (22.83 %)	10.0 ± 1.0 (32.61 %)	15.0 ± 2.64 (48.92 %)	18.6 ± 1.52 (61.96 %)	21.0 ± 2.00 (68.49 %)
CCE	*Acinetobacter baumannii*	–	–	–	–	–
	Bacillus subtilis	4.66 ± 1.52 (17.04 %)	7.0 ± 1.00 (23.86 %)	15.0 ± 2.64 (51.14 %)	17.0 ± 1.00 (57.96 %)	18.0 ± 2.64 (61.37 %
	Escherichia coli	–	7.0 ± 2.64 (28.38 %)	13.0 ± 2.00 (52.71 %)	15.3 ± 2.08 (60.82 %)	19.0 ± 3.00 (77.04 %)
	Pseudomonas aeruginosa	6.0 ± 1.00 (23.38 %)	8.33 ± 2.08 (31.17 %)	15.6 ± 2.08 (.55 %)	18.0 ± 2.00 (70.14 %)	19.0 ± 2.00 (74.04 %)
	Staphylococcus aureus	–	–	–	–	–
DF	*Acinetobacter baumannii*	7.0 ± 2.64 (29.58 %)	9.66 ± 1.52 (42.26 %)	17.0 ± 1.00 (71.85 %)	19.0 ± 2.64 (80.30 %)	21.0 ± 2.00 (88.75 %)
	Bacillus subtilis	7.0 ± 2.00 (23.86 %)	15.0 ± 1.00 (51.14 %)	18.3 ± 1.52 (61.37 %)	20.0 ± 3.00 (68.18 %)	22.0 ± 2.64 (75.0 %)
	Escherichia coli	20.33 ± 1.52 (81.10 %)	21.0 ± 2.00 (85.15 %)	22.0 ± 1.00 (89.21 %)	22.6 ± 1.2 (93.26 %)	24.0 ± 2.00 (97.32 %)
	Pseudomonas aeruginosa	14.0 ± 1.00 (54.55 %)	19.0 ± 3.00 (74.04 %)	20.0 ± 2.64 (77.94 %)	22.0 ± 2.00 (85.73 %)	22.6 ± 2.08 (89.63 %)
	Staphylococcus aureus	18.66 ± 2.08 (61.96 %)	21.0 ± 2.64 (68.49 %)	22.0 ± 2.00 (71.75 %)	23.0 ± 1.00 (75.01 %)	25.0 ± 1.00 (81.53 %)
MF	*Acinetobacter baumannii*	–	–	–	–	–
	Bacillus subtilis	–	6.66 ± 2.08 (23.86 %)	14.0 ± 3.00 (47.73 %)	16.0 ± 2.00 (54.55 %)	19.0 ± 1.00 (64.78 %
	Escherichia coli	–	7.0 ± 1.00 (28.38 %)	15.3 ± 2.08 (60.82 %)	17.0 ± 2.64 (68.93 %)	19.0 ± 2.00 (77.04 %)
	Pseudomonas aeruginosa	8.0 ± 2.64 (31.17 %)	16.0 ± 2.00 (62.35 %)	18.0 ± 1.00 (70.14 %)	19.3 ± 2.08 (74.04 %)	21.0 ± 3.00 (81.83 %)
	Staphylococcus aureus	17.0 ± 1.00 (54.44 %)	20.33 ± 1.5 (65.23 %)	22.02.00 (71.75 %)	23.0 ± 1.00 (75.01 %)	24.3 ± 1.52 (78.27 %)
Ciprofloxacin (50 μg/mL)	For positive control: *Acinetobacter baumannii*= 23.66 mm, *Bacillus subtilis*= 29.33 mm, *Escherichia coli*= 24.66 mm, *Pseudomonas aeruginosa*= 25.66, *Staphylococcus aureus*= 30.66 mm					
Absolute Methanol	For negative control: *Acinetobacter baumannii*= 0.0 mm, *Bacillus subtilis*= 0.0 mm, *Escherichia coli*= 0.0 mm, *Pseudomonas aeruginosa* = 0.0, *Staphylococcus aureus*= 0.0 mm					

Abbreviations: RCE=Rhizome Crude Extract, CCE=Callus Crude Extract, DF=Dichloromethane Fraction, MF=Methanol Fraction

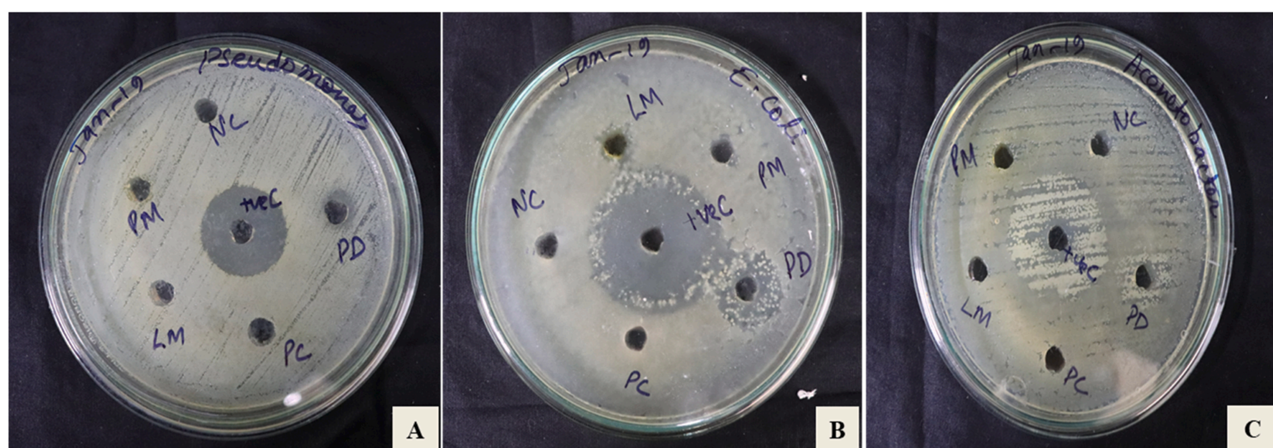

Fig. 2. Zone of Inhibition (ZOI) shown by rhizome and callus of *P. polyphylla* at 10 mg/mL: a) ZOI on *Pseudomonas aeruginosa*; b) ZOI on *E. coli*; c) ZOI on *Acinetobacter baumannii*. Abbreviations: Methanol fraction (PM), Dichoromethane fraction (PD), Callus extract (PC), Positive control (+veC), and Negative control (NC).

radical form and ease of the assay, the DPPH radical is a frequently employed substrate for the quick detection of the radical scavenging activity of plant extract [54]. Antioxidants were responsible for the decline in DPPH radical absorbance because antioxidant molecules react with free radicals to either its reduction or scavenge them by donating them hydrogen or electrons, which is indicated by the progressive fading of the color from violet to yellow. The absorbance value was decreased with the increase in the concentration of plant extract and ascorbic acid.

3.3. Total phenolic contents (TPC) of callus and rhizome

The total phenolic content of the methanolic crude extract of rhizome was found to be higher (13.07 ± 0.23 mg of GAE/g dry weight) than the methanolic crude extract of callus (6.62 ± 0.23 mg of GAE/g dry weight). Similarly, the DCM fraction of the rhizome had higher total phenolic content (26.40 ± 0.61 mg of GAE/g dry weight) than the methanol fraction (16.18 ± 0.84 mg of GAE/g dry weight) (Table 2). The high phenolic contents in the DCM fraction may be owing to phenolic purification and concentration during the fractionation process, which is likely responsible for its significant antioxidant activity. Moreover, the DCM fractions of rhizome had a higher phenolic content than the methanol fractions. It could be because phenolic compounds have a higher affinity for DCM than methanol in *P. polyphylla.* Plants contain phenolic compounds that have redox characteristics that permit them to act as antioxidants for breaking chain reactions [55]. The total phenolic content of the methanolic crude extract and fractions of crude extracts of *P. polyphylla* was determined using the Folin-Ciocalteu reagent (FCR) in terms of gallic acid equivalent (mg of GAE/g of the dry weight of extract) by using the calibration curve of gallic acid (10 μg/mL-60 μg/mL) [$y = 0.018x$, $R^2 = 0.9807$, where, y = absorbance (nm), x = gallic acid concentration, R^2 =correlation coefficient]. The total phenolic content varied from 26.40 ± 0.61–6.62 ± 0.23 mg of GAE/g dry weight.

This test includes the oxidation of phenolic compounds in an alkaline solution by the yellow F-C (molybdotungstophosphoricheteropolyanion) reagent and colorimetric detection of the resultant blue product (molybdotungustophosphate). Polyphenols in the plant extracts react with a specific redox reagent (FCR) to form a complex blue product that shows a broad light absorption depending on the qualitative and quantitative composition of the phenol mixture and can be quantified by the UV–visible spectrometry at 765 nm. The amount of phenols present affects how much light at that wavelength is absorbed.

3.4. Total flavonoid contents (TFC) of callus and rhizome

The total flavonoid content of the methanolic crude extract of rhizome was higher (28.38 ± 0.71 mg of QE/g dry weight) than in the methanolic crude extract of callus (25.27 ± 0.37 mg of QE/g dry weight). Similarly, the total flavonoid content of the DCM fraction of the rhizome had higher (43.72 ± 0.61 mg of QE/g dry weight) than the methanol fraction (30.20 ± 0.81 mg of QE/g dry weight). (Table 2). The DCM fractions of rhizome had higher flavonoid content than the methanol fractions. It could be because flavonoid compounds have a higher affinity for DCM than methanol in *P. polyphylla.*

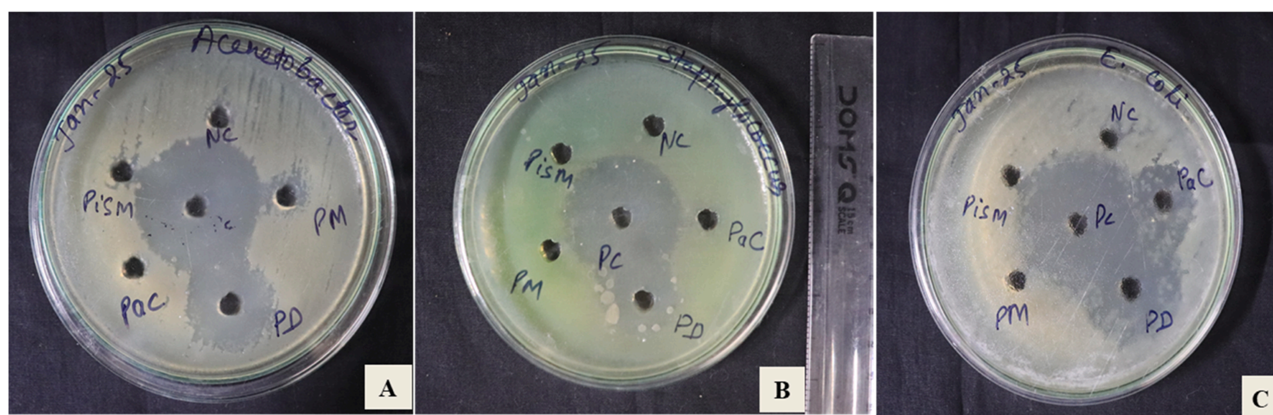

Fig. 3. Zone of Inhibition (ZOI) shown by rhizome and callus of *P. polyphylla* at 60 mg/mL: a) ZOI on *Acinetobacter baumannii*; b) ZOI on *Staphylococcus aureus*; c) ZOI on *E. coli*. Abbreviations: Methanol fraction (PM), Dichloromethane fraction (PD), Callus extract (PaC), Positive control (PC), and Negative control (NC).

Table 4
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of Rhizome & Callus in *P. polyphylla*.

Extract/Fraction	Values of MIC/and MBC (mg/mL)									
	Acinetobacter baumannii		*Bacillus subtilis*		*Escheriachia coli*		*Pseudomonas aeruginosa*		*Staphylococcus aureus*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
RCE	–	–	–		40	42.25	25	28.25	30	33.25
CCE	–	–	25	26.5	20	22.75	40	44.25	–	–
DF	30	33.5	20	24.5	5	7.5	10	13.25	15	17.25
MF	–	–	35	38.75	30	34.75	20	22.75	15	18.5

Abbreviations: RCE=Rhizome Crude Extract, CCE=Callus Crude Extract, DF= Dichloromethane Fraction, MF=Methanol Fraction

The aluminium chloride colorimetric test was used for the determination of total flavonoids present in the methanolic crude extracts and their DCM and methanol fractions in *P. polyphylla* using quercetin as standard. The flavonoids of the plant extract in the presence of aluminium chloride form acid liable complexes that have an immense yellow fluorescence and can be detected under a microplate reader spectrophotometer at 415 nm. The content of flavonoids is directly proportional to the intensity of light absorption at that wavelength. The Calibration curve was generated by using Quercetin (10–80 μg/mL) with the line of equation [$y = 0.0256x$, $R^2 = 0.9838$, where, y = absorbance (nm), x = quercetin concentration, R^2 =correlation coefficient] and using this equation, the flavonoid content of the various plant extracts was estimated.

3.5. Antibacterial activity

3.5.1. Zone of inhibition (ZOI) and relative percentage inhibition

At five different concentrations (5, 10, 20, 40, and 60 mg/mL), methanolic crude extracts of rhizome and callus inhibited the growth of 60 % of tested bacteria (*Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Bacillus subtilis*). Similarly, the DCM fraction of rhizome inhibited the growth of 100 % of tested bacteria, while the methanol fraction of 80 % of tested bacteria at five different concentrations (Table 3). A comparison was made with the standard drug Ciprofloxacin (50 μg/mL). It showed that the DCM fraction of rhizome was more potent than methanol fraction and other crude extracts against the tested bacteria, which could be attributed to the presence of more active antimicrobial compounds. Additionally, the multidrug-resistant *A. baumannii*, which is the primary cause of pneumonia, bacteremia, urinary tract infection, and wound infection, was inhibited by the DCM fraction of the rhizome. The DCM fraction of *Premna resinosa* had the best antibacterial activity with a MIC of 31.25 μg/mL against Methicillin-resistant *S. aureus* [56]. The DCM fractions of plant extracts were more effective in ethnopharmacological research than other fractions and crude extracts, according to studies on antiprotozoal properties [57,58]. Moreover, the antibacterial activity of *in vitro*-raised callus was comparable to the activity of *in vivo*-grown rhizome and inhibited the growth of one gram-positive (*Bacillus subtilis)* and two gram-negative (*Pseudomonas aeruginosa,* and *E. coli*) bacteria. The callus may have produced antibacterial compounds in MS media that prevented *B. subtilis, P. aeruginosa,* and *E. coli* from growing in MHA media, and could be employed to sustain wild-grown *P. polyphylla's* rhizome for long-term use. All the extracts showed a concentration-dependent zone of inhibition (ZOI) and relative percentage inhibition against the tested bacteria. At 5 mg/mL, the DCM fraction of rhizome showed a relative percentage inhibition of 81.10 %, which increased to 97.32 % at 60 mg/mL in *E. coli.* The lower intestine of warm-blooded creatures frequently harbors the rod-shaped *E. coli*, which is a gram-negative, facultatively anaerobic, coliform bacterium. Some *E. coli* strains can cause diarrhea, stomachaches, and a low-grade fever, and *P. polyphylla* rhizome and *in vitro*-raised callus could be used as a possible natural drug against *E. coli* infections. In the field of infectious disease, 82 (58 %) of the 141 small compounds licensed as antibacterial agents between 1981 and 2014 came from natural sources [59]. The highest antibacterial activity was found against *E. coli* where the diameter of the zone of inhibition was >31 mm and the relative percentage inhibition of the methanol extract of rhizome in *E. coli* and *S. aureus* was 95.58 % at 5 mg/mL [41]. The season in which rhizomes were collected, the habitat and environment in which they grew, the solvents used to prepare the extract, the kinds of bacterial strains used, and other variables could all be contributing reasons to the results of this study differing from those of the previous study (Figs. 2 and 3).

3.5.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Callus and Rhizome

All the methanolic crude extracts (rhizome and callus) and fractions of rhizome were evaluated for MIC and MBC against two gram-positive (*S. aureus* and *B. subtilis)* and three gram-negative bacteria (*A. baumannii, E. coli,* and *P. aeruginosa*). The lowest MIC (5 mg/mL) and MBC (7.5 mg/mL) were found in the DCM fraction of rhizome against *E. coli* (Table 4 & Fig. 4).

In addition, the methanolic crude extract of *in vitro* callus showed growth inhibition against *Bacillus subtilis, E. coli,* and *Pseudomonas aeruginosa,* and the lowest MIC and MBC were found to be 25 mg/mL and 26.5 mg/mL respectively in *Bacillus subtilis*. Even though the MIC and MBC of callus extract were found to be higher than anticipated, it may be significant for future researchers for further investigation against other useful microbes. Elicitors/precursors in cell suspension culture could be used to increase the amount of antibacterial compound synthesized in calli in a shorter period that widens the application of callus in the pharmacological industry. Compounds found in the callus of *Withania somnifera* were more effective than those found in the natural root in inhibiting the growth of *Pseudomonas aeruginosa, Proteus vulgaris*, and *Klebsicela pneumonia,* whereas compounds found in the natural root inhibited the growth of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis,* and *Salmonella typhimurium* [60].

This study showed that most of the extracts and fractions acted as bacteriostatic agents with a lower MIC than the MBC value against tested bacterial strains. Bacteriostatic agents prevent bacterial growth whereas bactericidal agents kill bacteria [61]. There are no previous records of *in vitro*-raised callus of *P. polyphylla* having any antibacterial properties. However, previous research on *P. polyphylla*, especially *in vivo* parts, showed antibacterial activity against various bacterial strains. Total steroidal saponins of above-ground parts (TSSAPs) and total steroidal saponins of rhizomes (TSSRs) extracted from ethanol extract showed antibacterial activities against *Escherichia coli* [62]. The MIC and MBC values or antibacterial activities of plants depend on the difference in the solvent used for extraction, extraction procedure, the plant part tested, soil or climate, post-harvest treatment or collection, and the microorganisms tested [63]. The steroidal saponins, which are predominantly concentrated in the rhizomes, are the primary compounds responsible for their antibacterial activity [64]. A substantial number of phenolics and flavonoids have also been found in the plant species, which could be the cause of its antibacterial activity [41].

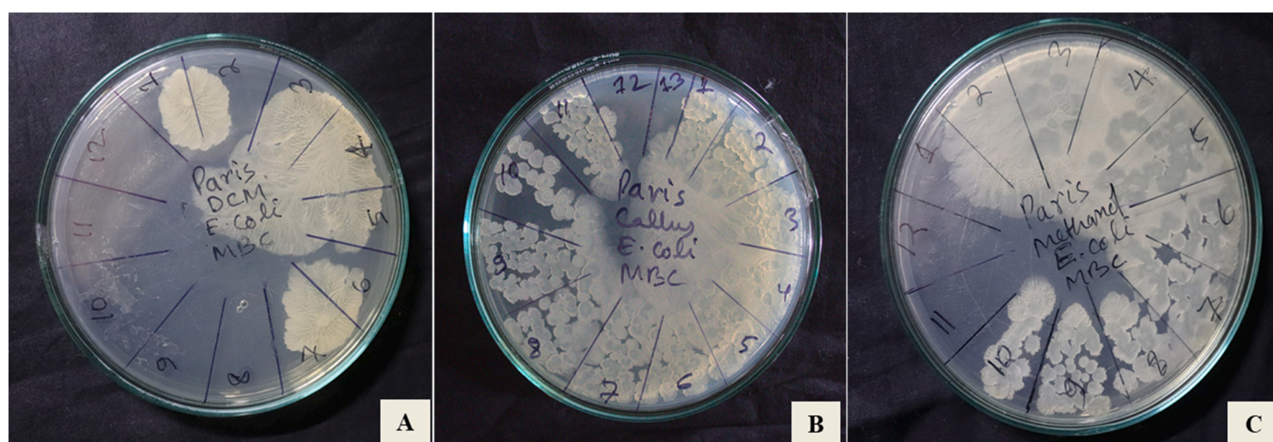

Fig. 4. Minimum bactericidal concentration (MBC) shown by rhizome and callus in *P. polyphylla*: **a)** MBC of Dichloromethane (DCM) fraction on *E. coli*; **b)** MBC of Callus extract on *E. coli*; **c)** MBC of Methanol fraction on *E. coli*. (1–13 numbers on Mueller Hinton Agar (MHA) Petri plate represent various extract concentrations greater than the minimum inhibitory concentration (MIC) starting with the MIC of the particular extract on tested bacteria).

3.6. Cytotoxic Activity of Callus and Rhizome of P. polyphylla in HeLa, MCF-7, and Normal Epithelial Cell Lines

Methanolic crude extracts of *in vivo*-grown rhizome and *in vitro*-raised callus, as well as fractions of *in vivo*-grown rhizome, were evaluated for their potential anticancer activity. All the crude extracts and fractions in HeLa and MCF-7 cells, except crude extract of callus in MCF-7 cells, showed significant cytotoxic activity when compared to the synthetic anticancer drug cisplatin (Table 5). The DCM fraction showed a stronger cytotoxic activity on HeLa cells, with an IC_{50} of 235.94 ± 0.720 μg/mL, and a high dose-dependent inhibition of cell growth, reaching > 62 % inhibition at 400 μg/mL after 48 h. The methanol fraction, on the other hand, showed a stronger cytotoxic activity on MCF-7 cells, with an IC_{50} of 211.36 ± 0.570 μg/mL, and a substantial dose-dependent reduction of cell growth, reaching > 68 % inhibition at 400 μg/mL within 48 h of treatment (Figs. 5 and 6). It shows the possibility that diverse chemical compounds, present in the DCM and methanol fractions at variable quantities, may have had different effects on HeLa and MCF-7 cells. In the extraction process, biological activity might be influenced by the polarity of the solvents. None of the crude extracts and fractions inhibited normal human epithelial cells (higher IC_{50} values than those of HeLa and MCF-7 cells), showing that they were not toxic to normal cells at concentrations of 50–400 μg/mL (Table 5). The callus extract was relatively less toxic (IC_{50} =1191.12 ± 6.91 μg/mL) than rhizome extract (IC_{50} =1107.05 ± 16.84 μg/mL), DCM fraction (IC_{50} =991.67 ± 4.48 μg/mL), and methanol fraction (IC_{50} = 880.79 ± 14.25 μg/mL) on normal cell lines. Higher dosages of all the treatments could have a negative impact on healthy cells.

Furthermore, the crude extract of callus had a weaker cytotoxic activity on HeLa cells than the crude extract of rhizome, with an IC_{50} of 548.49 ± 0.580 μg/mL and a significant dose-dependent reduction in cell growth, reaching > 40 % inhibition at 400 μg/mL after 48 h (Fig. 5). The crude extract of callus, on the other hand, showed no cytotoxicity in MCF-7 breast cancer cell lines. It could be because the characteristics of the HeLa and MCF-7 cancer cell lines may differ from one another, and there may be fewer cytotoxic compounds in the callus than in the rhizome. Though several studies have been published on the cytotoxicity of *P. polyphylla* rhizome and other parts, this is the first attempt to test the cytotoxicity of *in vitro*-generated callus. The ethanolic crude extract of the leaf was stronger than the ethanolic crude extract of callus in HeLa cells [65]. The inhibitory activity of samples produced from *in vivo*-raised plant material was higher than that of *in vitro*-raised samples, owing to differences in cytotoxic activities between wild and *in vitro* samples [45].Figs. 7 and 8.

This study revealed that the DCM and methanol fractions had comparatively stronger cytotoxicity than crude methanol extracts on HeLa and MCF-7 cell lines. The ethyl acetate fraction of *Pseudocedrela kotschyi* stem bark exhibited greater cytotoxicity on HeLa cells, and RD cells than the methanol crude extract on HeLa cells, and RD cells [66]. However, the isolated compound could have more significant cytotoxicity than the crude extract and fractions. The cytotoxicity of saponin extracted from ethyl acetate fraction was higher than that of fraction and standard drugs [66].

Previous research on *P. polyphylla* showed that isolated pure compounds had higher cytotoxicity than crude extracts on HeLa and MCF-7

Table 5
Cytotoxicity of Rhizome and callus extract with their IC_{50} value in HeLa, MCF-7, and Normal Epithelial cell lines.

Fraction/Extracts	Cell lines with IC_{50} (μg/mL)		
	HeLa Cells	MCF-7 Cells	Normal Epithelial Cells
RCE	473.39 ± 0.470	448.87 ± 0.670	1107.05 ± 16.84
CCE	548.49 ± 0.580	–	1191.12 ± 6.91
DF	235.94 ± 0.720	296.70 ± 0.835	991.67 ± 4.48
MF	296.48 ± 0.815	211.36 ± 0.570	880.79 ± 14.25
Positive control (Cisplatin)	24.56 ± 0.775	26.43 ± 0.530	–

Abbreviations: RCE=Rhizome Crude Extract, CCE=Callus Crude Extract, DF=DCM Fraction, and MF=Methanol Fraction

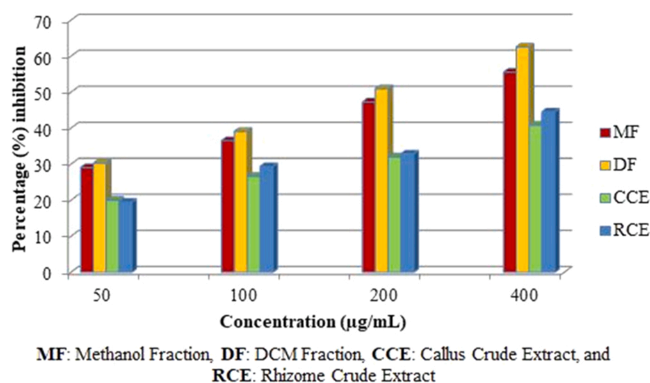

Fig. 5. Percentage inhibition of rhizome and callus of *P. polyphylla* in HeLa cells.

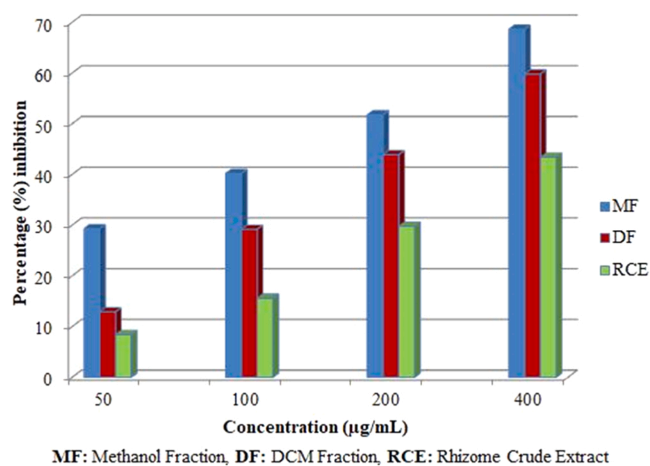

Fig. 6. Percentage inhibition of rhizome and callus of *P. polyphylla* in MCF-7 cells.

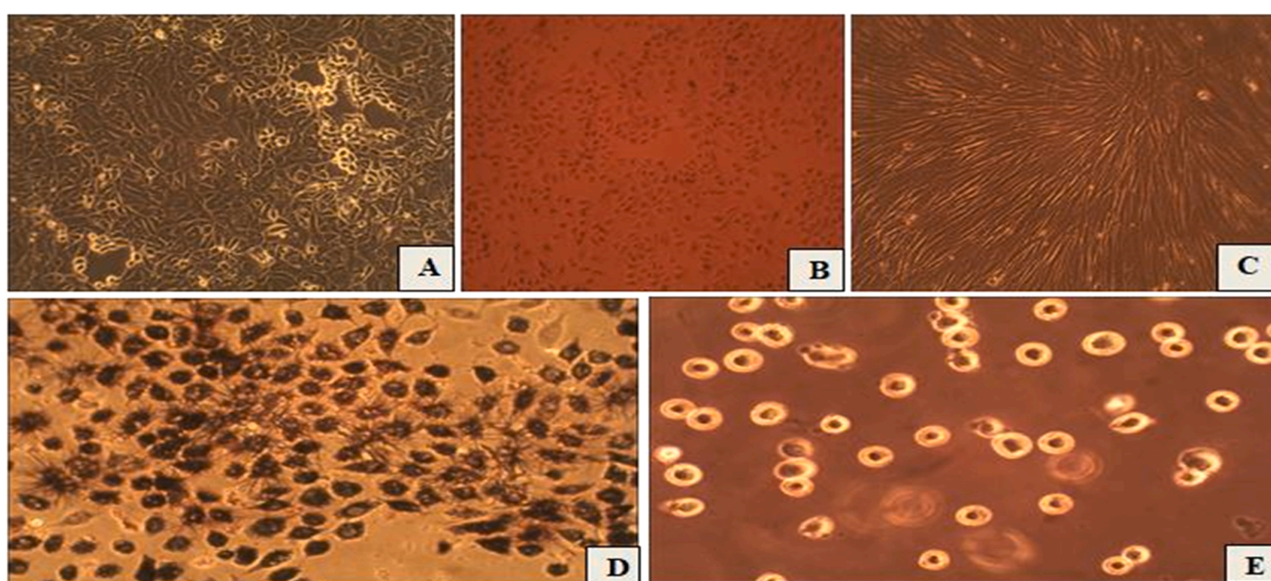

Fig. 7. Olympus Inverted Microscopic Images of cell lines at various stages: a) MCF-7 cells; b) HeLa confluent cells; c) Normal human epithelial cells; d) Formazan formation; e) Trypsinized cells (Cells cultured in EMEM media in a 5 % CO2 incubator at 37 °C).

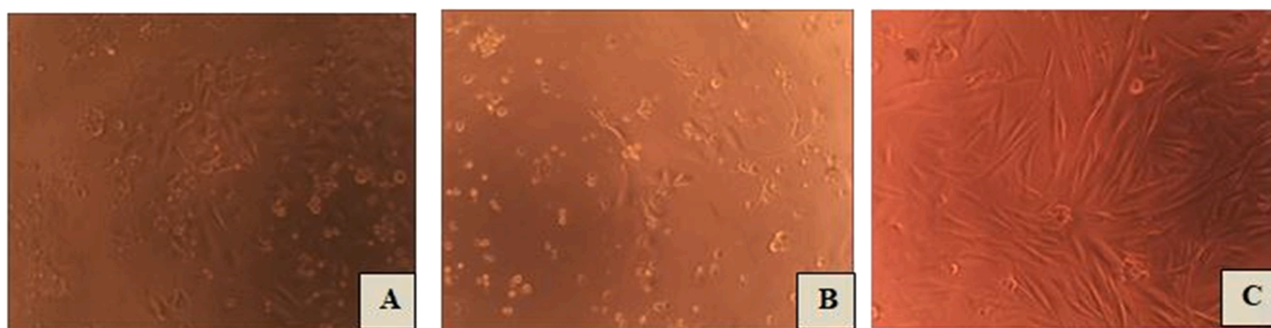

Fig. 8. Olympus Inverted Microscopic Images of cell lines after treatment of extracts: a) HeLa cells; b) MCF-7 cells; c) Normal epithelial cells.

cells [9,10,52,62] could be due the purity of targeted compounds. The results of this investigation on callus and rhizome crude extracts and rhizome fractions (apart from the methanol fraction in MCF-7 cells) of *P. polyphylla* revealed a positive correlation between the antioxidant, antibacterial, and cytotoxic activities. Because extracts such as the DCM fraction had increased antioxidant activity, they also had higher antibacterial and cytotoxic effects, and vice versa. Similar bioactive compounds may be responsible for all these three bioactivities.

4. Conclusions

In *P. polyphylla*, the results of this investigation demonstrated a

significant association between the antioxidant, antibacterial, and cytotoxic properties of crude extracts (rhizome and callus) and fractions (rhizome). *In vitro*-produced callus showed antioxidant, antibacterial, and anticancer activity comparable to *in vivo* rhizome, suggesting that it could be an alternative source of bioactive compounds for therapeutic drugs. Besides, the fraction of crude extracts of *P. polyphylla* rhizome showed higher free radical scavenging activity, and antibacterial activity against five bacteria (*Acinetobacter baumannii, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Bacillus subtilis*), and cytotoxicity against HeLa and MCF-7 cells than the methanolic crude extracts, possibly due to the presence of more antioxidant, antibacterial and anticancer compounds in fractions. Furthermore, the DCM fraction demonstrated stronger antioxidant and antibacterial activity than methanol fractions, which could be because phenolic compounds have a higher affinity for DCM than methanol. In the case of cytotoxicity, the DCM fraction was more cytotoxic to HeLa cells, while the methanol fraction was more cytotoxic to MCF-7 cells. These findings suggest that the bioactive components in the active extracts of *P. polyphylla's in vivo* rhizome and *in vitro* callus may help with the development of natural drugs for therapeutic purposes.

CRediT authorship contribution statement

CBT: Conceptualization, Data generation, and Analysis, Writing-Original draft preparation. **HDB:** Formal analysis, Review, and Editing. **KKP:** Review, Analysis & Editing. **PRJ:** Assisting in Data generation & Editing. **TLC:** Assisting in Data generation, **BP:** Writing-Review & Final Editing.

Declaration of Competing Interest

The authors and other parties do not have any competing interests.

Data availability

Data will be made available on request.

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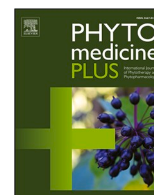

Comparative antioxidant, antibacterial, and antidiabetic activities of *in vitro*-grown callus and wild-grown various parts of *Piper longum* L

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A B S T R A C T

Background: Piper longum L. is a tropical and subtropical medicinal plant that has been used as antidiabetic, diarrhea, stomachache, cough, asthma, and bronchitis since ancient times. The *in vitro*-grown callus from the leaf, however, has not been tested and utilized for any of these conditions. Thus, this research pursues to assess the comparative study of antioxidant, antibacterial, and antidiabetic activities of *in vitro*-grown callus and various parts of *Piper longum*-grown in the wild.

Methods: The antioxidant activity of crude extracts and the fractions were determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) free-radical scavenging assay, the antibacterial activity by agar-well diffusion method, and antidiabetic activity by α-amylase and α-glucosidase inhibition assay. In addition, the total phenol content (TPC) and total flavonoid contents (TFC) were calculated using Folin-Ciocalteau reagent and aluminum chloride complex-forming assay respectively.

Results: The highest antioxidant activity (IC_{50}=134.81±1.16 μg/mL), TPC (41.22±0.50 mg of GAE/g dry weight), and TFC (73.41±0.53 mg of QE/g dry weight) were obtained in the dichloromethane (DCM) fraction of root than other crude extracts and fractions. The DCM fractions of root exhibited the minimum inhibitory concentration (MIC) at 5.0 mg/mL and minimum bactericidal concentration (MBC) of 8.35 mg/mL to the *Staphylococcus aureus.* Moreover, the DCM fraction of root showed the highest α-amylase and α-glucosidase inhibition (IC_{50}=365.21±31.02 and 489.07±27.96 μg/mL) than other crude extracts and fractions. To the best of our information, comparative antioxidant (IC_{50}=206.61±0.64 μg/mL), antibacterial (suppressed 80 % of tested bacteria), and antidiabetic (IC_{50}=1165.15±15.63 and 1304.76±12.43 μg/mL) properties of the *in vitro*-grown callus were recorded for the first time in *P. longum,* and were found to be comparative to those of the wild-grown parts.

Conclusions: These results showed that wild-grown *P. longum* roots, particularly the DCM fraction, could be an important source of natural antioxidants, antimicrobials, and antidiabetics for better curative uses than other wild-grown parts and *in vitro*-grown callus. *In vitro*-grown callus, however, could be used as a natural drug source and used to conserve *P. longum* in its natural habitats in the future.

Abbreviations

2,4-D	2,4-dichlorophenoxy acetic acid
ANOVA	analysis of variance
ATCC	American type culture collection
CFU	colony-forming unit
DPPH	(1,1-diphenyl-2-picrylhydrazyl)
FCR	Folin-Ciocalteau reagent
IC_{50}	half maximal inhibitory concentration
Kn	kinetin
MBC	minimum bactericidal concentration
MHA	Mueller Hinton agar
MIC	minimum inhibitory concentration
MS	Murashige and Skoog
NA	nutrient agar
NAA	naphthalene acetic acid
NB	nutrient broth
ROS	reactive oxygen species
RPI	relative percentage inhibition
RSA	radical scavenging activity

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TFC total flavonoid contents
TPC total phenol content
ZoI zone of inhibition

1. Introduction

Antioxidants are molecules that prevent oxidation, a chemical process that can cause the production of reactive oxygen species (ROS) or free radicals, and chain reactions that can injure cells if their levels in living organisms get too high, and can neutralize unstable free radicals. Reactive oxygen species have been shown to savage the cell membrane's unsaturated fatty acids, causing lipid peroxidation, decreased membrane fluidity, the decline of enzyme and receptor activity, and membrane protein degradation (Dean and Davies, 1993). When free radicals exceed antioxidants, oxidative stress develops that has been related to a variety of degenerating diseases, the most common of which are atherosclerosis, hypertension, and diabetes (Cai and Harrison, 2000), tissue ischemia, and premature aging (Halliwell and Gutteridge, 1990), heart problems (Kris-Etherton et al., 2002), Alzheimer's and Parkinson's disorders (Di Matteo and Esposito, 2003), irritation and cutaneous aging (Ames et al., 1993) as well as a variety of cancers (Ziech et al., 2010; Simon et al., 2000). Diabetes, a metabolic disorder, is mostly caused by oxidative stress, and taking antioxidant supplements and using medicinal plants with antioxidant properties has been shown to have hypoglycemic effects (Rajendiran et al., 2018). Diabetes is represented by high blood sugar levels, which over time seriously harm the kidneys, eyes, heart, and blood vessels. The most common kind of diabetes, type 2, typically strikes adults and arises when the body either stops generating adequate insulin or develops a resistance to it. According to data from the International Diabetes Federation (IDF), 1 in 11 people worldwide has diabetes, and the incidence of diabetes ranges from 7.66 % to 11 % (Muzammil et al., 2022). Biguanides, meglitinides, thiazolidinediones, acarbose, miglitol, and voglibose are being used as hypoglycemic and insulin-like drugs to reduce blood glucose levels in diabetes patients (Rahman et al., 2021). From ancient times, ethnic communities all over the world have used various parts—such as roots, stems, leaves, fruit, flowers, etc.—in the form of juices, infusions, decoctions, powders, and other preparations to treat antidiabetic disorders. According to Rahman et al. (2021), ethnic groups in Bangladesh use medicinal plants' leaves the most for antidiabetic purposes, followed by fruits, whole plants, roots, etc., however, they mostly use fruits of *P. longum* as antidiabetic agents.

Most antioxidants contain polyphenols, flavonoids, carotenoids, and vitamins. Polyphenols are widely identified as anti-inflammatory, antiviral, antibacterial, and antioxidant agents among these phytochemicals (Steinmetz and Potter, 1996). Some enzymes like glutathione peroxidase, superoxide dismutase (SOD), and catalase are examples of antioxidant mechanisms that can protect the body from harm produced by reactive oxygen species (ROS). To counteract free radicals, which bring about a range of cancers in mankind, several natural products are now accessible on the pharmaceutic market as antioxidants (Ali et al., 2015). Moreover, the failure of chemotherapy and antibiotic resistance is one of the most significant issues facing our time, which has prompted research into the antibacterial properties of numerous medicinal plants (Colombo and Bosisio, 1996). Since medicinal plants are believed to be a rich source of antibacterial compounds, their ability to combat germs is examined (Ahirwal et al., 2011). There might be an affirmative relationship between the antioxidant properties of compounds and their antibacterial and antidiabetic activities.

A callus is a homogeneous mass of parenchyma cells formed from proliferating cells in nutrient media that can be employed for micropropagation and secondary metabolite production. It is critical to provide the ideal growing conditions (chemical and physical settings) for the plant species utilized to develop callus and secondary metabolites. Callus cultures have developed economic promise for the production of therapeutically crucial secondary metabolites (Shrestha and Pant, 2011; Pant, 2014; Ogita, 2015; Wu et al., 2016). As they produce secondary metabolites *in vitro*, they can exhibit a variety of bioactivities including antioxidant, antibacterial, antidiabetic, etc.

Piper longum is a medicinal and aromatic plant that is distributed from tropical to subtropical parts of Srilanka, Nepal, India, Bhutan, Indonesia, Singapur, Malaysia, and Myanmar (Thapa et al., 2022). In Nepal, it is spread out east to west between Terai, and Churia hills up to 1000 m (Press et al., 2000). The Government of Nepal has emphasized this plant for research and development as well as agrotechnology development (DPR, 2012). Its dry fruits/spikes (Pipla) and thick root with stem segment (Piplamool) are traded illegally to India and other countries, which are used in cough, bronchitis, asthma, diabetes, diarrhea, stomachache, and indigestion (Thapa et al., 2022). It can be utilized in traditional Ayurvedic drug formulations and as an alternative medication for diabetes, oxidative stress-related diabetic complexities, and other conditions (Bansal et al., 2017). According to Zaveri et al. (2010) the alkaloid piperine, which makes up 3–5 % of *P. longum's* dry mass, is the main and active component. The compound piperine exhibits antifebrile, a pain reliever, antimicrobial, insecticidal, and anti-inflammatory effects (Kakegawa et al., 1992). The crude extracts, fractions, and pure compounds extracted from wild-grown parts of *P. longum* showed bioactivities against antibacterial, antidiabetic, anti-inflammatory, antimalarial, anticancer, antiasthmatic, immunomodulatory, hepatoprotective, antiulcer, antidepressant, analgesic, hypocholesterolemic, antiamoebic, antifungal, insecticidal, and acaricidal activities (Zaveri et al., 2010).

This research pursues to examine and compare the antioxidant, antibacterial, and antidiabetic characteristics of *in vitro*-grown callus from leaf and various parts of *P. longum* grown in the wild. If the callus induced from leaf explants demonstrates increased potential for certain bioactivities comparable to those of wild-grown plants, it would be conceivable to use it in place of wild plants, and that helps conserve valuable medicinal plants in their natural habitats. Many biologically active metabolites, especially those with antioxidant, antimicrobial, anti-diabetic, anti-stress, and anticancer characteristics, have been frequently reported from *in vivo* parts of *P. longum*. But, to the best of our information, the comparative antioxidant, antibacterial, and antidiabetic characteristics of *in vitro*-grown callus and wild-grown parts of *P. longum* have been reported for the first time.

2. Materials and method

2.1. Plant materials

P. longum at one and a half years old was collected from the Rupandehi district in western Nepal in November-December 2021 and a few plants were grown in pots at the Central Department of Botany, T.U. for callus induction from the leaf. The plant specimen was ascertained by comparing the herbarium of the Tribhuvan University Central Herbarium (TUCH), and the plant specimens were stored in the TUCH, Kirtipur (Vouchers No. 135 & 136).

2.2. Chemicals and reagents

The chemicals and reagents used for *in vitro* free radical scavenging activity and α-glucosidase inhibition assays were bought from Merck Company, Germany. The hormones (2,4-D, Kn, and NAA) utilized to induce callus, as well as the media utilized for antimicrobial assays such as NA (Nutrient Agar), NB (Nutrient Broth), and MHA (Mueller Hinton Agar), were bought from HiMedia, India. Alpha-amylase from porcine pancreases and 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) were purchased from Sigma-Aldrich (Germany). Antibiotic ciprofloxacin was bought from Lomus Pharmaceuticals, Kathmandu, Nepal. The reagents and chemicals utilized were of analyzing quality.

2.3. Bacterial strains

The National Public Health Lab (NPHL) in Kathmandu, Nepal provided the ATCC strains of bacteria used in this investigation. Antibacterial activity was tested using *Pseudomonas aeruginosa* (ATCC 9027), *Bacillus subtilis* (ATCC 6051-U), *Acinetobacter baumannii* (ATCC 19606), *Staphylococcus aureus* (ATCC baa977), and *Escherichia coli* (ATCC MA 35218).

2.4. Induction of callus and biomass production

With a few modifications, the standard protocol (Akbaş et al., 2009; Thapa et al., 2023) was followed for callus induction and biomass production in MS media from leaf explants. Surface sterilization of leaf explant with 70 % ethanol for 30 s was followed by decanting the ethanol, adding 0.1 % mercuric chloride ($HgCl_2$) for 3–4 min, and three thorough washes with sterile distilled water to remove any remaining mercuric chloride. Leaf explants (0.5 cm^2) were cultured on MS, 1/2 MS, and 1/4 MS (Murashige and Skoog, 1962) alone or with additional hormones (2,4-D and Kn). Culture tubes (20 × 150 mm^2) with explants were subjected to fluorescent lighting (3000–4000 lux) with a 16 h photoperiod at 25±2 °C in the culture room. At 12 weeks of culture, the callus was induced in MS medium fortified with and without hormones. The callus was proliferated for 8 weeks under the same condition to obtain a large amount of callus.

2.5. Preparation of extracts

2.5.1. Wild plant parts extract preparation

The root, stem, leaf, and fruits of *P. longum* were air dried before being put through an electric grinder (Electron) to make powder. About 200 g of powder of root, stem, leaf, and fruit were macerated separately in 800 mL of 90 % methanol for 72 h (Bhattarai et al., 2022). This process was repeated three times, each time filtering the mixture solution through cotton and filter paper. The filtrate was concentrated at 37 °C using a rotary evaporator (EYELA). Finally, the crude extract was weighed; kept in vials; and stored at 4 °C in the fridge for further biochemical analysis. The percentage yield of extracts was calculated using the following formula.

$$\text{Percentage Yield} = \frac{\text{Dry weight of plant extract}}{\text{Dry weight of plant powder}} \times 100$$

2.5.2. In vitro-grown callus extract preparation

In vitro-grown callus extract was prepared by following the standard protocol (Park et al., 2018; Thapa et al., 2023) with some modifications. Air-dried callus (15 g) was crushed with 50 mL of pure methanol in a mortar and pestle. The callus mixture was macerated for 72 h in 150 mL of methanol in the dark, stirring thoroughly every 12 h with a glass rod. The mixture solution was filtered with filter paper. This process was repeated three times. Then, the filtrate was concentrated at 37 °C using a rotary evaporator under reduced pressure. The crude extract was accumulated in vials and refrigerated at 4 °C for subsequent chemical analysis.

2.5.3. Crude extracts fractionation

The crude extracts of wild-grown parts were fractionated separately using the liquid-liquid partition method and a range of nonpolar to polar solvents, such as hexane, dichloromethane, and methanol (Bhattarai et al., 2022). About 20 g of crude extract was dissolved in 100 mL of distilled water, filtered with filter paper, and then combined in a separating funnel with hexane in a 1:2 ratio. The hexane and the aqueous layers were isolated by agitating the solution in a separating funnel. The aqueous layer was then combined with DCM at 1:2 ratios using a separating funnel, agitated thoroughly, and the DCM layer was separated from the aqueous layer. Under reduced pressure, the aqueous layer was concentrated at 45 °C on a rotary evaporator to produce an aqueous extract that was then mixed in 100 % methanol. The mixture was filtered with the filter paper to obtain the methanol solution/layer. All the separated layers/solutions were concentrated (at 20 °C for DCM, and 37 °C for hexane and methanol) individually to separate the hexane, dichloromethane, and methanol fractions in a rotary evaporator. Moreover, all the fractions were weighed, placed in vials, and maintained at 4 °C until needed. The hexane fraction, however, was disregarded because it displayed negligible bioactivities in response to the measured activities.

Moreover, the crude callus extract was also fractionated with hexane, DCM, and 100 % methanol, as previously described. However, on the evaluated assay, these fractions failed to yield a significant result. As a result, we excluded all fractions from antioxidant, antibacterial, and antidiabetic tests.

2.6. Determination of free radical scavenging activity, total phenolic contents, and total flavonoid contents

2.6.1. Free radical scavenging activity by DPPH assay

The free radical scavenging capacity of the crude extracts and fractions was assessed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay by following Blois (1958) and Desmarchelier et al. (1997) with slight modification. A volume of 100 μL of each sample (1 mg/mL) with various concentrations was blended with 100 μL of DPPH (0.1 mM of DPPH in methanol) in a 96-well plate. The microplate was left to stand in the dark for 30 min. For positive control, various concentrations of ascorbic acid (5 μg/mL to 50 μg/mL) and for negative control, absolute methanol were utilized. A 96-well microplate reader (Synergy LX) was used to measure the absorbance at 517 nm. The percentage of the DPPH free radical scavenging activity was computed using the given formula.

$$\%\ \text{Radical Scavenging Activity (RSA)} = \frac{Ab_{Control} - Ab_{Sample}}{Ab_{Control}} \times 100$$

Where $Ab_{Control}$= Absorbance of negative control methanol with DPPH

Ab_{sample}= Absorbance of extracts or samples with DPPH

The effective extract concentration needed to scavenge 50 % of the DPPH free radicals is designated as the IC_{50} (50 % inhibitory concentration).

2.6.2. Total phenolic content (TPC)

The quantity of total phenolic content in the crude extract and fractions was assessed using the Folin-Ciocalteau reagent (FCR) with gallic acid as the standard (Zhang et al., 2006). The volume of 100 μL Folin-Ciocalteu's reagent (1:10) was combined with 20 μL of extract (1 mg/mL) in a 96-well plate. The addition of 80 μL of sodium carbonate (1 M) solution was done after 6 min. The absorbance was measured at 765 nm following a 15-minute incubation in the dark. The concentration of all phenolic compounds was calculated as mg of gallic acid equivalents per g of dry extract (mg of GAE/g of E) utilizing a standard curve of gallic acid (10 μg/mL to 60 μg/mL). Three replicates of each measurement were made.

2.6.3. Total flavonoid contents (TFC)

With some modifications from Chang et al. (2002) an aluminum chloride complex-forming assay was performed to evaluate the total flavonoid contents of the samples. Absolute methanol was used to dissolve quercetin (1 mg/mL) and served as the standard. The complete test mixture containing 20 μL of plant extract (1 mg/mL) or standard quercetin, 5 μL 10 % $AlCl_3$, 5 μL potassium acetate (1 M), 60 μL ethanol, and 110 μL distilled water was kept in the dark for 30 min and the absorbance was measured at 415 nm. Using the quercetin standard curve (10 μg/mL to 80 μg/mL), the amount of TPC was reported as milligrams of quercetin equivalent per gram of dry weight (mg of QE/g) of the extract.

2.7. Determination of antibacterial activity

The agar well diffusion method was used to measure antibacterial activity (Mosquera et al., 2004; Lokhande et al., 2007) with minor modifications. Stock solution was prepared by dissolving 100 mg of extracts in 1 mL of pure methanol. The serial dilution method was then used to prepare extracts in different concentrations. To resurrect the bacteria and create a pure culture, all the microbial strains were spread on nutrient agar plates and cultured at 37 °C for 24 h. Then, 1.5×10^8 CFU/mL of bacteria was obtained by transferring a few similar bacterial colonies into normal saline and roughly diluting them to 0.5 McFarland standard turbidity. A sterile cotton swab was utilized to inoculate individual bacterial strains from the diluted bacterial solutions onto Mueller Hinton Agar (MHA) plates. Utilizing a sterile cork borer, 6 mm-diameter wells were made on the MHA plates. The MHA plates were then incubated at 37 °C for 24 h with 35 μL of extracts in each well. The zone of inhibition (ZoI) in mm surrounding the application site of each extract solution—5, 10, 20, 40, and 60 mg/mL—was used to assess the antibacterial activity. The antibiotic ciprofloxacin (50 μg/mL) was used as the positive control, while pure methanol was used as the negative control. The average for the ZoI was determined by averaging three duplicates of the extracts.

2.7.1. Relative percentage inhibition

The relative percentage inhibition of bacterial strains by each concentration of the extract was obtained by analyzing the zones of inhibition of positive (ciprofloxacin) and negative (methanol) controls (Mayirnao and Bhat, 2017).

$$\text{Relative percentage inhibition of test extract} = \frac{(m - n)}{(o - n)} \times 100$$

Where, m= Inhibition zone of the plant extracts (samples)
n= Inhibition zone of the methanol (negative control)
o= Inhibition zone of the ciprofloxacin (positive control)

2.7.2. Minimum inhibitory concentration (MIC) assay

To assess minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) values, only extracts that displayed an inhibitory zone with particular bacterial strains were employed. The MIC values of different extracts were assessed utilizing the micro-broth dilution method (Abu-Shanab et al., 2006) with some modifications. The extracts were serially diluted in methanol at concentrations of 1.0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, and 50 mg/mL. Each dilution (1 mL) of extracts was combined with 0.05 mL suspension of the tested bacterial strain before being incubated at 37 °C for 24 h. The plant extracts (0.05 mL) of each calculated concentration in 2 mL of liquid nutrient broth were used as the negative control, whereas 0.05 mL of bacterial suspension in 2 mL of liquid nutrient broth was used as the positive control. The MIC was determined visually as the lowest concentration of extract (mg/mL) which prevented bacteria from growing in a mixture of nutrient broth. Then, the MBC of each extract was determined by inoculation of each concentration of sample solution starting from MIC in Mueller Hinton Agar media and incubated for 24 h at 37 °C. The minimum concentration of an antibacterial agent (extracts) necessary to eradicate a particular bacterium is called the MBC.

2.8. In vitro alpha-glucosidase inhibition assay

The α-glucosidase inhibition method was used to assess the antidiabetic effect of crude extracts and fractions (Fouotsa et al., 2012). The volume of 20 μL of various extract concentrations (75–1200 μg/mL), 20 μL of alpha-glucosidase enzyme (0.5 unit/mL), and 60 μL of phosphate buffer (6.8 pH, 0.1 M) were all added in a 96-well plate. After the mixture solution had been pre-incubated at 37 °C for 15 min, the initial absorbance was determined at 405 nm in a spectrophotometer (Synergy LX). After that, 40 μL of p-NPG (5 mM) was mixed with the solution mixture, and it was left at 37 °C for incubation for 15 min. With the addition of 60 μL of 1 M Na_2CO_3, the reaction ceased, and the quantity of p-nitrophenol that p-NPG releases was calculated using the final absorbance measured at 405 nm. As a positive control acarbose was utilized, and the phosphate buffer served as a negative control. The percentage of alpha-glucosidase inhibition was computed utilizing the formula, and the required concentration of extract to block 50 % of alpha-glucosidase activity (IC_{50}) was determined using a linear regression equation.

$$\% \text{ Inhibition} = \frac{A_1 - A_2}{A_1} \times 100$$

Where, A_1=Absorbance of enzyme-substrate reaction with phosphate buffer

A_2= Absorbance of enzyme-substrate reaction with various extracts of the plant

2.9. In vitro alpha-amylase inhibition assay

With minor modifications, the α-amylase inhibition was assessed by following Senger et al. (2012). The various extract concentrations (20 μL) were mixed with 80 μL of an enzyme (1.5 units/mL) in 50 mM sodium phosphate buffer (pH 6.9, 0.9 % NaCl) and then the mixture was preincubated at 37 °C for 10 min. After adding 100 μL of CNPG3 (1.0 mM) as the substrate, the reaction mixture was incubated at 37 °C for 15 min. Sodium phosphate buffer was used as the negative control and acarbose as the positive control. The absorbance of the sample was measured in a spectrophotometer at 405 nm, and the enzyme inhibitory effect was calculated using the formula mentioned above.

2.10. Statistical analysis

For induction and proliferation of *in vitro* callus, the mean values of a callus fresh weight with standard deviation were calculated. The quantity of each plant extract that must be used for DPPH, α-glucosidase, and α-amylase enzyme inhibition activities by 50 % (IC_{50}) was calculated in Microsoft Excel 2010 using a linear regression equation. Using the R program, a one-way ANOVA test was carried out at a p-value of <0.05 to assess the significance of plant parts to free radical scavenging capacity (IC_{50}). Moreover, the outcomes of antibacterial tests including ZoI, MIC, and MBC were converted into percentages in Microsoft Excel 2010.

3. Result and discussions

3.1. Induction of callus and biomass production

Callus was induced in full MS, 1/2MS, and 1/4MS with and without the addition of plant growth regulators (Fig. 1). However, a large callus was found in MS media supplemented with 1.0 mg/L 2,4-*D* + 2.0 mg/L Kn and 10 % coconut water. One of the most essential elements controlling cell growth, metabolite synthesis, and differentiation is plant growth regulators (Liang et al., 1991). At the constant concentration of 2,4-D (1.0 mg/L), the callus biomass increased with Kn from 0.5 mg/L to 2.0 mg/L and then decreased. It could be because callus induction is favored by a medium concentration of auxin (2,4-D) to cytokinin (Kn) concentration. The callus was proliferated under the same condition for 8 weeks to obtain substantial callus biomass. The secondary metabolites synthesized by the callus (20-week-old callus) in a nutrient medium during this time were used for the determination of its antioxidant, antibacterial, and antidiabetic activities. The types of plant hormones used for callus induction and the age of the callus might affect the quality and quantity of metabolite production in the callus.

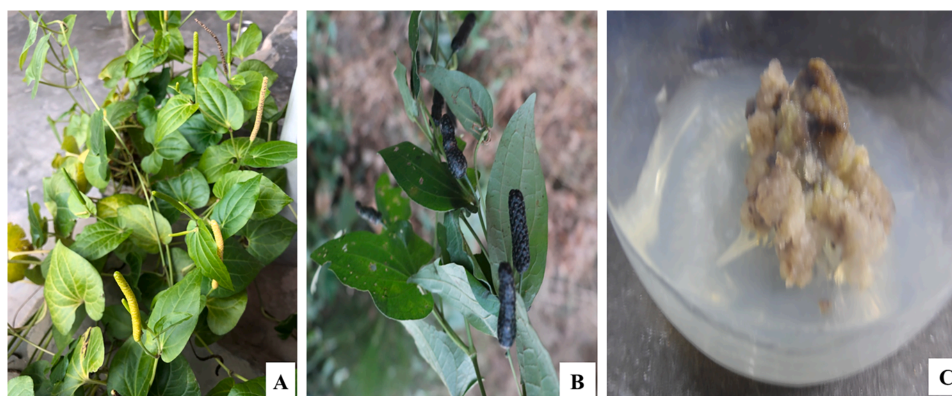

Fig. 1. *Piper longum:* (A) Wild-grown male plant, (B) Wild-grown female plant, (C) *In vitro*-grown Callus.

3.2. Yield of extraction and fractionation

Cold and hot maceration processes were used for the extraction of *in vitro*-grown callus and various parts of *P. longum* using 90 % methanol. The best extraction solvent is methanol since it can extract a variety of valuable bioactive compounds and produces the greatest extraction yields. Because methanol has a low boiling point (64.7 °C), a lower temperature is required to evaporate it to yield an extract. As a result, overheating is less likely to harm the extracted compounds. The highest extraction yield was obtained in fruit (21.54 %), followed by leaf (11.07 %), stem (6.17 %), root (5.75 %), and callus (2.05 %) (Table 2).

Moreover, various bioactive compounds with different polarity can be found in plants. The degree of polarity of the utilized solvent and the polarity of the bioactive compounds are key factors in the liquid-liquid partition method used to fractionate crude extract. Thus, the extraction solvent has to match the polar nature of the intended chemical compounds. The yields of the DCM and methanol solvents were considerably different, and the polarity-based fractionation revealed that the methanol yield was higher than the DCM yield in all the parts of *P. longum* such as leaf, stem, fruit, and root (Table 1). It might be due to the polar nature of methanol, which can dissolve a wide range of compounds. Zhang et al. (2018) state that an increased solvent-to-solid ratio corresponds to an increased extraction yield. This study demonstrated that the yields from polar solvents were greater than those from non-polar solvents.

Table 1
Callus induction & biomass production in MS media from leaf explant in *P. longum*.

S. N.	*Strength of Media	Plant Growth Regulator (mg/L)	Fresh wt (g) ±SD	Dry wt (g) ±SD
1	FMS	–	0.837±0.045	0.323 ±0.080
2	HMS	–	0.674±0.022	0.259 ±0.059
3	QMS	–	0.409±0.022	0.155 ±0.023
4	FMS	1.0 2,4-*D* + 0.5 Kn + 10 % CN	1.560±0.067	0.594 ±0.095
5	FMS	1.0 2,4-*D* + 1.0 Kn + 10 % CN	2.289±0.159	0.870 ±0.127
6	FMS	1.0 2,4-*D* + 1.5 Kn + 10 % CN	2.612±0.196	1.010 ±0.268
7	FMS	1.0 2,4-*D* + 2.0 Kn + 10 % CN	3.142±0.220	1.213 ±0.315
8	FMS	1.0 2,4-*D* + 3.0 Kn + 10 % CN	1.732±0.156	0.670 ±0.175

Condition for culture: 16 h photoperiod with cool white fluorescent light, 25±2 °C temperature, at 12 weeks of primary culture.

* Abbreviations: FMS=Full MS, HMS=Half MS, QMS=Quarter MS, CN=Coconut water, Kn=Kinetin, 2,4-*D* = 2,4-dichlorophenoxy acetic acid.

Table 2
Crude extract/and Fraction yield (%) of various parts and callus of *Piper longum*.

Plant parts	Extraction method	Crude extract/ Fraction	Solvent used	Yield (%)
Leaf	Cold & hot maceration	Crude Extract	90 % methanol	11.07
	Liquid-liquid partition	DCM Fraction	DCM	1.69
	Liquid-liquid partition	Methanol Fraction	100 % methanol	12.77
Stem	Cold & hot maceration	Crude Extract	90 % methanol	6.17
	Liquid-liquid partition	DCM Fraction	DCM	2.20
	Liquid-liquid partition	Methanol Fraction	100 % methanol	78.77
Fruit	Cold & hot maceration	Crude Extract	90 % methanol	21.54
	Liquid-liquid partition	DCM Fraction	DCM	2.05
	Liquid-liquid partition	Methanol Fraction	100 % methanol	35.66
Root	Cold & hot maceration	Crude Extract	90 % methanol	5.75
	Liquid-liquid partition	DCM Fraction	DCM	0.57
	Liquid-liquid partition	Methanol Fraction	100 % methanol	16.19
Callus	Cold & hot maceration	Crude Extract	90 % methanol	2.05

3.3. Free radical scavenging activity of crude extracts and fractions by DPPH assay

DPPH is a generally used substrate for the rapid evaluation of the antioxidant activity of plant extract due to its stableness in the radical form and convenience of the test (Bozin et al., 2008). The impact of antioxidants on DPPH was considered to be due to their ability to donate hydrogen (Brighente et al., 2007). In this assay, the absorbance value decreases as the concentration of antioxidants (such as plant extract or ascorbic acid) increases in the reaction mixture.

Among the tested crude extracts of wild-grown parts and *in vitro*-grown callus, the root exhibited the maximum antioxidant activity, with an IC_{50} of 193.31±0.39 μg/mL, followed by callus (IC_{50}=206.61±0.64 μg/mL) (Table 3). Similarly, the root had the maximum free radical scavenging capacity (IC_{50}=193.31±0.39 μg/mL) among the tested methanolic crude extracts of wild-grown parts, followed by leaf (IC_{50}=311.72±0.49 μg/mL), fruit (IC_{50}=391.74±0.57 μg/mL), and stem (IC_{50}=576.32±0.83 μg/mL). It might be due to the presence of a higher amount of antioxidant compounds in the crude root extracts than in other extracts. However, Jobi and Seju (2020) reported that the methanolic crude extract of the leaf possessed higher antioxidant

Table 3
Antioxidant activity, total phenol and flavonoid content of the various extracts and fractions of P. longum.

Plant parts	Crude Extract /Fraction	Antioxidant activity (IC_{50}: μg/mL)	Total phenol content (mg of GAE/g dry wt)	Total flavonoid content (mg of QE/g dry wt)
Root	Crude extract	193.31±0.394	137.33±0.120	113.18±0.532
	DCM fraction	134.81±1.162	41.22±0.509	73.41±0.531
	Methanol fraction	487.38±0.251	12.59±0.925	7.94±0.196
Leaf	Crude extract	311.72±0.490	118.32±0.105	98.17±0.093
	DCM fraction	186.11±0.487	27.62±0.848	24.15±0.491
	Methanol fraction	346.82±0.586	14.37±0.925	8.82±0.281
Fruit	Crude extract	391.74±0.577	113.94±0.193	82.10±0.054
	DCM fraction	194.89±0.514	22.25±0.169	20.75±0.315
	Methanol fraction	520.30±0.587	10.55±0.962	7.60±0.325
Stem	Crude extract	576.32±0.835	94.80±0.231	62.60±0.301
	DCM fraction	310.04±0.600	21.66±0.509	17.81±0.434
	Methanol fraction	584.28±0.497	9.59±0.570	7.10±0.281
Callus	Crude extract	206.61±0.649	115.87±0.58	95.81±0.156
Ascorbic acid (positive control)		7.40±0.533	–	–

potential and total phenolic content than the fruits in *P. longum.* Similarly, compared to ethanol and acetone extracts, Muzammil et al. (2022) found that the crude methanol leaf extract of *Coronopus didymus* had higher polyphenol contents and antioxidant activity. The ability of methanolic crude extracts of wild-grown parts of *P. longum* to scavenge free radicals in this study was found to be different than that of various crude extracts reported by previous researchers. It could be caused by factors including the purity of the extraction solvent, the use of polar or non-polar solvents, location, the environment where the plant is growing, and other environmental factors. On the other hand, pure molecules isolated from various crude extracts and fractions can have significant antioxidant activity.

Among the tested fractions of wild-grown parts, the dichloromethane fraction of root showed the highest antioxidant activity, with an IC_{50} of 134.81±1.16 μg/mL, followed by the DCM fraction of leaf (IC_{50}=186.11 ±0.487.60 μg/mL) compared to ascorbic acid (IC_{50}=7.40±0.53 μg/mL) (Table 3). According to this research, fractions have a greater ability to scavenge free radicals than their crude extracts. The overall concentration of phenols, which are known to have antioxidant capabilities, may be related to this activity. Several studies have suggested that the presence of bioactive compounds such as phenols and flavonoids may be liable for the plants' free radical scavenging potential, implying that they might be used as a natural antioxidant source (Subedi et al., 2014). Sannigrahi et al. (2010) noted that ethyl acetate fraction was found more effective than methanolic crude extracts, as well as their chloroform and n-butanol soluble fractions of *Enhydra fluctuans* for antioxidant activities. According to Silveira et al. (2017) fractions including n-butanol, ethyl acetate, and DCM were more effective than the crude extract of stem bark in *Tabernaemontana catharinensis*. It could be because the crude extract contains impurities or contaminants like lipids, carbohydrates, or a variety of other non-polar inactive substances. Moreover, this research demonstrates the accumulation of higher total phenolic and flavonoid contents in DCM fractions, as a result, it demonstrated a higher antioxidant capacity than methanol fractions. According to Thapa et al. (2023) the strong antioxidant and bioactivity of the dichloromethane fraction may be due to the synergistic interaction of both polar and non-polar molecules, as it can dissolve both polar and nonpolar organic compounds. On the other hand, the type of solvent used has a significant impact on the contents of the main bioactive compounds obtained from the plant, suggesting that the type of solvent may enhance the concentration (Muzammil et al., 2022). The p-value from one way ANOVA test of crude extracts of *in vitro*-grown callus and wild-grown plant parts such as root, stem, leaf, and fruit *versus* their IC_{50} value showed a significant difference (p-value <0.05, i.e.=$<2e^{-16}$). This study showed that the free radical scavenging activity of extracts depends on plant parts. The presence of bioactive metabolites varies by plant part and the amount of such metabolite accumulated (Pant et al., 2021).

Furthermore, compared to methanolic crude extracts of some wild-grown parts (fruit, stem, and leaf), the methanolic crude extract of *in vitro*-grown callus had stronger antioxidant activity (IC_{50}=206.61±0.64 μg/mL). Some reports have been found to support the significant antioxidant properties of the callus. According to Sagharyan et al. (2020) antioxidants such as phenolic and flavonoid compounds are synthesized and accumulated in *in vitro*-grown calluses to exhibit stronger antioxidant activity. A considerable potential to scavenge free radicals was also noted by other researchers for *in vitro*-grown callus. In *Habenaria edgeworthii*, callus grown on half MS medium fortified with 3.0 μM BAP (6-benzylamino purine) demonstrated significant phenolic content as well as raised antioxidant activity (Giri et al., 2012). Similarly, the stable free radical 2,2-diphenyl-1-picrilhydrazyl (DPPH) was decreased by methanol extracts of both light- and dark-grown calluses in *Inula crithmoides* (Bucchini et al., 2013).

3.4. Total phenolic contents (TPC) of crude extracts and fractions

We obtained the calibration curve of gallic acid to be $y = 0.018x$, and R^2=0.9807 using linear regression, where, R^2 is the correlation coefficient, y is the absorbance (nm), and x is gallic acid concentration. The quantity of TPC in the extracts/fractions was calculated using this curve.

Among the tested methanolic crude extracts, the maximum TPC was obtained in the root (137.33±0.12 mg of GAE/g dry weight) and the least was obtained in the stem (94.80±0.23 mg of GAE/g dry weight) (Table 3). The second-highest TPC (115.87±0.58 mg of GAE/g dry weight) was found in *in vitro*-grown calli, which may be the cause of their stronger antioxidant capacity. Similarly, among the tested fractions, the maximum TPC was obtained in the DCM fraction of root (41.22±0.50) and the least was found in the methanol fraction of stem (9.59±0.57 mg of GAE/g dry weight) (Table 3). The substantial free radical scavenging activity of the dichloromethane fraction is probably due to the high concentration of polyphenolics therein, which may have been purified and concentrated during the fractionation process. It might be because phenolic compounds have a greater affinity for dichloromethane than methanol. Antioxidants that break chain reactions are known as phenolic compounds. The total phenolic content of crude extracts and fractions has been linked to the capacity of these substances to scavenge free radicals, meaning that extracts/fractions with higher phenolic contents exhibited stronger antioxidant properties.

3.5. Total flavonoid contents (TFC) of crude extracts and fractions

We obtained the calibration curve of quercetin to be $y = 0.0256x$, and R^2=0.9838 using linear regression, where, R^2 is the correlation coefficient, y is the absorbance (nm), and x is quercetin concentration. The quantity of TFC in the extracts/fractions was calculated using this curve.

Among the tested methanolic crude extracts, the maximum TFC was obtained in the root (113.18±0.53 mg of QE/g dry weight) and the least was found in the stem (62.6 ± 0.30 mg of QE/g dry weight) (Table 3). The second-highest TFC (105.81±0.15 mg of QE/g dry weight) was found in *in vitro*-grown callus, which may be the cause of their stronger

antioxidant capacity. Among the tested fractions, the highest total flavonoid content was found in the dichloromethane fraction of root (73.41±0.53 mg of QE/g dry weight) and the least was found in the methanol fraction of stem (7.10±0.28 mg of GAE/g dry weight) (Table 3). In comparison to the methanol fractions, the dichloromethane fractions possessed more amount of flavonoid. It might be because flavonoid compounds have a stronger affinity for dichloromethane than methanol. This study demonstrated that extracts and fractions with higher total flavonoid content have stronger antioxidant properties.

3.6. Zone of inhibition (ZoI) and relative percentage inhibition (RPI)

Methanolic crude extracts of root and fruit prevented the growth of all (100 %) examined gram-positive (*S. aureus* and *B. subtilis*) and gram-negative (*P. aeruginosa, E. coli,* and *A. baumannii*) bacteria at five various concentrations (5, 10, 20, 40, & 60 mg/mL); leaf and callus crude extract 80 % of examined bacteria; and stem crude extract 60 % of examined bacteria. It was compared to the antibiotic ciprofloxacin (50 μg/mL), which prevented the growth of all bacterial strains that were investigated. It might be due to the presence of a higher amount of antibacterial compound in crude extracts of root and fruit. The two gram-positive and three gram-negative bacteria were used for the antibacterial test in this study because their responses to various extracts and antibiotics could vary. Gram-negative bacteria, for instance, usually possess an outer covering with a lipopolysaccharide component that shields themselves from antibiotics, making them less susceptible to them than gram-positive bacteria, and gram-negative bacteria also produce various toxins than gram-positive bacteria. Additionally, the crude extract of callus grown *in vitro* had antibacterial activity comparable to that of other wild-grown parts, and it prevented the growth of both gram-positive (*B. subtilis,* and *S. aureus*) and gram-negative (*P. aeruginosa*) bacteria. The presence of antimicrobial molecules in callus generated *in vitro* is dependent upon the type of plant and growth conditions, such as the age of the callus, the type of growth hormone used, etc. For instance, the antibacterial compounds phenol, 4H-pyran-4-one, 1,2-benzene dicarboxylic acid, and octadecanoic acid were found in the callus of *Canthium parviflorum* (Kala and Ammani, 2017). Further examination of the *in vitro-grown* callus of *P. longum* for antibacterial activity on other bacterial strains could yield encouraging results.

Furthermore, the dichloromethane fraction of root and fruit prevented the growth of 100 % of examined bacteria; the dichloromethane fraction of leaf 80 % of examined bacteria; the dichloromethane fraction of stem 40 % of examined bacteria; the methanol fraction of root and fruit 60 % of examined bacteria; and methanol fractions of stem and leaf 40 % of examined bacteria at five concentrations (5, 10, 20, 40, & 60 mg/mL) (Tables 4 & 5). Compared to gram-positive bacteria, gram-negative bacteria exhibited a smaller zone of inhibition and were more resistant to the used extracts and fractions due to the presence of their protective outer membrane. We found that crude extracts and dichloromethane fractions of root and fruits were more potent than other extracts and fractions on the examined bacterial strains, which might be connected with having more potent antibacterial compounds. Moreover, all the extracts and fractions demonstrated a zone of inhibition (ZoI) and relative percentage inhibition (RPI) that were concentration-dependent on the examined bacterial strains (Fig. 2).

This study demonstrated that crude extracts and dichloromethane fractions of *P. longum* roots and fruits could be used to cure a wide range of illnesses and disorders caused by *P. aeruginosa, A. baumannii, E. coli, B. subtilis, and S. aureus,* because these extracts and fractions prevented the growth of the bacteria *in vitro* condition. *A. baumannii is a* gram-negative, multidrug-resistant bacterium that can cause wound infections, pneumonia, bacteremia, and urinary tract infections. Similarly, *E. coli* causes sepsis, infantile stomach flu, traveler's diarrhea, and gastrointestinal bleeding; *P. aeruginosa* causes pneumonia, infections in the blood, or other body parts after surgery; *S. aureus* causes skin infections and sometimes pneumonia, inflammation of the heart, and

Table 4
Inhibition zone (ZoI) and relative percentage inhibition (RPI) of methanolic crude extracts *of P. longum* on various bacterial strains.

Sample/ Extract	Test Bacteria	ZOI (Inhibition zone) in mm±SD/% RPI (Relative percentage inhibition) (Omitting the diameter of the well)				
		5 mg/ mL	10 mg/ mL	20 mg/ mL	40 mg/ mL	60 mg/ mL
Root (Crude methanol)	**Ab*	2.0 ± 1.52 (8.11 %)	4.0 ± 1.00 (16.22 %)	6.0 ± 2.00 (24.33 %)	7.0 ± 2.08 (28.38 %)	9.0 ± 2.64 (36.49 %)
	**Bs*	4.0 ± 2.00 (14.11 %)	6.0 ± 1.00 (28.17 %)	7.0 ± 2.64 (24.7 %)	9.0 ± 1.73 (31.76 %)	15.0 ± 1.52 (52.94 %)
	**Ec*	6.0 ± 2.08 (23.38 %)	8.0 ± 2.64 (31.17 %)	10.0 ± 1.00 (38.97 %)	12.0 ± 2.64 (46.76 %)	14.0 ± 1.00 (54.55 %)
	**Pa*	3.0 ± 2.00 (10.84 %)	5.0 ± 1.00 (18.07 %)	7.0 ± 1.15 (25.30 %)	10.0 ± 2.64 (36.15 %)	12.0 ± 1.00 (43.38 %)
	**Sa*	6.0 ± 1.15 (18.37 %)	7.0 ± 1.52 (21.43 %)	11.0 ± 2.08 (33.68 %)	13.0 ± 1.52 (39.80 %)	20.0 ± 2.00 (61.23 %)
Stem (Crude methanol)	**Ab*	–	–	–	–	–
	**Bs*	–	–	6.o ± 2.00 (21.17 %)	8.0 ± 1.00 (37.78 %)	10.0 ± 0.57 (35.29 %)
	**Ec*	5.0 ± 2.64 (19.48 %)	6.0 ± 1.52 (30.8 %)	9.0 ± 1.00 (35.97 %)	10.0 ± 2.00 (38.97 %)	13.0 ± 1.0 (50.66 %)
	**Pa*	–	4.0 ± 1.73 (14.46 %)	6.0 ± 1.00 (21.69 %)	9.0 ± 2.00 (32.52 %)	11.0 ± 1.52 (39.76 %)
	**Sa*	–	–	–	–	–
Leaf (Crude methanol)	**Ab*	-	-	4.0 ± 1.00 (16.22 %)	5.0 ± 1.52 (20.64 %)	8.0 ± 2.08 (32.44 %)
	**Bs*	–	–	–	–	–
	**Ec*	–	6.0 ± 2.00 (23.38 %)	9.0 ± 1.73 (35.07 %)	11.0 ± 0.57 (42.86 %)	13.0 ± 1.00 (50.66 %)
	**Pa*	–	–	5.0 ± 1.00 (18.07 %)	8.0 ± 0.57 (28.92 %)	10.0 ± 2.08 (36.15 %)
	**Sa*	–	7.0 ± 2.00 (21.43 %)	10.0 ± 0.57 (30.61 %)	11.0 ± 1.52 (33.68 %)	19.0 ± 2.08 (58.17 %)
Fruit (Crude methanol)	**Ab*	1.0 ± 0.57 (4.05 %)	3.0 ± 2.00 (12.16 %)	5.0 ± 1.00 (20.27 %)	6.0 ± 2.08 (24.33 %)	8.0 ± 1.52 (32.88 %)
	**Bs*	3.0 ± 1.73 (10.58 %)	5.0 ± 2.00 (17.64 %)	6.0 ± 1.00 (21.17 %)	8.0 ± 2.08 (28.23 %)	14.0 ± 1.52 (49.41 %)
	**Ec*	5.0 ± 2.00 (19.48 %)	7.0 ± 1.52 (27.27 %)	9.0 ± 1.00 (35.07 %)	11.0 ± 0.57 (42.86 %)	13.0 ± 1.52 (50.66 %)
	**Pa*	2.0 ± 1.00 (7.23 %)	4.0 ± 0.57 (14.46 %)	6.0 ± 2.08 (21.69 %)	9.0 ± 1.73 (32.53 %)	11.0 ± 2.00 (39.76 %)
	**Sa*	5.0 ± 0.57	6.0 ± 2.00	9.0 ± 1.00	12.0 ± 1.73	15.0 ± 1.52

(*continued on next page*)

Table 4 (*continued*)

Sample/ Extract	Test Bacteria	ZOI (Inhibition zone) in mm±SD/% RPI (Relative percentage inhibition) (Omitting the diameter of the well)				
		5 mg/ mL	10 mg/ mL	20 mg/ mL	40 mg/ mL	60 mg/ mL
		(15.30 %)	(18.37 %)	(27.55 %)	(36.74 %)	(45.92 %)
Callus (Crude methanol)	*Ab	–	–	–	–	–
	*Bs	–	6.0 ± 2.00 (21.17 %)	10.0 ± 0.57 (35.29 %)	14.0 ± 1.73 (49.41 %)	17.0 ± 1.00 (60.0 %)
	*Ec	–	–	6.0 ± 2.64 (23.38 %)	8.0 ± 1.52 (31.17 %)	15.0 ± 1.00 (58.45 %)
	*Pa	–	5.0 ± 1.73 (18.07 %)	7.0 ± 1.73 (25.3 %)	10.0 ± 2.64 (36.15 %)	15.0 ± 1.00 (54.22 %)
	*Sa	8.0 ± 1.00 (24.49 %)	10.0 ± 0.57 (30.61 %)	14.0 ± 2.00 (42.86 %)	19.0 ± 1.73 (58.17 %)	22.0 ± 2.64 (67.30 %)
Ciprofloxacin (50 µg/mL)	For positive control: *Ab*= 24.66 mm, *Bs*=28.33 mm, *Ec*=25.66 mm, *Pa*=27.66, *Sa*=32.66 mm					
Absolute Methanol	For negative control: *Ab*= 0.0 mm, *Bs*=0.0 mm, *Ec*=0.0 mm, *Pa*=0.0, *Sa*=0.0 mm					

**Ab=Acinetobacter baumannii, *Ec=E. coli, *Pa=Pseudomonas aeruginosa, *Sa=Staphylococcus aureus, *Bs=Bacillus subtilis.*

infection of bone; and *B. subtilis* can spoil food and be considered an opportunist pathogen among the persons having an impaired immune system. However, before these extracts and fractions may be used as natural drugs or antibacterial agents for various bacterial infections, *in vivo* research is required for their validation.

3.7. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

All the methanolic crude extracts (except the crude callus extract) were not evaluated for MIC and MBC due to their smaller zones of inhibition and little relative percentage inhibition than their fractions on examined bacterial strains. Thus, only methanolic crude extract of *in vitro*-grown callus and all fractions of wild-grown parts were evaluated for the determination of MBC and MIC on the three gram-negative and two gram-positive bacterial strains. The minimum MIC (5.0 mg/mL) and MBC (8.35 mg/mL) were obtained in the dichloromethane fraction of root on *S. aureus*, which is a gram-positive bacteria. It could be due to the presence of higher concentrations of antibacterial compounds in DCM than in other fractions and crude extract of *in vitro*-grown callus (Table 6 & Fig. 3). However, pure compounds isolated from *P. longum* may have more potent antibacterial activity. According to Lokhande et al. (2007) the isolated constituents (piperin) from the root extract of *P. longum* showed better antibacterial activity (MIC=12.5 mg/mL) against *B. cereus* and *E. coli* than crude extracts.

Moreover, the methanolic crude extract of *in vitro*-grown callus also exhibited the minimum MIC (10 mg/mL) and MBC (13.5 mg/mL) in *S. aureus* (gram-positive), which may be related to the presence of significant antibacterial compounds in the callus and the more susceptible nature of the bacterial strain. Adhikari et al. (2013) found that compounds identified in the *in vitro*-grown callus of *Withania somnifera* were more potent than those obtained in the wild-grown roots in stopping the growth of *Klebsiella pneumonia, Proteus vulgaris, and Pseudomonas aeruginosa* whereas *Salmonella typhimurium, Bacillus subtilis, E. coli,* and *Staphylococcus aureus* were all inhibited by the compounds identified in the wild-grown roots. Therefore, the *in vitro*-grown callus of *P. longum* also might possess many antibacterial compounds that need to be

Table 5
Zone of inhibition (ZOI) and relative percentage inhibition (RPI) of fractions of crude extracts of *P. longum* on various bacterial strains.

Sample/ Extract	Test Bacteria	ZOI (Inhibition zone) in mm±SD/% RPI (Relative percentage inhibition) (Omitting the diameter of the well)				
		5 mg/ mL	10 mg/ mL	20 mg/ mL	40 mg/ mL	60 mg/ mL
Root (DCM Fraction)	*Ab	9.0 ± 1.73 (36.49 %)	12.0 ± 2.00 (48.66 %)	15.0 ± 1.00 (60.82 %)	17.0 ± 2.64 (68.93 %)	20.0 ± 0.57 (81.1 %)
	*Bs	10.0 ± 2.00 (35.29 %)	12.0 ± 2.08 (42.35 %)	18.0 ± 1.52 (63.53 %)	20.0 ± 1.00 (70.59 %)	23.0 ± 1.73 (81.18 %)
	*Ec	14.0 ± 1.00 (54.55 %)	16.0 ± 2.00 (62.35 %)	20.0 ± 2.08 (77.94 %)	21.0 ± 1.00 (81.83 %)	23.0 ± 0.57 (89.63 %)
	*Pa	12.0 ± 1.73 (43.38 %)	17.0 ± 0.57 (61.46 %)	20.0 ± 2.00 (72.3 %)	22.0 ± 1.00 (79.53 %)	24.0 ± 1.15 (86.76 %)
	*Sa	20.0 ± 2.08 (61.23 %)	22.0 ± 1.00 (67.36 %)	25.0 ± 1.15 (76.54 %)	27.0 ± 2.00 (82.66 %)	30.0 ± 0.57 (91.85 %)
Root (Methanol Fraction)	*Ab	–	8.0 ± 1.00 (32.44 %)	11.0 ± 2.00 (44.6 %)	13.0 ± 1.15 (52.71 %)	15.0 ± 0.57 (60.82 %)
	*Bs	–	–	–	–	–
	*Ec	8.0 ± 0.57 (31.17 %)	10.0 ± 2.64 (38.97 %)	12.0 ± 1.00 (46.76 %)	16.0 ± 2.00 (62.35 %)	18.0 ± 1.73 (70.14 %)
	*Pa	–	8.0 ± 1.52 (28.92 %)	11.0 ± 1.00 (39.76 %)	16.0 ± 1.73 (57.84 %)	18.0 ± 2.00 (65.07 %)
	*Sa	–	–	–	–	–
Stem (DCM Fraction)	*Ab	–	–	–	–	–
	*Bs	10.0 ± 2.64 (35.29 %)	12.0 ± 2.00 (42.35 %)	18.0 ± 1.00 (63.53 %)	20.0 ± 2.08 (70.59 %)	21.0 ± 1.73 (74.12 %)
	*Ec	8.0 ± 2.00 (31.17 %)	10.0 ± 0.57 (35.29 %)	13.0 ± 1.15 (50.66 %)	15.0 ± 1.00 (58.45 %)	18.0 ± 2.08 (70.14 %)
	*Pa	–	9.0 ± 0.57 (32.53 %)	13.0 ± 2.00 (46.99 %)	18.0 ± 1.00 (65.07 %)	20.0 ± 1.15 (72.3 %)
	*Sa	–	–	–	–	–
Stem (Methanol Fraction)	*Ab	–	–	–	–	–
	*Bs	5.0 ± 1.15 (17.64 %)	8.0 ± 2.00 (28.23 %)	12.0 ± 1.00 (42.35 %)	17.0 ± 0.57 (60.0 %)	19.0 ± 2.08 (67.06 %)
	*Ec	6.0 ± 1.73 (23.38 %)	8.0 ± 2.00 (31.17 %)	12.0 ± 1.00 (46.76 %)	17.0 ± 2.64 (66.25 %)	19.0 ± 0.57 (74.04 %)
	*Pa	–	–	9.0 ± 1.00 (32.53 %)	15.0 ± 2.00 (54.22 %)	18.0 ± 0.57 (65.97 %)
	*Sa	–	–	–	–	–
Leaf (DCM Fraction)	*Ab	–	5.0 ± 2.00 (20.27 %)	12.0 ± 2.08 (48.66 %)	19.0 ± 2.64 (77.04 %)	21.0 ± 1.00 (85.15 %)
	*Bs	–	–	–	–	–
	*Ec	6.0 ± 1.15	8.0 ± 1.00	13.0 ± 1.73	14.0 ± 2.00	16.0 ± 2.64

(*continued on next page*)

Table 5 (*continued*)

Sample/ Extract	Test Bacteria	ZOI (Inhibition zone) in mm±SD/% RPI (Relative percentage inhibition) (Omitting the diameter of the well)				
		5 mg/ mL	10 mg/ mL	20 mg/ mL	40 mg/ mL	60 mg/ mL
		(23.38 %)	(31.17 %)	(50.66 %)	(54.55 %)	(62.35 %)
	*Pa	8.0 ± 1.00 (28.92 %)	9.0 ± 2.00 (32.53 %)	15.0 ± 1.00 (54.22 %)	17.0 ± 0.57 (61.46 %)	19.0 ± 2.08 (68.69 %)
Leaf (Methanol Fraction)	*Sa	–	–	–	–	–
	*Ab	–	–	–	–	–
	*Bs	–	–	–	–	–
	*Ec	5.0 ± 1.73 (19.48 %)	7.0 ± 1.15 (27.27 %)	12.0 ± 1.00 (46.76 %)	13.0 ± 1.52 (50.66 %)	15.0 ± 1.00 (58.45 %)
	*Pa	–	–	–	–	–
	*Sa	7.0 ± 2.08 (25.20 %)	9.0 ± 2.64 (32.53 %)	13.0 ± 1.00 (46.99 %)	18.0 ± 2.00 (65.07 %)	20.0 ± 1.73 (61.23 %)
Fruit (DCM Fraction)	*Ab	7.0 ± 2.00 (28.38 %)	9.0 ± 1.00 (36.49 %)	12.0 ± 1.15 (48.66 %)	15.0 ± 1.00 (60.82 %)	18.0 ± 1.73 (72.99 %)
	*Bs	10.0 ± 1.00 (35.29 %)	12.0 ± 1.15 (42.35 %)	16.0 ± 2.00 (56.47 %)	18.0 ± 1.52 (63.53 %)	20.0 ± 1.73 (70.59 %)
	*Ec	10.0 ± 1.00 (38.97 %)	16.0 ± 2.00 (62.35 %)	20.0 ± 0.57 (77.94 %)	22.0 ± 2.08 (85.73 %)	23.0 ± 1.15 (89.63 %)
	*Pa	12.0 ± 1.15 (43.38 %)	15.0 ± 1.00 (54.22 %)	17.0 ± 0.57 (61.46 %)	20.0 ± 2.08 (72.3 %)	22.0 ± 2.00 (79.53 %)
	*Sa	13.0 ± 1.00 (39.8 %)	16.0 ± 2.00 (48.98 %)	20.0 ± 1.00 (61.23 %)	25.0 ± 2.08 (76.54 %)	27.0 ± 1.73 (82.66 %)
Fruit (Methanol Fraction)	*Ab	–	–	–	–	–
	*Bs	5.0 ± 2.00 (17.64 %)	10.0 ± 1.00 (35.29 %)	12.0 ± 0.57 (42.35 %)	18.0 ± 1.15 (63.53 %)	20.0 ± 2.08 (70.59 %)
	*Ec	5.0 ± 1.00 (19.48)	10.0 ± 1.15 (38.97 %)	12.0 ± 1.00 (46.76 %)	16.0 ± 2.64 (62.35 %)	20.0 ± 1.73 (77.94 %)
	*Pa	–	–	–	–	–
	*Sa	14.0 ± 0.57 (42.86 %)	17.0 ± 1.00 (52.05 %)	20.0 ± 2.00 (61.23 %)	25.0 ± 1.73 (76.54 %)	27.0 ± 2.64 (82.66 %)
Ciprofloxacin (50 μg/mL)	For positive control: *Ab*= 22.66 mm, *Bs*=26.33 mm, *Ec*=24.66 mm, *Pa*=28.66, *Sa*=36.66 mm					
Absolute Methanol	For negative control: *Ab*= 0.0 mm, *Bs*=0.0 mm, *Ec*=0.0 mm, *Pa*=0.0, *Sa*=0.0 mm					

**Ab=Acinetobacter baumannii, *Ec=E. coli, *Pa=Pseudomonas aeruginosa, *Sa=Staphylococcus aureus, *Bs=Bacillus subtilis.*

investigated further.

Minimum bactericidal concentration (MBC) is a confirmatory test for the MIC. The MIC and MBC values rely on the type of extraction solvent, the plant portion that was tested, and the microorganisms that were examined. Barua et al. (2014) found the MBC values to be 20.23, 33.43, 36.23, and 64.09 mg/mL for chloroform, hexane, ethanol, and ethyl acetate extracts of seeds of *P. longum* against *Mycobacterium smegmatis*. Moreover, the present study exhibited that all the extracts of *P. longum* were bacteriostatic against the tested bacterial strains as their MBC values were higher than the MIC values. Bactericidal agents eliminate 99.99 % of bacteria whereas bacteriostatic agents stop bacterial growth or maintain them in the stationary phase of growth.

3.8. *In vitro α-amylase and α-glucosidase inhibition assays*

The complex compound carbohydrates are broken down by α-amylase into oligosaccharides or disaccharides, and the disaccharides are broken down into simple glucose molecules by α-glucosidase, which raises the postprandial glucose level in the blood. Inhibiting the enzymes α-amylase and α-glucosidase reduces the decomposition of food polysaccharides in the digestive tract into simple saccharides, which lowers postprandial hyperglycemia (Kumar et al., 2011a). Among the tested crude methanolic extracts, the highest α-amylase and α-glucosidase inhibitory activity was found in the root, which had IC_{50} of 1066.60 ±56.18 and 1255.90±2.76 μg/mL respectively, followed by fruit and callus, and that was compared with positive control acarbose (IC_{50}=125.73±13.54 μg/mL) (Table 7). However, Muzammil et al. (2022) found that the crude ethanol leaf extract of *Coronopus didymus* exhibited a greater inhibition of α-glucosidase in comparison to methanol and acetone extracts. Methanol crude leaf extract of *Lannea coromandelica* at 200 and 400 mg/kg body weight also showed substantial antidiabetic activity in mice with diabetes induced by alloxan (Islam et al., 2022). Moreover, crude methanolic extract of *in vitro-grown* callus exhibited a greater α-amylase and α-glucosidase inhibitory activity (IC_{50}=1165.15±15.63 and 1304.76±12.431 μg/mL) than crude extracts of leaf and stem (Table 7). It might be because *in vitro*-grown callus produced biologically active secondary metabolites for the inhibition of α-amylase and α-glucosidase. Several researchers isolated bioactive compounds from the callus, such as catechin from *Camellia sinensis* (Sutini et al., 2020), piperin from *Piper longum* (Siddique et al., 2019) and *Piper nigrum* (Ahmad et al., 2014), terpenoid compounds from *Piper retrofractum* (Faramayuda et al., 2021), flavonoid, terpenoid, octadecanoic acid from *Piper betle* (Junairiah et al., 2019). Tropane alkaloids, reserpine, flavonoids, α-tocopherol, ajmaline, serpentine, stilbene, scopolamine, paclitaxel, resveratrol, and anthocyanins have all been reported to be produced by callus cultures (Efferth, 2019). Therefore, from *in vitro*-grown callus, valuable bioactive compounds can be produced that could serve as a source of natural drugs for diabetes and other disorders. Callus can be utilized in place of wild-grown plants to aid in the conservation of wild plant species in their natural habitats.

Similarly, among the tested fractions of wild-grown parts, the highest α-amylase and α-glucosidase inhibitory activity were obtained in the DCM fraction of root, which had IC_{50} of 365.21±31.02 and 489.07 ±27.96 μg/mL respectively, followed by the DCM fraction of leaf (IC_{50} of 831.14±18.14 and 922.37±21.34 μg/mL respectively) (Table 7). The methanol fractions of all crude extracts of wild-grown parts had comparatively weaker α-glucosidase and α-amylase inhibitory activities than the DCM fractions. The DCM fraction may contain more α-glucosidase and α-amylase inhibitory compounds than other fractions and methanolic crude extracts. Similarly, the higher antioxidant activity, total phenolic content, and total flavonoid content of the DCM fraction compared to other fractions and crude extracts may also account for its higher antidiabetic activity. According to research on the olive mill plant waste (Mwakalukwa et al., 2020), *Clerodendrum volubile* (Erukainure et al., 2018), and *Syzygium cumini* leaf (Franco et al., 2020), the DCM fraction has stronger anti-diabetic activity than other fractions. Through the methylation and acetylation of hydroxyl groups, phenolic compounds like flavonoids' antioxidant characteristics have been shown to significantly reduce *in vitro* diabetes mellitus (Sarian et al., 2017).

We found the degree of enzyme inhibition of the same samples different in two types of enzymes, such as crude extracts and methanol fractions of leaf and stem had a lower α-amylase inhibition, but higher α-glucosidase inhibition (Table 7). This result was supported by the observation of other researchers. Alqahtani et al. (2019) revealed differences in the inhibitory capacity of α-amylase and α-glucosidase in the same samples, such as alpha-amylase inhibition was higher in crude

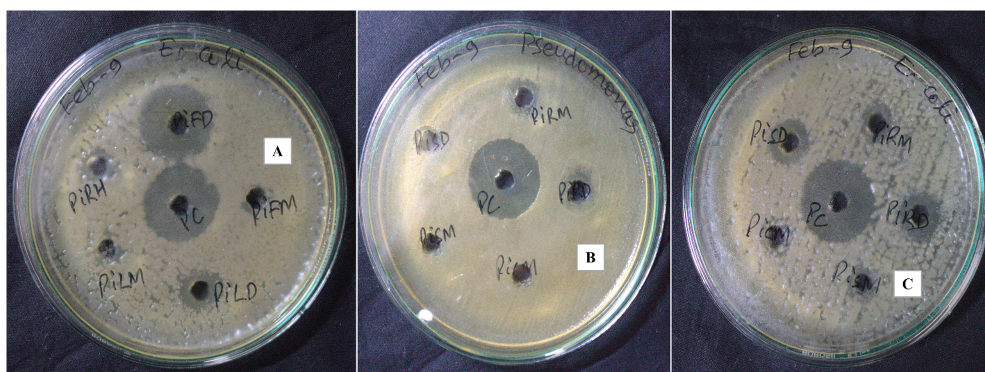

Fig. 2. Zone of Inhibition (ZoI) by various fractions of *P. longum*: (A) on *E. coli* at 20 mg/mL, (B) on *P. aeruginosa* at 5 mg/mL, (C) on *E. coli* at 10 mg/mL. (PiRM=Methanol fraction Root, PiRD=DCM fraction Root, PiSM=Methanol fraction Stem, PiSD=DCM fraction Stem, PiFD=DCM fraction Fruit, PiFM= Methanol fraction Fruit, PiLD= DCM fraction Leaf, PiLM= Methanol fraction Leaf, PiCM=Crude Methanol extract Callus, PC=Positive control).

Table 6
MIC and MBC values of various fractions and crude callus extract in *P. longum*.

Plant parts	Extract/Fraction	Values of MIC/and MBC (mg/mL)									
		*Ab		*Bs		*Ec		*Pa		*Sa	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Root	DCM	25	27.65	15	18.55	10	12.45	10	13.25	5	8.35
	Methanol	40	42.65	–	–	35	37.55	30	31.55	–	–
Fruit	DCM	25	28.45	15	17.25	10	13.85	20	24.35	30	32.25
	Methanol	–	–	40	41.75	35	36.95	–	–	20	22.65
Stem	DCM	–	–	20	21.55	25	26.85	40	43.35	–	–
	Methanol	–	–	40	42.95	25	27.35	50	53.55	–	–
Leaf	DCM	30	32.55	–	–	20	22.15	25	26.25	15	16.65
	Methanol	–	–	–	–	20	23.35	–	–	40	42.25
Callus	Crude methanol	–	–	15	16.85	30	32.75	25	26.55	10	13.55

* *Ab=Acinetobacter baumannii.*
* *Ec=E. coli.*
* *Pa=Pseudomonas aeruginosa.*
* *Sa=Staphylococcus aureus.*
* *Bs=Bacillus subtilis.*

extract, n-butanol, and chloroform fractions than α-glucosidase inhibition; but, α-glucosidase inhibition was higher in n-hexane than in α-amylase. According to Kwon et al. (2008) there was less or no α-amylase inhibition in the same samples, such as four different types of teas, and red and white wines, however, there was higher α-glucosidase inhibition. Yilmazer-Musa et al. (2012) reported that tea extracts and its constituent catechin 3-gallates were less efficient α-amylase inhibitors, however powerful α-glucosidase inhibitors. Similarly, Saini and Gangwar (2022) found that ethyl acetate extract of an endophytic fungal strain isolated from *Aegle marmelos* significantly inhibited α-glucosidase but only moderately to weakly by α-amylase. The reason for this could be that alpha glucosidase and alpha amylase have different roles and specificities for the breakdown of carbohydrates. Alpha-amylase will primarily act on starch molecules to produce larger carbohydrate fragments whereas alpha-glucosidase will degrade these carbohydrate fragments into simple sugars. Distinct inhibitors might have distinct modes of activity and specificity for enzymes (Wang et al., 2022). Consequently, when applied to the same sample, alpha-amylase and alpha-glucosidase may not show equal activity.

In vivo study using animal models (rats) conducted by earlier researchers on the fruit extract (Kumar et al., 2011b), root extract (Nabi et al., 2012, 2013), and oil (Kumar et al., 2013) of *P. longum* revealed possible antidiabetic activities. However, it is the first report on an *in vitro* study of the antidiabetic activity of wild-grown various parts of *P. longum* and an *in vitro*-grown callus. Though, root has higher antidiabetic activity *in vitro* than leaf, fruit, and stem, especially its dichloromethane fraction, leaf and fruit may also be a source of antidiabetic agents because of their significant α-amylase and α-glucosidase inhibition *in vitro*.

4. Conclusions

The finding of this study suggests that the roots, particularly the DCM fraction of *P. longum* could be a potential candidate for developing natural drugs for antidiabetic disorder and antibacterial diseases caused by *P. aeruginosa, B. subtilis, E. coli, A. baumannii,* and *Staphylococcus aureus* than other crude extracts and fractions of wild-grown parts and *in vitro*-grown callus. Similarly, the evaluation of *P. longum* against microbial pathogens and as a potential source of pharmaceuticals can assist in increasing treatment choices in developing nations with a high incidence of infectious and parasitic diseases. Moreover, the *in vitro*-grown callus might be utilized in place of wild-grown parts in the future to develop natural drugs in addition to being used to conserve *P. longum* in its natural habitat. Because, the *in vitro*-grown callus of *P. longum* demonstrated comparable antioxidant, antibacterial, and antidiabetic activities to those of wild-grown stem, fruit, and leaf. Therefore, a more thorough investigation of *in vitro*-grown callus including its phytochemical, genetic, pharmacological, and physiological characteristics is required for their use in pharmaceutical industries.

Author's agreement

We are pleased to inform you that all of the listed authors have read and approved the edited version of the manuscript for further processing or publication. The authors and other parties have no conflict of interest.

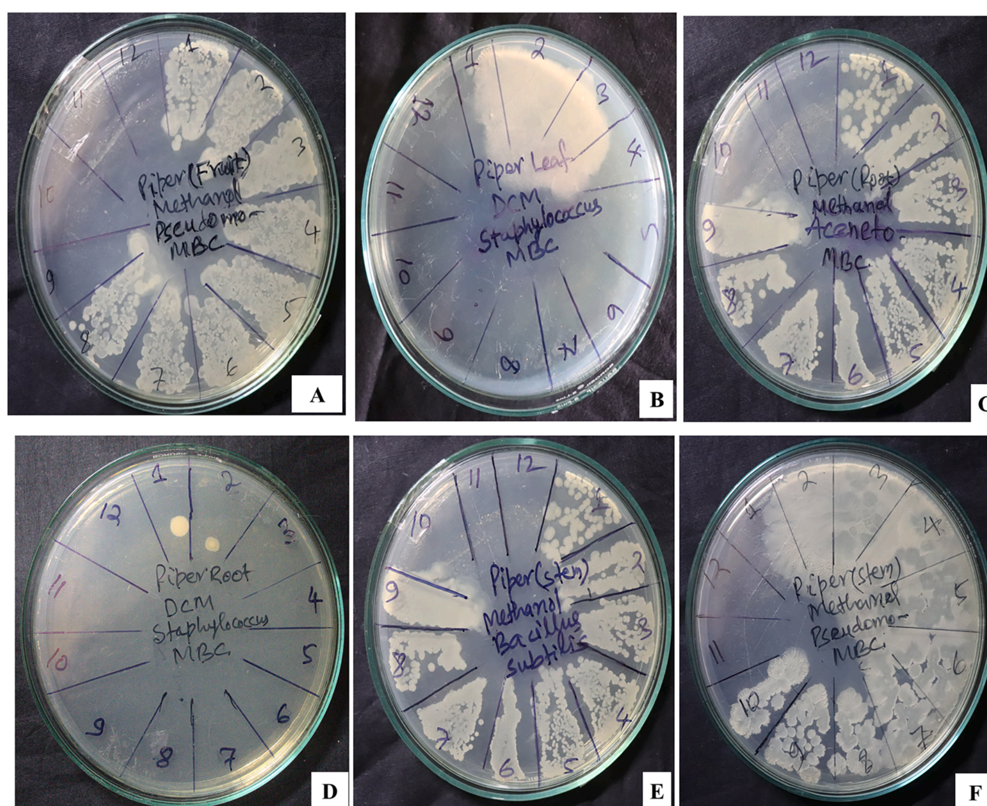

Fig. 3. Minimum Bactericidal Concentration (MBC) of *P. longum*: (A) on *P. aeruginosa* by methanol fraction of fruit, (B) on *S. aureus* by DCM fraction of leaf, (C) on *A. baumannii* by methanol fraction of root, (D) on *S. aureus* by DCM fraction of root, (E) on *B. subtilis* by methanol fraction of stem, (F) on *P. aeruginosa* by methanol fraction of stem. [Numbers 1–12 on MHA plates represent various concentrations of extracts starting from MIC].

Table 7
α-amylase and α-glucosidase inhibition activity by various extracts and fractions in *P. longum*.

S. N.	Plant parts	Extract/ Fractions	α-amylase inhibition (IC_{50}: (μg/mL)	α-glucosidase inhibition (IC_{50}: (μg/mL)
1.	Root	Crude	1066.60±56.186	1255.90±2.764
		DCM	365.21±31.021	489.07±27.966
		Methanol	3434.53±57.853	4248.05±35.637
2.	Leaf	Crude	1514.18±70.127	1360.20±13.948
		DCM	831.14±18.140	922.37±21.347
		Methanol	3348.48±149.183	3223.11±13.419
3.	Fruit	Crude	1123.05±13.724	1467.17±43.418
		DCM	921.38±59.881	1058.14±4.45
		Methanol	3199.02±134.046	4331.27±51.216
4.	Stem	Crude	1855.68±38.085	1731.85±20.989
		DCM	1138.77±35.339	1380.85±15.696
		Methanol	2534.98±73.510	2324.01±57.118
5.	Callus	Crude	1165.15±15.637	1304.76±12.431
6.	Acarbose (Positive control)		118.23±27.321	125.73±13.54

CRediT authorship contribution statement

Chandra Bahadur Thapa: Writing – original draft, Data curation, Formal analysis, Conceptualization, Writing – review & editing. **Hari Datta Bhattarai:** Validation, Formal analysis, Writing – review & editing. **Krishna Kumar Pant:** Writing – review & editing, Investigation, Formal analysis. **Bijaya Pant:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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In vitro propagation and evaluation of genetic homogeneity using RAPD, ISSR, and SCoT markers in *Piper longum* L.

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ABSTRACT

Piper longum L. has been widely used to treat digestive disorders, insomnia, diabetes, bronchitis, and cough since ancient times, which has caused a decline in its population in natural habitat. This study aims to develop a protocol for micropropagation in *P. longum* using direct organogenesis using nodal explant as well as to compare genetic uniformity between the mother plant and *in vitro*-grown plants using Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), and Start Codon Targeted (SCoT) markers. Nodal explants were cultured on Murashige & Skoog (MS) medium added with α-naphthaleneacetic acid (NAA), Kinetin (KN), Thidiazuron (TDZ), 6-Benzylaminopurine (BAP), and 10 % coconut water. The highest number of shoots (5.33±1.15) and longest shoot length (6.16± 0.65 cm) were found in MS media added with 1.0 mg/L TDZ, while the highest number of roots (7.0 ± 1.0) and the longest root length (5.53±0.35 cm) were found in MS media added with 1.0 mg/L Indole-3-butyric acid (IBA) in nodal explants. The evaluation of genetic homogeneity using 12 specific RAPD, ISSR, and SCoT markers revealed that the *in vitro*-raised plants are genetically very similar, or even identical, to the wild mother plants, and there is no polymorphism between the mother plant and *in vitro*-raised plants for the specific markers tested. Twelve of twenty-four RAPD, ISSR, and SCoT primers amplified a total of 39 loci varying in size from 150 to 1400 base pairs. This study showed that an effective protocol developed for the *in vitro* propagation of *P. longum* by direct organogenesis can be used to produce true-to-type plants, which may aid in homozygous breeding programs and *ex-situ* conservation and could be used for commercial propagation.

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1. Introduction

P. longum (Piperaceae), commonly called "long pepper" is a medicinal plant, that is distributed in the tropical and subtropical world including India, Bangladesh, Cambodia, China, Laos, Myanmar, Nicobar Island, Thailand, Vietnam, Malaya, Nepal, Philippines, Sri Lanka, and America (Kumar et al., 2016; Biswas et al., 2022). It grows wild in tropical wet rainforests between 100 and 1000 m above sea level (Press et al., 2000; Sumy et al., 2000; Bhatt et al., 2017). It is a trailing undershrub that spreads on the ground. Since ancient times, ripe fruits and thick roots with short stem segments (Piplamul) are commonly utilized in traditional medicine and Ayurveda for the medication of cough, cold, bronchitis, asthma, insomnia, diabetes, stomachic, and diarrhea, as well as for snake bite and scorpion sting antidote. Fruit is additionally utilized as a culinary spice, and while it is uncommon in European cuisines, it may be utilized in Indian vegetable pickles, various North African spice mixes, and Indonesian and Malaysian meals (Kumar et al., 2016). It contains a variety of bioactive compounds, which includes alkaloids (piperine and piperlongumine), essential oil, flavonoids, lignan, bitter principle, arbutin, coumarins, amide, sterols (sitosterol and stigmasterol), and steroids (Yadav et al., 2019). Piperine alkaloid is the primary constituent of *P. longum*, accounting for 3−5 % of the plant (Zaveri et al., 2010). Although it is a prioritized plant in Nepal for research and development as well as agrotechnology development (DPR, 2012, 2017), its population is declining in its natural habitat due to extensive collection for traditional medicinal use, climate change, invasive alien species, and illegal trade to neighboring countries. Moreover, plant regeneration by seeds in natural habitat, on the other hand, is challenging due to poor seed viability and a low rate of seed germination (Sarasan et al. 1993). The standard method of vegetative propagation in *P. longum* is

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difficult due to the prolonged time for root formation in vegetative cuttings (Soniya and Das, 2002). Therefore, it needs *ex-situ* conservation utilizing *in vitro* culture techniques.

Micropropagation is a useful technology for the *ex-situ* conservation of valuable plant species since it allows for the speedy multiplication and conservation of numerous rare, threatened, and endangered plant species from a small amount of plant material with little negative influence on wild populations (Fay, 1992, 1994; Rao, 2004; Pant et al., 2019). Similarly, tissue culture allows for faster vegetative propagation and the upkeep of free of pathogens plants (Saito and Nakano, 2002). Micropropagation of *P. longum* utilizing nodal explants through direct organogenesis is common in practice (Bhat et al., 1992; Soniya and Das, 2002; Rani and Dantu, 2012) and may produce true-to-type plants or with less somaclonal variation in some plants between the mother plant and *in vitro*-raised plants due to chemical, physical, and other culture stresses in explants during *in vitro* culture. Therefore, producing true-to-type plants is challenging by tissue culture technique, and there may be genetic heterogeneity between *in vitro*-raised plants and mother plants. When an *in vitro* culture technique is used for plant multiplication, there is always the possibility of somaclonal variation occurring in the propagated plants due to gene mutations caused by single nucleotide substitutions, changes comprising additions or deletions, DNA segment replication, translocations and inversions, and faults in tandemly repeat replication of DNA (Amiteye, 2021).

There are several methods of assessing genetic uniformity between the *in vitro*-grown plantlets and mother plants utilizing various types of molecular markers. Some widely used PCR amplification-based molecular markers such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), and Start codon targeted (SCoT) polymorphism can be employed to analyze plant genetic diversity (Collard and Mackill, 2009; Sen et al., 2010; Dos Santos et al., 2011; Chowdhury et al., 2014; Ahmad et al., 2022). Molecular approaches have an advantage over traditional techniques since they are not affected by environmental influences and can produce accurate and consistent findings (Peredo et al., 2009). RAPD is a dominant marker that employs short, random primers, which may bind to any location in the genome and generate a variety of band patterns. RAPD provides several advantages, including ease of application, a low price, and the usage of only a small quantity of plant materials (Guasmi et al., 2012). Similarly, ISSR is a dominant marker that targets microsatellite repeat areas without using DNA sequencing, which are repetitive sequences of 2–6 nucleotides in length and produces more reproducible and informative bands than RAPD (Lagercrantz et al. 1993; Zietkiewicz et al. 1994). Moreover, SCoT is also a dominant marker that targets the start codon ATG, which is found in the majority of genes, to generate more specific and consistent bands than RAPD and ISSR (Joshi et al. 1997; Collard and Mackill, 2009). Since a single primer is employed as both the forward and reverse primers, the SCoT method is analogous to RAPD, ISSR, or single primer amplification reactions (Gupta et al. 1994; Williams et al. 1990). SCoT approach was confirmed in rice utilizing a genetically heterogeneous collection of alleles and a backcross population (Collard and Mackill, 2009). This research aims to establish an efficient protocol for the *in vitro* propagation of *P. longum* through direct organogenesis and to assess the genetic homogeneity of *in vitro*-raised plants and mother plants using RAPD, ISSR, and SCoT markers.

2. Materials and methods

2.1. *In vitro propagation of P. longum through nodal explants*

2.1.1. *Collection of plant material and sterilization*

P. longum was collected from the Rupandehi district, western Nepal at an altitude of 160 m above sea level during September-October 2021 and a few plants were planted at the garden of Central Department of Botany, T.U., Kirtipur. Nodal segments (stem) were washed thoroughly in tap water to remove dust particles, dipped in Tween-20 (2 % v/v) for half an hour, and then washed in running tap water. Nodal segments were dipped in 0.2 % Bavistin (w/v) for 1 h, 70 % alcohol (v/v) for 1 min, and 0.1 % mercuric chloride ($HgCl_2$) (w/v) for 7 min. Before autoclaving, 50 mg/L activated charcoal was added to the media to control media browning. After sterilization, explants were rinsed thrice with sterile distilled water to eliminate any residual mercuric chloride. The media were adjusted to a pH of 5.8, distributed into sterile 78 mm × 122 mm jars (30 mL each) and 25 mm × 150 mm culture tubes (18 mL each) that were closed with aluminum foil, and then were autoclaved for 15 min at 121 °C temperature and 15 pounds pressure.

2.1.2. *Shoot multiplication from nodal explant and shoot elongation*

For shoot multiplication and elongation from nodal explants, nodal explants (0.5 cm) were cut off and cultured in MS (Murashige and Skoog, 1962) medium in the absence of plant growth regulators (PGRs) and 5–10 % coconut water (CW) (v/v) as well as in MS media added with 0.5–3.0 mg/L BAP alone, 0.5–3.0 mg/L TDZ alone, and combination of 0.5–3.0 mg/L BAP and NAA at constant concentrations of 0.25 or 0.5 mg/L. The culture was maintained in a 16-hour light period, 3000–4000 lux cool-white fluorescent light, and at 25± 2 °C temperature.

2.1.3. *Root formation from* in vitro *shoots*

In vitro-regenerated shoots were then cultured on MS media without PGRs as well as fortified with 0.25–2.0 mg/L IBA, 0.25–2.0 mg/L IAA, and 0.25–2.0 mg/L NAA for the development of adventitious roots. Cultures were grown at 25 ± 2 °C in a culture room with a 16-hour light period using cool-white fluorescent bulbs (3000–4000 lux).

2.2. *Assessment and comparison of genetic homogeneity of* in vitro *plantlets and the mother plant*

2.2.1. *Sample selections for isolation of DNA*

For DNA extraction, immature and healthy leaves from a mother/wild plant growing in its natural habitat, as well as four types of *in vitro*-raised plantlets from nodal explants, each type plantlet grown in MS + 1.0 mg/L TDZ + 10 % CW, MS + 2.0 mg/L BAP + 10 % CW, MS + 2.0 mg/L BAP + 0.25 mg/L NAA + 10 % CW, and MS + 2.0 mg/L BAP + 0.5 mg/L NAA + 10 % CW respectively, were chosen.

2.2.2. *DNA extraction by CTAB method*

The CTAB (Cetyltrimethylammonium bromide) approach (Doyle, 1991) was used to isolate genomic DNA from leaves of wild and *in vitro*-raised plants with some modifications. About 0.2 g of leaves were washed with distilled water to eliminate dust particles, shade-dried, and then crushed into powder in a mortar and pestle with liquid nitrogen. In a microcentrifuge tube, 1 mL of pre-warm CTAB buffer (1 M Tris–HCl, 5 M NaCl, 2 % CTAB, 0.2 % β-mercaptoethanol, pH 8.0) was mixed to make a smooth paste. The microcentrifuge tube containing the samples was incubated in a water bath at 65 °C for 30 min. After cooling for 4–5 min, the samples were spun up at 10,000 rpm for 5 min in a centrifuge, and the supernatant was placed into clean, sterilized microcentrifuge tubes. The supernatant was mixed with an equal volume of chloroform and isoamyl alcohol (24:1) and flipped upside down multiple times for 8–10 min. It was spun up again at 12,000 rpm for 8–10 min in a centrifuge, resulting in three layers. The top layer was shifted to a clean, sterilized centrifuge tube, and an equivalent volume of ice-cold absolute ethanol was added. It was left at 20 °C for 30 min before being spun up at 12,000 rpm for 5 min in a centrifuge to produce the DNA pellet. The supernatant was removed, and the pellet was treated with 500 μL of

70 % ethanol. It was spun up again for 5 min at 10,000 rpm in a centrifuge, and the residual ethanol was pipetted out. The pellet was dried for 30 min in a laminar airflow cabinet before being redissolved in 1X TE buffer (40 μL). Finally, the isolated DNA concentration was adjusted to 20−30 ng and kept at −20 °C.

2.2.3. Amplification of DNA and evaluation of RAPD, ISSR, SCoT markers

A total of twenty-four RAPD, ISSR, and SCoT markers (GenoDirex Inc., Taiwan) were evaluated and only those with distinct and replicable bands were included for evaluation. Eight 10-mer RAPD primers, eight 15−18-mer ISSR primers, and eight 18-mer SCoT primers were utilized to amplify the genomic DNA (Table 3). The RAPD, ISSR, and SCoT testing were performed in a thermal cycler with a total reaction volume of 15 μL in a PCR tube containing 5.5 μL nuclease-free water, 6.5 μL master mix [Taq PCR polymerase (0.5 U l), dNTPs (0.2 mM)], 1 μL primer (1 mM), and 2 μL genomic DNA (30 ng). Cycling conditions such as pre-heating, denaturation, annealing, extension, and final extension were also adjusted to determine the ideal cycling conditions. The cycling situation was adjusted in a Pro Flex PCR (Thermo Fisher Scientific, USA) as pre-heating at 94 °C for 5 min followed by 45 cycles of denaturing at 94 °C for 3 min, annealing at 36−40 °C for 1 min for RAPD, 50−53 °C for ISSR for 1 min, and 51−55 °C for 1 min for SCoT, extension at 72 °C for 1 min, and then a final extension at 72 °C for 5 min. To evaluate the primers' usefulness, the products of PCR were identified on 1.5 % agarose gels at a constant power of 70 Vs for 1.5 h using 1X TBE (Tris base, boric acid, and EDTA) buffer. DNA was stained with 1 μg/mL ethidium bromide for easy identification when shot under UV light in a gel documentation system (UVITEC, Cambridge). The amplified product's size was assessed by comparing it to a 100 bp DNA ladder marker (0.1 μg/μL) (Solis BioDyne, Estonia). For calculating alleles, distinct and repeatable bands were examined.

The polymorphic information content (PIC) of a marker is an indicator of its genetic variation. For dominant markers, it ranges between 0 and 0.5, with higher values suggesting greater diversity and lower ones indicating greater homogeneity. PIC of the RAPD, ISSR, and SCoT markers were determined using the number and frequency of alleles at a particular locus. The $PIC_{(Dominant\ markers)} = 2f(1-f)$, or $1-[f^2+(1-f)^2]$, where 'f ' is the average frequency of marker in the set of data (De Riek et al., 2001).

2.3. Statistical analysis

All experiments of *in vitro* culture were carried out in triplicate, and the average value with standard deviation (SD) was computed using Microsoft Excel 10. The impact of various combinations of plant growth regulators (PGRs) at each concentration and the effect of various concentrations of PGRs on each PGR group for the development of shoot number as well as shoot length were analyzed by utilizing one-way analysis of variance (ANOVA) and Duncan's test at 0.05 significance level using IBM SPSS version 20. The effect of PGRs and their concentrations for the development of shoot number and shoot length were also evaluated by a two-way ANOVA test at 0.05 significance level using IBM SPSS (Statistical Package for Social Sciences) version 20.

3. Results

3.1. *In vitro propagation of* P. longum *from nodal explants (direct organogenesis)*

The multiple shoots and roots were regenerated from the nodal explants directly in MS media added with different concentrations of TDZ, BAP, and a combination of BAP+NAA, and 10 % CW. The maximum number of shoots (5.33±1.15) per explant and the longest shoot length (6.16±0.65 cm) were found in MS+1.0 mg/L TDZ+10 % CW than in MS+BAP+10 % CW (3.33±0.57) or the combination of MS +BAP+NAA+10 % CW (4.0 ± 1.0) (Table 1). However, the MS media without the addition of plant growth regulators (PGRs) and 10 % CW (control) produced the least number of shoots (1.00±00) per explant and a shorter shoot length (1.83±0.26 cm). When BAP was combined at a constant concentration of 0.25 mg/L or 0.5 mg/L NAA with 10 % CW in MS media, the average number of shoots (4.0 ± 1.0) per explant and the average length of shoots (5.2 ± 0.30 cm) was found to be higher at 0.25 mg/L of NAA than at 0.5 mg/L of NAA.

The test of one-way ANOVA for the effect of various PGRs (BAP, TDZ, BAP+NAA 0.25, and BAP+NAA 0.5) at each concentration *versus* the shoot number and shoot length, as well as the effect of various concentrations (0.5, 1.0, 2.0, and 3.0) of PGRs on each PGR *versus* shoot number and shoot length, were found to be significant at 0.05 significant level (Figs. 1 and 2). Moreover, the test of two-way ANOVA for the effect of various PGRs at different concentrations *versus* the number of shoots produced was found to be significant at 0.05 significant level (p-value of PGRs *vs* shoot number was <0.001, concentration *vs* shoot number was <0.001, and the interaction between PGRs and concentration was 0.002). Similarly, the test of two-way ANOVA for the effect of various PGRs at different concentrations *versus* the shoot length was found to be significant at 0.05 significant level (p-value of PGRs *vs* shoot length was 0.001, concentration *vs* shoot length was <0.001, and the interaction between PGRs and concentration was <0.001).

Table 1
The average shoot number and shoot length at various concentrations of PGRs in *P. longum*.

S.N.	Plant Growth Regulators (PGRs)				Average shoot number per explant (M±SD)	Average shoot length in cm (M±SD)
	BAP (mg/L)	TDZ (mg/L)	NAA (mg/L)	Coconut water (%)		
1	0	0	0	0	1.0 ± 0.0	1.83±0.28
2	0.5	0	0	10	1.33±0.57	2.76±0.25
3	1	0	0	10	2.33±0.57	3.8 ± 0.7
4	2	0	0	10	3.33±0.57	5.46±0.87
5	3	0	0	10	2.33±0.57	2.7 ± 0.72
6	0	0.5	0	10	1.66±0.57	3.26±0.68
7	0	1	0	10	5.33±1.15	6.16±0.65
8	0	2	0	10	3.66±0.57	3.96±0.50
9	0	3	0	10	2.33±0.57	2.93±0.45
10	0.5	0	0.25	10	1.0 ± 0.0	2.83±0.30
11	1	0	0.25	10	2.33±0.57	3.76±0.25
12	2	0	0.25	10	4.0 ± 1.0	5.2 ± 0.30
13	3	0	0.25	10	2.66±0.57	3.8 ± 0.43
14	0.5	0	0.5	10	1.66±0.57	3.23±0.15
15	1	0	0.5	10	2.33±0.57	4.53±0.35
16	2	0	0.5	10	2.0 ± 1.0	2.7 ± 0.2
17	3	0	0.5	10	1.33±0.57	2.33±1.5

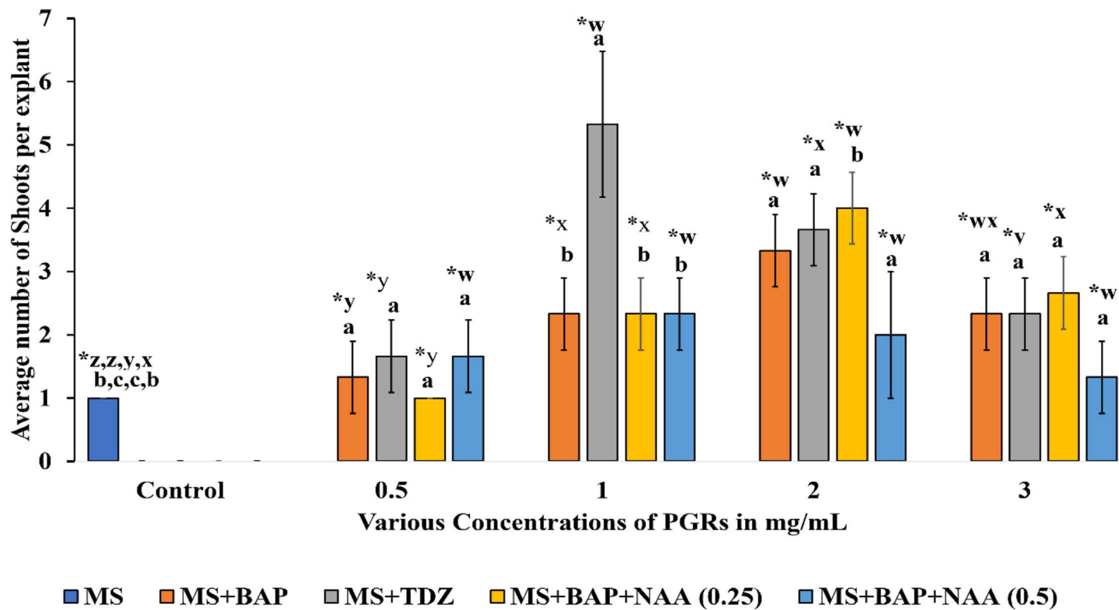

Fig. 1. The average number of shoots in various combinations of PGRs at different concentrations and in MS media (control) in *P. longum*. Note: The alphabets 'a', 'b', 'c', and 'd' above error bars suggest significant differences at 0.05 significance level with control to the various combinations of PGR groups at each concentration. The alphabets '*w', '*x', '*y', and '*z' above error bars indicate significant differences at 0.05 significance level with control to the various concentrations of PGRs on each PGR.

After 4 weeks of growth in the same media, the *in vitro*-developed shoots were cut off and shifted to MS media enriched with 0.25 to 2.0 mg/L of IAA, IBA, or NAA. The maximum number of roots (7.0 ± 1.0) per explant and the longest roots (5.53±0.25 cm) were found in MS +1.0 mg/L IBA than in MS+1.5 mg/L IAA (maximum number of roots 4.66±0.57; the longest roots 3.9 ± 0.23 cm) after the 4 weeks of culture (Table 2 and Fig. 3). The NAA was unable to regenerate roots on *in vitro* shoots.

The one-way ANOVA test of the control group and various PGRs with different concentrations *versus* regeneration of root number showed no significant difference (p-value 0.867) at $p < 0.05$. Similarly, the one-way ANOVA test of the control group and various PGRs with different concentrations *versus* root lengths also showed no significant difference (p-value 0.981) at $p < 0.05$.

3.2. *Genetic homogeneity study and comparison of* in vitro-*grown plantlets and mother plant using RAPD, ISSR, and SCoT markers*

A total of 24 markers were screened for their reproducibility using genomic DNA isolated from one mother plant (wild) and 4 types of *in vitro*-grown plants for the evaluation of genetic stability; however, only 12 markers, each 4 from RAPD, ISSR, and SCoT, were amplified in PCR that displayed clear bands on agarose gel (Table 3). Similarly, a total of 39 bands (12 bands in RAPD, 8 bands in ISSR, and 19 bands in SCoT primers) were obtained from the 12 markers when compared with the ladder marker (100 bp) for evaluation. The genetic stability test revealed 100 % monomorphism in all markers between the mother plant and the *in vitro*-grown plants, and the size of the amplified DNA fragment varied approximately from 150 to 1400 base pairs

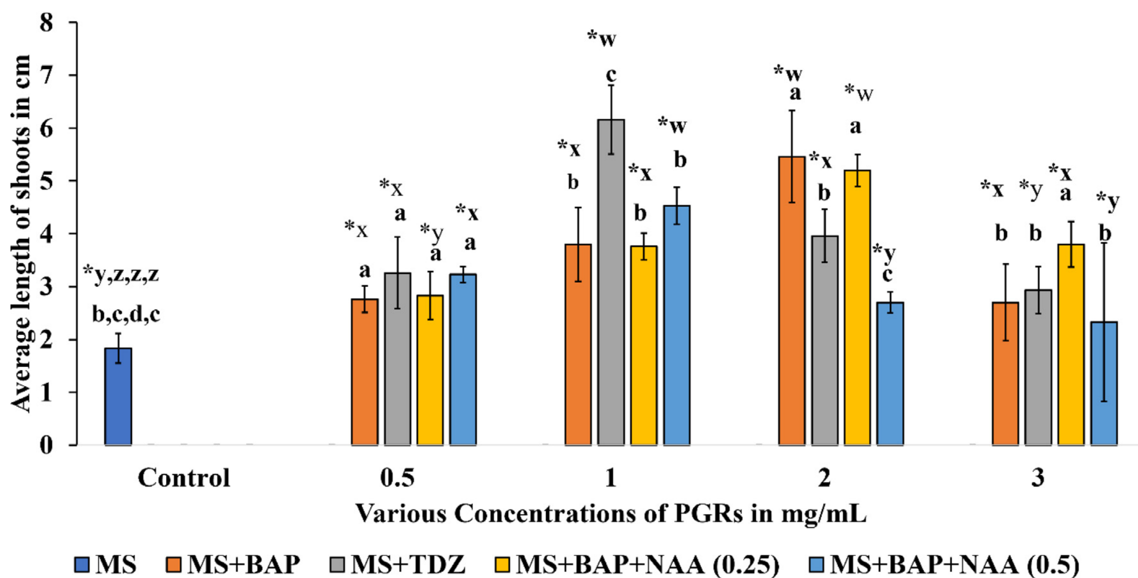

Fig. 2. The average length of shoots in various combinations of PGRs at different concentrations and in MS media (control) in *P. longum*. Note: The alphabets 'a', 'b', 'c', and 'd' above error bars suggest significant differences at 0.05 significance level with control to the various combinations of PGR groups at each concentration. The alphabets '*w', '*x', '*y', and '*z' above error bars indicate significant differences at 0.05 significance level with control to the various concentrations of PGRs on each PGR.

Table 2
The average root number and root length at various concentrations of PGRs in *P. longum*.

S.N.	Concentrations of PGRs			Average number of roots per explant (M±SD)	Average root length in cm (M±SD)
	IAA	IBA	NAA		
1	0	0	0	0.0	0.0
2	0	0.25	0	3.00 ± 1.0	1.46 ± 0.25
3	0	0.5	0	3.66 ± 0.57	1.8 ± 0.26
4	0	1	0	7.00 ± 1.0	5.53 ± 0.25
5	0	1.5	0	5.66 ± 0.57	3.8 ± 0.2
6	0	2	0	4.33 ± 0.47	3.4 ± 0.3
7	0.25	0	0	2.0 ± 1.0	1.43 ± 0.15
8	0.5	0	0	3.00 ± 1.0	2.06 ± 0.37
9	1	0	0	5.33 ± 1.15	2.3 ± 0.2
10	1.5	0	0	4.66 ± 0.57	3.9 ± 0.23
11	2	0	0	4.33 ± 0.94	3.23 ± 0.30
12	0	0	0.25	0.0	0.0
13	0	0	0.5	0.0	0.0
14	0	0	1.0	3.0 ± 1.0	1.66 ± 0.20
15	0	0	1.5	3.66±0.57	2.2 ± 0.25
16	0	0	2.0	4.0 ± 0.81	4.0 ± 0.43

(Table 4). The amplified DNA fragments ranged from 200 to 1100 bp in RAPD, 150–1100 bp in ISSR, and 200–1400 bp in SCoT primers. The smallest size of amplified DNA fragment was found in UBC-880 (ISSR) markers, while the largest size was identified in S30 (SCoT) markers. The polymorphic information (PIC) value of all markers was found to be zero since there was 100 % genetic monomorphism between the mother and *in vitro*-raised plants.

4. Discussion

P. longum is an important medicinal plant that requires *ex-situ* conservation due to overharvesting of the roots and fruits for traditional medicinal purposes, habitat loss due to climate change, and a lack of awareness about the effective conservation of plants in natural habitats. Because of the presence of less viable seeds and the limited life span for seed germination, vegetative multiplication by seeds is problematic in *P. longum* (Sarasan et al., 1993). Therefore, *in vitro* culture techniques aid in the multiplication of true-to-type plants as well as the *ex-situ* conservation of plants. In this study, plants were regenerated in MS media utilizing nodal explants (direct organogenesis) in MS media. However, micropropagation from nodal explant

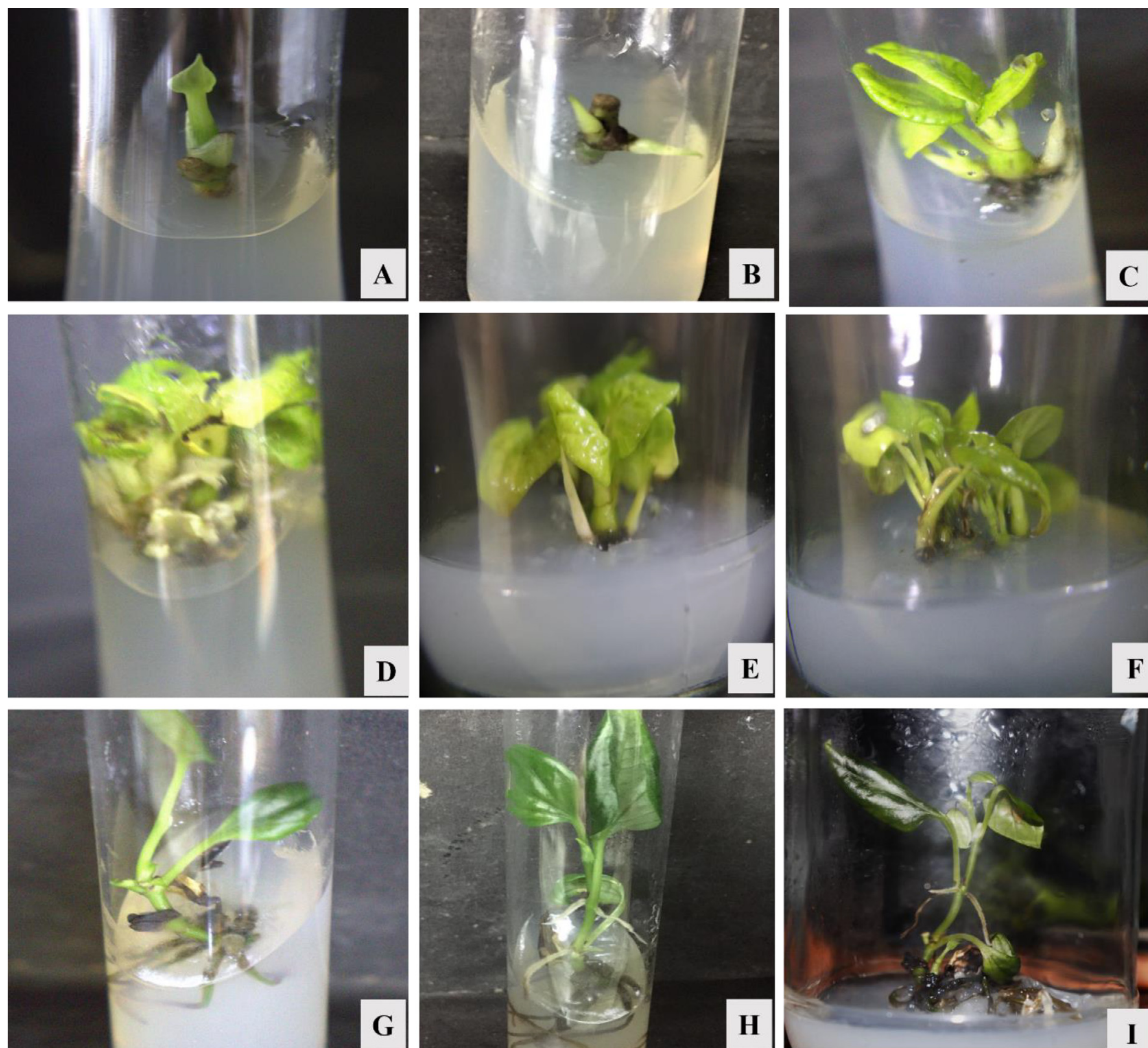

Fig. 3. Various stages of shoot and root development in *P. longum* from node explants. (A-B) MS+ 2.0 mg/L BAP+ 0.25 mg/L NAA; (C) MS+2.0 mg/L BAP; (D-F) MS+1.0 mg/L TDZ; (G) MS+2.0 mg/L NAA; (H) MS+1.5 mg/L IAA (I) MS+1.0 mg/L IBA.

Table 3
Three types of primers, their sequences, length of primer, and results of DNA amplification.

Primer groups	Primer name	Sequence of primer (5′–3′)	Length (bp)	DNA amplification
RAPD	OPA-01	CAGGCCCTTC	10	No clear band
	OPA-02	TGCCGAGCTG	10	Visible bands
	OPA-03	AGTCAGCCAC	10	Visible bands
	OPA-06	GGTCCCTGAC	10	No clear band
	OPA-07	GAAACGGGTG	10	Visible bands
	OPA-08	GTGACGTAGG	10	No clear band
	OPA-09	GGGTAACGCC	10	No clear band
	OPP-01	GTAGCACTCC	10	Visible bands
ISSR	UBC-810	GAGAGAGAGAGAGAGAT	17	No clear band
	UBC-814	CTCTCTCTCTCTCTCTA	17	No clear band
	UBC-815	CTCTCTCTCTCTCTCTG	17	Visible bands
	UBC-834	AGAGAGAGAGAGAGAGYT	18	No clear band
	UBC-835	AGAGAGAGAGAGAGTC	16	No clear band
	UBC-843	CTCTCTCTCTCTCTCTRA	18	Visible bands
	UBC-844	CTCTCTCTCTCTCTCTRC	18	Visible bands
	UBC-880	GGAGAGGAGAGGAGA	15	Visible bands
SCoT	S5	CAACAATGGCTACCACGA	18	No clear band
	S6	CAACAATGGCTACCACGC	18	Visible bands
	S9	CAACAATGGCTACCACGT	18	No clear band
	S10	CAACAATGGCTACCAGCC	18	No clear band
	S12	ACGACATGGCGACCAACG	18	No clear band
	S25	ACCATGGCTACCACCGGG	18	Visible bands
	S30	CCATGGCTACCACCGGCG	18	Visible bands
	S35	CATGGCTACCACCCGCCC	18	Visible bands

Table 4
Three types of markers, annealing temperature, percentage of monomorphism, PIC value, and the approximate size of amplified DNA segments.

Primer groups	Primer name	Annealing temp (°C)	Total bands observed	Monomorphism (%)	Marker PIC value	Approx. amplified range of base pair
RAPD	OPA-02	36 °C	3	100 %	0	500–100
	OPA-03	37 °C	3	100 %	0	600–1100
	OPA-07	40 °C	3	100 %	0	400–1000
	OPP-01	38 °C	3	100 %	0	200–600
ISSR	UBC-815	52.5 °C	3	100 %	0	300–1100
	UBC-843	50 °C	2	100 %	0	250–500
	UBC-844	53 °C	1	100 %	0	200–300
	UBC-880	51.5 °C	2	100 %	0	150–300
SCoT	S6	53.5 °C	4	100 %	0	200–1200
	S25	55 °C	4	100 %	0	300–1100
	S30	54.5 °C	6	100 %	0	300–1400
	S35	52.5 °C	5	100 %	0	300–1200

through direct organogenesis is challenging in *P. longum* due to the presence of systemic endogenous bacteria that frequently cause contamination in culture (Bhat et al., 1995; Parida and Dhal, 2011; Sathelly et al., 2016) and due to the browning of media with the release of metabolites. Direct organogenesis was observed in the MS medium without the use of PGRs (BAP, NAA, and TDZ) and CW as control, which produced a single shoot from each nodal explant. In MS treated with 1.0 mg/L TDZ and 10 % CW, however, multiple shoots (5.33±1.15) and longest shoot length (6.16±0.65) were obtained from each nodal explant (Table 1). Some reports in *P. longum* indicate the regeneration of more shoots than the number of shoots produced from nodal explants in this study. They used shoot apex, leaf, nodal, internodal, and petiolar explants with different combinations of PGRs in MS media, such as BA+ KN, BA alone, BA + IAA, and BA + NAA (Soniya and Das, 2002; Parida and Dhal, 2011; Rani and Dantu, 2012; Padhan, 2015; Saravanan, 2019), but this study used TDZ + 10 % CW water to regenerate higher number of shoots from the nodal explant. The number of shoot regeneration from the explants may depend on the size of the explants, environmental factors (temperature, photoperiod, light quality, light intensity), and types of used PGRs. According to Guo et al. (2011), TDZ shows both cytokinin- and auxin-like activities, and the lower concentrations of TDZ induce axillary shoot regeneration, whereas higher concentrations induce adventitious shoot formation. We initially used 5 % CW instead of 10 % CW in MS medium supplemented with BAP alone, NAA alone, TDZ alone, and a combination of BAP+NAA, however, this did not result in multiple shoot formation. According to Gnasekaran et al. (2010) coconut water functions as a growth regulator (cytokinin) comprising various nutritional and hormonal components, including diphenyl urea, and encourages cell division, growth, and shoot differentiation in culture. In comparison to BAP alone and the combination of BAP+NAA in MS media, TDZ in MS media showed better results for multiple shoot differentiation, multiplication, and longer shoot length formation (Table 1). This finding supports the results of Nautiyal et al. (2022) who demonstrated *in vitro* regeneration of multiple shoots in *Oryza sativa*. They found that the number of shoots regenerated from TDZ-treated seedlings in media was more than that of BAP-treated seedlings. In addition, it was observed that TDZ produced a stronger response than BAP in peanut shoot regeneration (Victor et al., 1999; Gairi and Rashid, 2004). Similarly, MS media supplied with 2.0 mg/L BAP+0.25 mg/L NAA+10 % CW produced more shoots (4.0 ± 1.0) and longer shoots (5.2 ± 0.30 cm) than MS media containing 2.0 mg/L BAP+10 % CW (Table 1). It could be because of the synergistic effect of auxin (NAA) and cytokinin (BAP) for shoot regeneration. Moreover, a higher number of shoots and longer shoot length were obtained in MS medium added with 0.5–3.0 mg/L BAP at a constant

concentration of 0.25 mg/L NAA than in MS media supplemented with 0.5–3.0 mg/L BAP at a constant concentration of 0.5 mg/L NAA (Table 1). It could be because higher concentrations of NAA (0.5) with various concentrations of BAP have less influence on shoot regeneration.

Furthermore, *in vitro* shoots, regenerated from nodal explants were transferred in MS media supplemented with IBA, IAA, or NAA for adventitious root regeneration. *In vitro* generation of plantlets with abundant roots is critical for the effective establishment of newly developed plants in soils (Ohyama, 1970). Roots were not regenerated in MS media without supplemented PGRs (IBA, IAA, and NAA). However, the maximum number of roots (7.0 ± 1.0) and longest roots (5.53±0.25 cm) were found in MS media containing 1.0 mg/L IBA. This finding was supported by the observation that IBA was able to induce roots from *in vitro* shoots in *P. longum* (Soniya and Das, 2002; Sharon and Maurya, 2004; Rani and Dantu, 2012). Similarly, a significant number of roots (5.33±1.15) per explant and longer roots (3.8 ± 0.2 cm) were produced in MS media fortified with 1.5 mg/L IAA. This finding was supported by the findings that IAA regenerated roots from *in vitro* shoots in MS media fortified with IAA in *P. longum* (Bhat et al., 1992). We were also able to regenerate roots from *in vitro* shoots in MS media containing 1.0–2.0 mg/L NAA. The findings of Philip et al. (1992) in *P. nigrum* and Sianipar et al. (2016) in *P. crocatum* supported this result.

The *in vitro* propagation technique utilizes various types of plant growth regulators (PGRs) and stresses in a controlled environment for the regeneration of plants. As a result, somaclonal variation may occur among micro-propagated plantlets due to several reasons such as changes in DNA methylation, gene replication, chromosomal aberration, polyploidy, and gene mutation (Saker et al., 2000), and prolonged culture and usage of higher concentrations of PGRs (Palama et al., 2010). Therefore, testing for genetic homogeneity of mother plants and *in vitro*-raised plants guarantees that the plants are genetically consistent and identical. Moreover, to guarantee the successful application of *in vitro* technology, whether for breeding programs or market distribution, molecular markers are utilized to observe somaclonal variation and certify the genetic stability of *in vitro* propagated plants (Da Silva et al., 2007). In this study, the genetic fidelity was evaluated between one mother plant (MP) and 4 types of *in vitro*-raised plants regenerated from nodal explants (TC1, TC2, TC3, and TC4) in different media compositions utilizing three types of dominant molecular markers: RAPD, ISSR, and SCoT (Figs. 4–6). Although dominant markers frequently show the presence or absence of a specific band without distinguishing between heterozygous and homozygous states in individuals, they are routinely used to evaluate the genetic homogeneity of *in vitro*-grown plants and mother plants.

This study reported a total of 39 scorable marker bands from selected 12 markers of RAPD, ISSR, and SCoT that did not reveal polymorphic bands among the tested markers. For the particular markers evaluated, the *in vitro*-raised plants are genetically very similar, or even identical, to their wild mothers. This shows that the tissue culture technique did not cause appreciable genetic variation at these loci. Similarly, the selected markers (RAPD, ISSR, and SCoT) revealed no polymorphism between the samples. This suggests that *in vitro* propagation has preserved genetic integrity at these loci. This finding was supported by some researchers' findings in other medicinal plants (Pandey et al., 2020; Oliya et al., 2021; Joshi et al., 2023; Pandey et al., 2023), but some researchers found a few percentages of genetic heterogeneity between the mother plant and *in vitro*-raised plants (Dhungana et al., 2022; Pradhan et al., 2023). It could be owing to the use of genetically homogeneous explants, subculturing of explants in the medium in the proper way and at the suitable time, use of low amounts of PGRs in tested *in vitro*-raised plants, proper sterilizing processes, and proper media composition. The size of the scorable band ranged between 150 and 1400 bp, and the smallest size of the band was observed in the ISSR marker (UBC 880) while the largest size was found in the SCoT marker (S30). Several variables can influence the size of the amplified DNA fragment, such as primer design, PCR reaction parameters, and DNA template properties. Furthermore, the SCoT primers produced the most scorable bands or multiple PCR products (19) than the other markers. The number of amplified bands per specific marker may depend on the quality of template DNA, primer design, presence of contaminants, suboptimal Mg^{2+} concentration, and DNA polymerase fidelity. Therefore, the

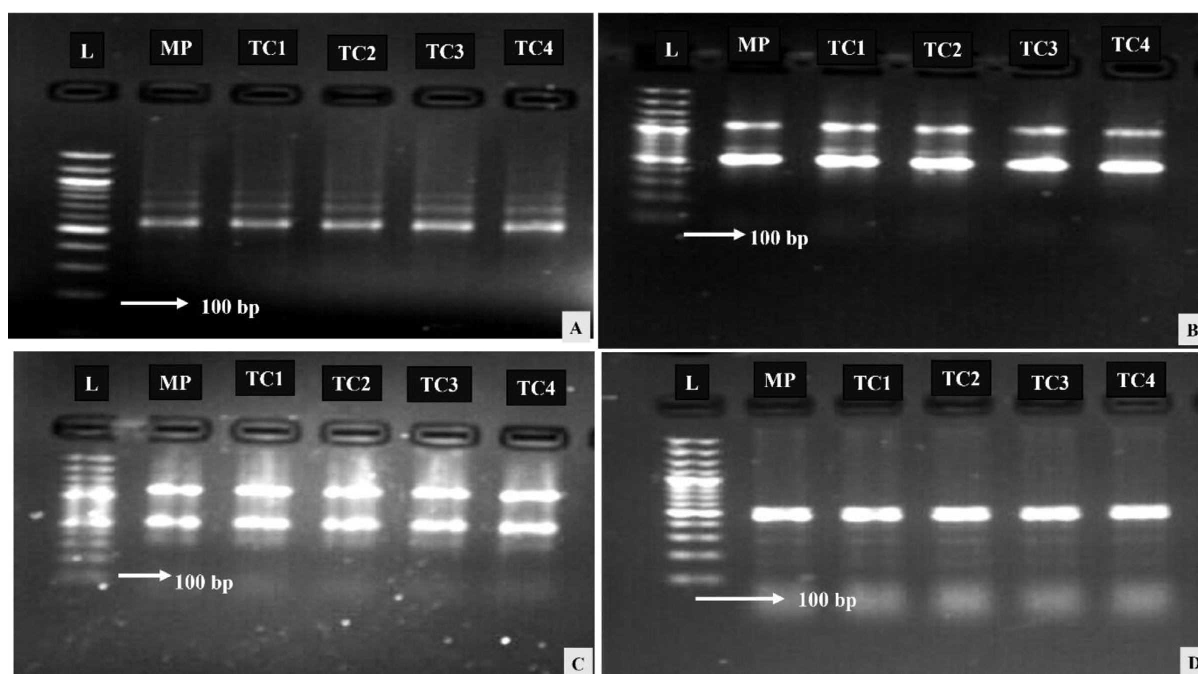

Fig. 4. RAPD markers for the test of genetic homogeneity between parent and *in vitro*-grown plants of *P. longum*. (A) Banding pattern of OPA-02, (B) Banding pattern of OPA-03, (C) Banding pattern of OPA-07, and (D) Banding pattern of OPP-01 (Note: *L*=Ladder DNA, MP=Mother plant, TC1, TC2, TC3, and TC4= *In vitro*-raised plants).

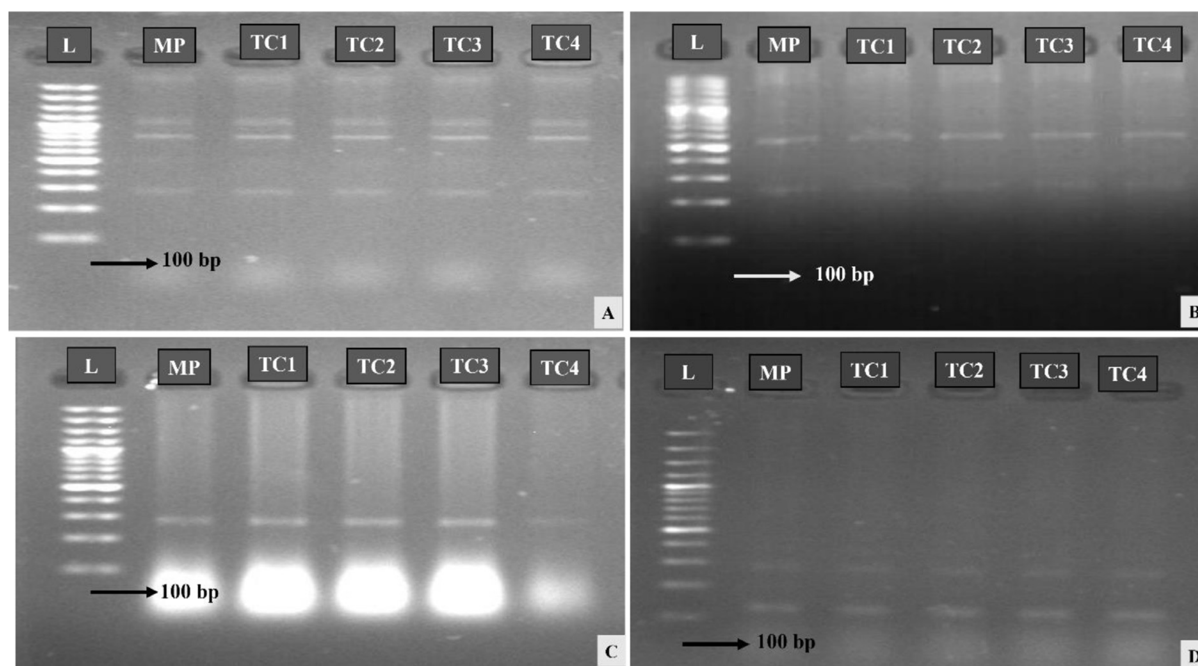

Fig. 5. ISSR markers for the test of genetic homogeneity between parent and *in vitro*-grown plants of *P. longum*. (A) Banding pattern of UBC-815, (B) Banding pattern of UBC-843, (C) Banding pattern of UBC-844, and (D) Banding pattern of UBC-880 (Note: *L*= Ladder DNA, MP=Mother plant, TC1, TC2, TC3, and TC4= *In vitro*-raised plants).

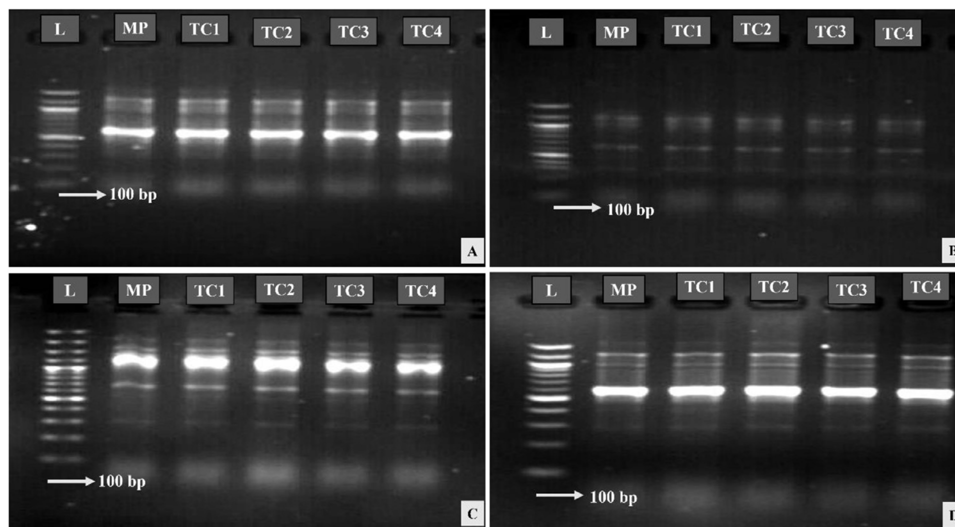

Fig. 6. SCoT markers for the test of genetic homogeneity between parent and *in vitro*-grown plants of *P. longum*. (A) Banding pattern of S-6, (B) Banding pattern of S-25, (C) Banding pattern of S-30, and (D) Banding pattern of S-35 (Note: *L*=Ladder DNA, MP=Mother plant, TC1, TC2, TC3, and TC4= *In vitro*-raised plants).

RAPD, ISSR, and SCoT analyses indicated that the *in vitro*-raised plants of *P. longum* showed the identical pattern of banding as the mother plants demonstrating that there was no genomic variation took place in the DNA of *in vitro*-raised plantlets. There are a few studies on genetic diversity among different wild *Piper* species and among the wild variety of *P. longum* using RAPD, ISSR, and SRAP markers; however, to the best of our information, it is the first report that evaluated genetic stability between the mother plant (wild) and *in vitro*-raised plant of *P. longum* using RAPD, ISSR, and SCoT markers.

5. Conclusion

In vitro culture technique can be used for the propagation of *P. longum* using nodal explants in MS media. This study demonstrated that an effective protocol developed for the *in vitro* propagation of *P. longum* by direct organogenesis can be used to produce true-to-type plants, which may aid in homozygous breeding programs and *ex-situ* conservation and could be used for commercial propagation. However, more research is needed to analyze the genetic homogeneity of *in vitro*-grown plants cultured in different conditions of PGRs using a larger number of markers and stress conditions to the mother plant to validate and production of the true-to-type plant in *P. longum*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Chandra Bahadur Thapa: Writing – original draft, Investigation, Data curation, Conceptualization. **Krishna Kumar Pant:** Writing – review & editing, Formal analysis. **Hari Datta Bhattarai:** Writing – review & editing, Methodology, Formal analysis. **Manisha Ghimire:** Methodology, Investigation, Data curation. **Anil Kumar Sah:** Methodology, Investigation, Data curation. **Bijaya Pant:** Writing – review & editing, Supervision, Conceptualization.

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In vitro Induction and Proliferation of Callus in *Piper longum* L. through Leaf Culture

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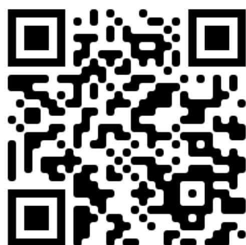

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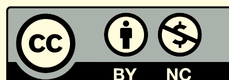

ABSTRACT

Piper longum L. (Family Piperaceae) is a well-known health promoter used to treat cough, chronic bronchitis, asthma, and diabetes mellitus. The study is aimed to develop a protocol for callus induction and proliferation in *P. longum*. The leaf explants from mature plants were cultured on an MS basal medium supplemented with various concentrations of plant growth hormones, viz. 2,4-Dichlorophenoxyacetic acid (2, 4-D), Kinetin (KN), 6-Benzylaminopurine (BAP), and α-Naphthalene acetic acid (NAA), as well as 10% coconut water. In primary culture, the callus was compact and light white. The best callus induction and growth were observed in the MS basal medium containing1.0 mg/L 2,4-D+ 2.0 mg/L KN at 12 weeks of primary culture. At eight weeks of secondary culture, the MS medium containing 2.0 mg/L BAP alone and 0.5 mg/L NAA + 2.0 mg/L BAP and 10% coconut water had the best callus proliferation. Compared to 2,4-D and KN alone, BAP alone supported rapid callus growth in the MS medium. In *P. longum*, large-scale callus formation from leaf explants could be exploited to produce, isolate, and increase bioactive secondary metabolites for therapeutic purposes.

Keywords: 6-Benzylaminopurine, Growth index, Kinetin, MS media, α-Naphthalene acetic acid, 2,4-Dichlorophenoxyacetic acid

1. INTRODUCTION

Piper longum L. is a valuable medicinal plant, called long pepper in English, Pipla in Nepali, Pipali in Sanskrit, Pippali in Hindi, & Thippili in Srilankan languages. *P. longum* is an endemic plant in the Indo-Malaya region. It is found growing wild in the tropical and subtropical forests of India, Bhutan, Srilanka, Malaysia, Indonesia, Nepal, Singapur, Myanmar, America, etc. In Nepal, it is distributed east to west Terai and Siwalik hills up to 1000 m asl (Press *et al.* 2000). It is a popular medicinal plant in South Asia and the Islamic regions of East and North Africa. Commercially, it is also known as "Pipla" and "Piplamul"; the Pipla is a dried spike (fruit), while a thick root with a stem segment is called Piplamul. The three grades of piplamool are available for use and trade: Grade I with thick roots and underground stems, Grade II and III consisting of thin roots and stems, or broken fragments with a low price compared to grade I (Kumar *et al.* 2011). Dried fruits are also used as seasoning and spices. The alkaloid piperine (3-5%) is the major and active constituent in *P. longum* (Zaveri *et al.* 2010). Fruit (mature female spike), roots (piplamul), leaves, & stem are used for several ailments in the form of juice, decoction, paste, or infusion as a mode of traditional drug preparation, viz. fruits for cough & cold (Muller-Boker 1993; Singh & Maheshwari 1994; Ghimire & Bastakoti 2009), for cough, asthma, & bronchitis (Sigdel & Rokaya 2011; Das *et al.* 2013; Kumar & Bharati 2014; Thapa 2020); stem and leaves for cough (Dangol & Gurung 1991); & roots for cough (Singh 2017), gastritis (Kumar *et al.* 2011), as antitoxin in scorpion sting and snake biting (Chopra *et al.* 1956), and jaundice (Singh *et al.*, 1997). It is also used in stomachic, laxative, anthelmintic, carminative, bronchitis, fever, cold, asthma, urinary discharge, tumors, piles, insomnia, jaundice, leprosy, gout, & rheumatism (IUCN 2004). Plants are decreasing in their natural habitats due to overexploitation for pharmaceutical and traditional medicinal use, habitat destruction, and illegal trade.

The traditional vegetative propagation of *P. longum* is insufficient due to poor seed viability, low percentage of seed germination, and delayed root formation in vegetative cuttings (Sarasan *et al.* 1993). Plant regeneration through seeds is difficult even in vitro conditions in *P. longum*. The callus culture is widely applied in basic research and industrial applications for the regeneration of entire plants and the production of secondary metabolites using precursors and elicitors. Callus and cell cultures have been successfully utilized to produce important pharmaceuticals in relatively large quantities (Pant 2014). Thus, induction of callus using various types of explants in culture is useful for regenerating entire plants through organogenesis or embryogenesis and for producing valuable secondary metabolites. The callus is also used for establishing cell suspensions, as good source material for protoplast isolation, and for extracting pure bioactive chemicals (Jhang *et al.* 1974; Furuya *et al.* 1983).

The callus induction and plant regeneration from leaf and nodal explants in *P. longum* was studied by Sarasan and Nair (1991); Bhat *et al.* (1992); Soniya and Das (2002); Sharon and Maurya (2004); Parida and Dhal (2011); Padhan (2015); Tiwari (2016); Sathelly *et al.* (2016); Fonseka and Wickramaarachchi (2018); Prajapati *et al.* (2019), Wasti and Pant (2019). This paper aims to analyze the callus induction and proliferation in different types of hormones at different concentrations and combinations using leaf explants. It will help to form the basis for the production of secondary metabolites from in vitro raised callus using precursors and elicitors and the phytochemical analysis of callus.

2. MATERIALS AND METHODS

2.1 Plant Material

Plants of *P. longum* were established in pots at the Central Department of Botany, TU, collected from the Rupandehi district, western Nepal, during October-November, 2020. The plant was identified by tallying the herbarium specimen in the Tribhuvan University Central Herbarium (TUCH), Kirtipur (Voucher specimen no. 135 & 136).

2.2 Explants Preparation

The young leaves were carefully excised from the stock plants and washed thoroughly in running tap water, adding a few drops of Tween-20 (Qualigens) for one and a half an hour. It was

again washed four times with distilled water. Then, the explants were surface sterilized with 70% ethyl alcohol for 30-35 seconds and with 0.1 % mercuric chloride solution for 4-5 min. It was washed four times with sterile distilled water to remove the traces of mercuric chloride. Then, leaf discs (0.8–1.0 cm diam.) were prepared with the help of a sterile cork borer in the sterile leaves.

2.3 Culture Medium Preparation

MS (Murashige & Skoog 1962) basal media was prepared from the stock solutions for all the in vitro cultures of leaf explant. For primary culture, the media was added with various concentrations of hormones viz. kinetin (KN) (0.25, 0.5, 1.0, 1.5, 2.0, & 3.0 mg/L) and 2,4-Dichlorophenoxy acetic acid (0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and, 5.0 mg/L) alone as well as in combination, 3% (w/v) sucrose, 10% coconut water (v/v) and 0.8% (w/v) agar. The pH was adjusted to 5.6 before autoclaving. For callus sub-culture, the MS media was supplemented with KN, BAP, & 2,4-D (0.5, 1.0, 1.5, & 2.0 mg/L) alone, and in combination of BAP and NAA (0.5, 1.0, 1.5, & 2.0 mg/L). For control, full, half, and quarter strengths of MS media were prepared without supplementing with the hormones and 10% coconut water. About 25 mL media were dispensed in autoclaved 400 mL sterile jars (78 mm × 122 mm) and closed with aluminium foil. All the media were autoclaved at 121ºC for 20 min at 15 lb/sq. inch.

2.4 Establishment of Cultures

The inoculation of leaf discs was carried out in the laminar airflow chamber. Before the inoculation, the laminar airflow chamber was made sterile by cleaning it with spirit or cotton soaked with 70% ethyl alcohol. The culture tubes and jars containing media, sterile metal instruments, and glassware were exposed to ultraviolet (UV) radiation for 45 minutes to remove the possible contaminants in and around the transfer area. After turning off the UV light, the blower was kept running during the inoculation process. Then, leaf discs (0.8–1.0 cm diam.) were inoculated on the MS medium aseptically. Similarly, the regenerated callus was subcultured on the MS medium after 12 weeks of primary culture. The cultures were maintained at 25 ± 2 °C and 12-16 h photoperiods. Each treatment consists of 3 replicates, and the experiment was repeated three times. Calli of 8 weeks of age were harvested, their growth indices and moisture contents were calculated (Adhikari & Pant 2013) and used for further study.

$$\text{Moisture (\%) of callus} = \frac{\text{Fresh wt of a callus-Dry wt of callus}}{\text{Fresh wt of callus}} \times 100$$

$$\text{Increase (\%)of callus (Growth index)} = \frac{\text{Fresh wt of callus-Fresh wt of callus explant}}{\text{Fresh wt of callus explant}} \times 100$$

3. RESULTS AND DISCUSSION

Callus induction, growth, and proliferation were obtained by inoculating sterile leaf discs of *P. longum* on MS media without addition and supplemented with different concentrations of 2,4-D and KN (0.25, 0.5, 1.0, 1.5, 2.0, & 3.0 mg/L) plus 10% coconut water. All the media did not respond positively to callus induction and growth. All the full, half, and quarter strength of MS basal media without the supplementation of hormones induced callus, but full strength of MS media (45%) induced a large callus than half (30%) and a quarter (25%) strength of MS media. The MS basal media added with various concentrations of kinetin alone did not induce callus. Soniya and Das (2002) reported that MS media supplemented with kinetin alone did not induce callus or direct plant regeneration in leaf explants. However, they induced callus from leaf explant in the MS media supplemented with 2.0 to 5.0 mg/L 2,4-D alone and combined with 2,4-D and KN.

The calli are loosely arranged undifferentiated parenchyma cells arising from the meristematic cells of the parent tissue, mostly from the cambium cells forming over a wounded or cut plant surface. In nature, callus is developed in plants by wounds, tumor-causing bacteria (Ti gene), and genetic tumours (Bhatiya 2015), while it can be produced in vitro culture of a leaf or shoot explants artificially in a suitable nutrient medium. In general, auxin alone and combined auxin and cytokinin induce callus in various plant species. However, callus induction occurs with an intermediate ratio of auxin and cytokinin; root formation occurs with a high ratio of auxin-to-cytokinin, and shoot regeneration occurs with a high ratio of cytokinin-to-auxin (Skoog & Miller 1957). In some species, abscisic acid and brassinosteroid hormones induce callus and might be used instead of auxin or cytokinin for

callus formation (Goren *et al.* 1979; Hu *et al.* 2000). However, auxin and cytokinin are widely studied and used in growth hormones for callus formation, organ regeneration, and metabolite production. The callus can be maintained and preserved for an indefinite period in culture by successive sub-culturing it on the fresh medium.

The best callusing media were MS supplemented with 1.0 mg/L 2,4-D + 2.0 mg/L KN (75%), followed by 0.25 mg/L 2,4-D + 1.0 mg/L KN (65%), 0.25 mg/L2,4-D + 2.0 mg/L KN (65%), 0.5 mg/L 2,4-D + 1.0 mg/L KN (55%), 0.5 mg/L2,4-D + 2.0 mg/L KN (52%), and 1.0 mg/L 2,4-D + 1.0 mg/L KN (45%) at 12 weeks of primary culture. Similarly, MS media added with 2,4-D at higher concentrations (3.0 mg/L to 5.0 mg/L) were found to be in good condition for callus induction (Table: 1). Hussain *et al.* (2011) also induced callus from leaf explant in MS media with a higher concentration of 2,4-D (2.0 mg/L to 3.0 mg/L) in *P. nigrum*. It shows that a low to a high concentration of 2,4-D (2.0 mg/L to 5.0 mg/L) in MS media favors the callus induction in *P. longum*. Similarly, by increasing the concentration of 2,4-D from 0.25 mg/L to 1.0 mg/L at the constant 1.0 mg/L to 2.0 mg/L KN in MS media, the induction and proliferation of callus were increased and then decreased. All the calli were compact and light white in primary culture. The callus may be friable or compact and may or may not contain somatic embryos (embryonic callus), roots (rooty callus), and shoots (shooty callus), which depends on the explants, plant hormones, culture condition, and plant species. The earlier workers found that the best condition for callus induction and growth from leaf segment was MS media added with 1.0 mg/L IAA + 1.0 mg/L BAP (Sathelly *et al.* 2016); with 0.5 mg/L TDZ (Prajapati *et al.* 2019); with 1.0 mg/L 2,4-D + 1.5 mg/L BAP (Malthi *et al.* 2016); with 1.0-2.0 mg/L 2,4-D + 1.0 mg/L BA (Sarasan *et al.* 1993) in *Piper longum*. Similarly, the best condition for callus induction and growth from leaf segment was MS media supplemented with 3.0 mg/L NAA + 0.05 mg/L BAP in *Piper betle* (Johri *et al.*1996); with 0.5 or 1.5 mg/L BA + 1.0 mg/L NAA in *Piper nigrum* (Ahmad *et al.* 2010); with 1.0 mg/L BA + 0.5 mg/L GA3 in *Piper nigrum* (Ahmad *et al.* 2013); with 2.0 mg/L 2,4-D + 1.5 mg/L KN in *Piper auritum* (Dominguez 2006); with 1.0 mg/L NAA + 1.0 mg/L BAP in *Bergenia ciliata* (Shrestha & Pant 2011); with 1.5 mg/L NAA + 10.0 µM SNP (Sodium Nitroprusside) + 10% coconut water in *Valeriana jatamansi* (Pandey *et al.* 2020); with 1.0 mg/L 2,4-D + 0.5 mg/L BAP in *Sonchus arvensis* (Wahyuni *et al.* 2020). It shows that the callus induction occurs from the leaf explants in MS media added with various hormones, viz. IAA, BAP, BA, NAA, TDZ, 2,4-D, and GA3 alone and in combination. Callus induction within a plant species depends on the type of explants (leaf, stem, or rhizome), the orientation of the explants, growth hormones, maturity of the plant and explants (juvenile or old), medium composition, metabolic condition of the plant, temperature, the growth conditions, and donor plant variety (Klimek-Chodacka *et al.* 2020). However, the plant species depends on successful callus induction, growth, and proliferation.

Table. 1 Callus induction in various concentrations of 2,4-D and Kinetin.

2,4-D/→ KN↓	**0.0 mg/L**	**0.25 mg/L**	**0.5 mg/L**	**1 mg/L**	**1.5 mg/L**	**2 mg/L**	**3 mg/L**	**4 mg/L**	**5 mg/L**
0.0 mg/L	LC	-	-	-	-	LC	MC	HC	HC
0.25 mg/L	-	-	-	-	LC	LC	LC	MC	LC
0.5 mg/L	-	-	MC	MC	MC	MC	MC	LC	LC
1.0 mg/L	-	HC	HC	HC	MC	LC	-	-	-
1.5 mg/L	-	MC	MC	MC	MC	LC	-	-	-
2.0 mg/L	-	HC	HC	HC	MC	LC	-	-	-
3.0 mg/L	-	HC	HC	MC	MC	LC	-	-	-

MS media, Culture condition: 25 ± 2 °C, 12-16 h Photoperiods, 12 weeks

*LC=Low callus (<1.0 g fresh wt.), *MC=Medium callus (1.0-2.0 g fresh wt.), *HC=High callus (>2.0 g fresh wt.)

The induced calli from 1.0 mg/L 2,4-D + 2.0 mg/L KN were maintained by subculturing in the same concentration of 2,4-D and KN in MS media, and then the calli were subcultured in MS basal media added with different concentrations of 2,4-D, Kinetin and BAP alone, as well as in the combination of NAA and BAP, and 10% coconut water. The proliferation of callus was found in all concentrations. However, the best callus proliferation was found in MS basal media supplemented with 2.0 mg/L BAP alone, which was determined based on the fresh weight and dry weight of calli after eight weeks of subculture (Table 2). At this concentration, 2.149±0.521 g fresh weight callus was obtained from the 0.219 ± 0.030 g initial fresh weight of callus explant, which was a 674 % increase of callus growth after eight weeks of subculture. After the subculture, callus morphology changed from compact light white to slightly friable yellow, compact yellow, and compact green. Calli in MS media added with auxin (2,4-D) were slightly friable yellow, but calli in MS media supplemented with cytokinins (KN and BAP) were compact green and compact yellow. Change in callus morphology after subculture might be due to genetic heterogeneity in callus and hormonal composition in media. In tissue culture, explants with different genotypes show different responses under similar growing conditions (Nehara *et al.* 1989 & 1990). Plant hormones affect cell growth, organogenesis, and metabolite synthesis (Lian *et al.* 1991). The proliferation of callus reduced, decreasing the concentration of all hormones, viz. 2,4-D, KN, and BAP alone in MS basal media.

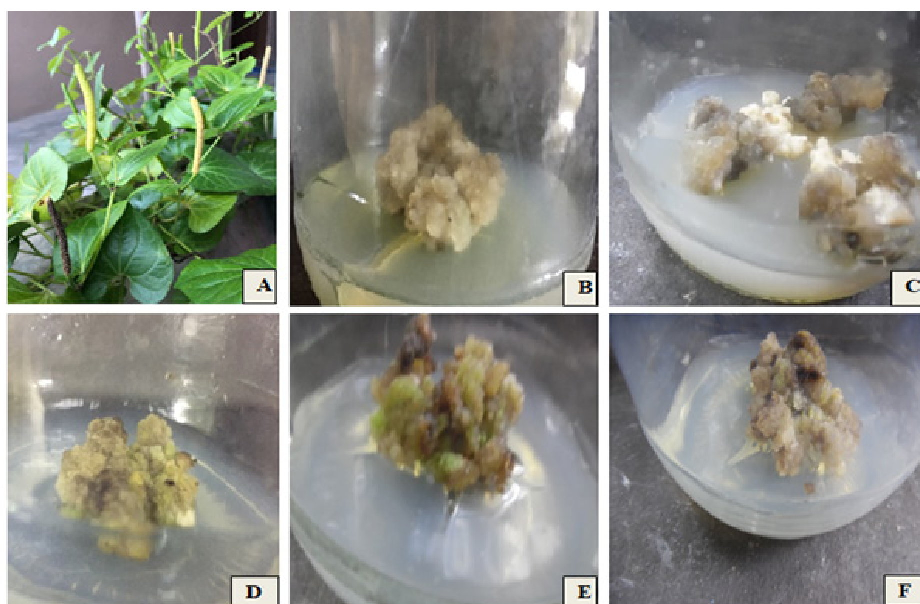

Fig. 1 (A): *Piper longum* plant; (B): Callus: MS + 1.0 mg/L2,4-D + 2.0 mg/L KN; (C): MS + 0.25 mg/L 2,4-D + 1.0 mg/L KN; (D): MS + 0.5 mg/L 2,4-D + 1.0 mg/L KN; (E): MS + 0.5 mg/L2,4-D + 2.0 mg/L KN; (F): MS + 1.0 mg/L 2,4-D + 1.0 mg/L KN

Cytokinins (Kinetin and BAP) alone were more effective than auxins (2,4-D) for callus proliferation in MS media in *P. longum*. The p-value from two way ANOVA test of callus growth (g) versus plant growth hormones (p-value<0.05, i.e.=0.0116) and various concentrations of 2,4-D, KN, and BAP (p-value<0.05, i.e.= 0.0289) showed significant difference It shows that the callus proliferation depends on types of plant hormones and concentrations supplemented in MS media. The callus proliferation increases by increasing the concentration of 2,4-D alone, KN alone, and BAP alone from 0.5 mg/L to 2.0 mg/L in MS media.

Table: 2 Individual effects of 2, 4-D, KN, & BAP on the growth and development of callus after secondary culture (observation at eight weeks)

S. N.	2,4-D (mg/L)	KN(mg/L)	BAP(mg/L)	Fresh wt (g) of callus explant	Fresh wt (g) of callus	Dry wt (g) of callus	Increase (%) of callus
1	0.5			0.220±0.029 CW	0.401±0.015 FLY	0.102±0.013	182
2	1			0.232±0.070 CW	0.544±0.057 FLY	0.122±0.020	234
3	1.5			0.235±0.073 CW	0.571±0.025 FLY	0.129±0.015	243
4	2			0.227±0.078 CW	0.950±0.093 FLY	0.213±0.047	419
5		0.5		0.222±0.076 CW	1.006±0.082 CY	0.262±0.059	312
6		1		0.213±0.033 CW	1.223±0.330 CG	0.227±0.013	359
7		1.5		0.224±0.029 CW	1.141±0.071 CG	0.189±0.012	352
8		2		0.209±0.053 CW	1.677±0.021 CG	0.317±0.019	543
9			0.5	0.236±0.030 CW	0.702±0.090 CY	0.139±0.028	209
10			1	0.226±0.077 CW	1.057±0.113 CG	0.204±0.013	324
11			1.5	0.257±0.041 CW	1.751±0.262 CG	0.346±0.048	430
12			2	0.219±0.030 CW	2.149±0.521 CG	0.367±0.106	674

MS media, Culture condition: 25 ± 2 °C, 12-16 h Photoperiods, eight weeks

*CW: compact light white, CG: compact green, CY: compact yellow, FLY: friable light yellow. Data were obtained from a total of 36 explants with repeated three experiments.

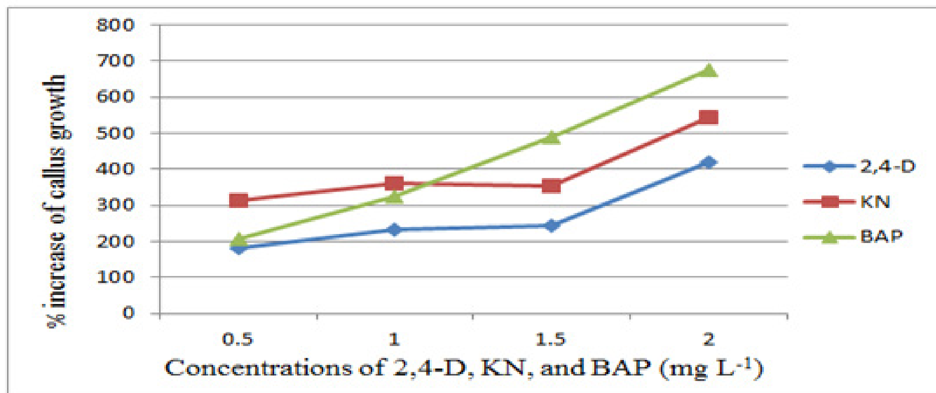

Fig. 2 Callus proliferation in MS media added with different concentrations of 2,4-D, KN, and BAP

Similarly, the best callus proliferation was obtained in MS media added with 0.5 mg/L NAA + 2.0 mg/L BAP in terms of fresh weight and dry weight after eight weeks of subculture (Table 3). At this concentration, 1.843±0.134 g fresh weight of callus was obtained from the 0.316 ± 0.011 g initial fresh weight of callus explant after eight weeks of subculture. All the calli after subculture were changed in morphology from compact light white to compact white brown, compact yellow, compact green, and slightly friable light yellow, which may be due to the release of secondary metabolites, change in hormonal composition in MS media, or vitrification. At a constant concentration of BAP (cytokinin), increasing the concentration of NAA (auxin) from 0.5 mg/L to 2.0 mg/L in MS media resulted in a decrease in callus growth and development. It shows that the higher concentration of auxins in combination with cytokinins does not favor the callus growth and proliferation of *P. longum*.

Table: 3 Combined effect of NAA + BAP on the growth and development of callus following secondary culture (observation at eight weeks)

S. N.	NAA (mg/L)	BAP (mg/L)	Fresh wt (g) of callus explants	Fresh wt (g) of callus	Dry wt (g) of callus	Increase (%) of callus
1	0.5	0.5	0.379±0.047 CW	2.089±0.185 CWB	0.541±0.067	551
2	1	0.5	0.340±0.042 CW	1.732±0.266 CG	0.431±0.078	509
3	1.5	0.5	0.309±0.055 CW	0.856±0.079 CY	0.206±0.028	277
4	2	0.5	0.363±0.061 CW	0.727±0.045 CY	0.171±0.008	200
5	0.5	1	0.331±0.037 CW	1.642±0.150 CG	0.426±0.049	496
6	1	1	0.394±0.014 CW	1.178±0.052 CG	0.290±0.039	298
7	1.5	1	0.338±0.023 CW	0.716±0.176 CG	0.162±0.027	211
8	2	1	0.386±0.015 CW	0.630±0.071 FLY	0.156±0.023	172
9	0.5	1.5	0.344±0.007 CW	1.465±0.098 CG	0.361±0.045	425
10	1	1.5	0.314±0.017 CW	1.132±0.147 CY	0.258±0.011	360
11	1.5	1.5	0.308±0.020 CW	0.744±0.096 FLY	0.183±0.029	241
12	2	1.5	0.387±0.014 CW	0.628±0.120 FLY	0.157±0.040	162
13	0.5	2	0.316±0.011 CW	1.843±0.134 CWB	0.428±0.004	583
14	1	2	0.363±0.011 CW	1.561±0.064 CY	0.390±0.051	430
15	1.5	2	0.397±0.025 CW	0.644±0.131 CY	0.158±0.042	162
16	2	2	0.344±0.045 CW	0.543±0.104 CG	0.146±0.031	157

MS media, Culture condition: 25 ± 2 °C, 12-16 h Photoperiods, eight weeks

*CW: compact light white, CG: compact green, CY: compact yellow, FLY: friable light yellow, CWB: compact white brown. Data were obtained from a total of 36 explants with repeated three experiments.

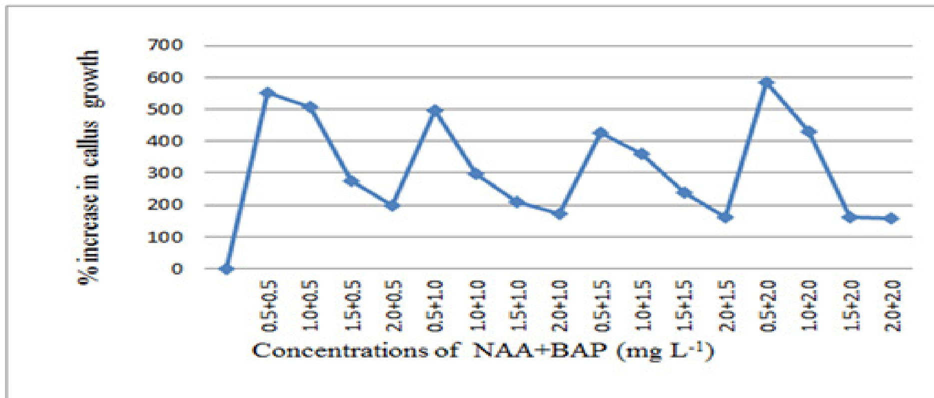

Fig: 3 Callus proliferation in MS media supplemented with various concentrations of NAA + BAP

Using an organic additive (10% coconut water) in MS media was effective for the induction and proliferation of calli in the leaf explant of *P. longum*. The callus induction in control was less than the supplemented hormones, viz. 45% (full MS), 30% (half MS), & 25% (quarter MS) less compared to MS media supplemented with 10% coconut water and plant hormones. Gnasekaran *et al.* (2010) argued that coconut water acts as a cytokinin-like growth regulator containing many nutritional and hormonal substances that induce cell division and growth in culture.

3. CONCLUSION

Callus can be induced from the leaf segments in MS media containing 2,4-D alone and in the combination of 2,4-D and KN. The callus induction in *P. longum* was favored by a high concentration of 2,4-D (auxin) alone and a comparatively low concentration of 2,4-D with a high concentration of KN (cytokinin) in conjunction with MS media. Increased concentrations of 2,4-D alone, BAP alone, and KN alone in MS media increased callus growth and proliferation. However, no definite callus growth and proliferation pattern was observed in MS media in combination with NAA and BAP at secondary callus culture. The protocol developed for the in vitro induction, growth, and proliferation of callus from leaf segment in *P. longum* could be useful for producing and enhancing valuable secondary bioactive compounds using elicitors. Plant regeneration in *P. longum* can also be accomplished from the callus via organogenesis and somatic embryogenesis, and it is in progress.

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Research Article

EVALUATION OF ANTIOXIDANT, ANTIDIABETIC, AND CYTOTOXIC ACTIVITIES OF *Lilium nepalense* D. DON

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ABSTRACT

Lilium nepalense, a temperate medicinal plant, is used as a diuretic, antipyretic, tonic, flavoring agent, and heart pain treatment. This research aimed to evaluate the *in vitro* antioxidant and antidiabetic activities of the methanol, dichloromethane (DCM), and hexane fractions, and *in vivo* cytotoxic activities of the crude extracts of the bulb. The antioxidant activity was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, antidiabetic activity by α-glucosidase inhibitory assay, and cytotoxic activity in terms of LC_{50} (median lethality concentration) by Brine shrimp assay. The DCM fraction showed the strongest antioxidant activity (IC_{50}=134.99±9.75 μg/mL) and highest antidiabetic activity (IC_{50}=182.01±20.50 μg/mL) than other fractions. Similarly, the DCM fraction had the highest total phenolic contents (243.97±33.78 mg of gallic acid equivalent per gram dry weight) and highest flavonoid contents (7.68±0.85 mg of quercetin equivalent per gram dry weight) than other fractions. Moreover, the crude extract of the bulb was not found to be cytotoxic to the Brine shrimp nauplii (LC_{50}=3.83 mg/mL). It is the first report to date describing the antioxidant, antidiabetic, and cytotoxic properties of *L. nepalense.* This study concludes that the DCM fraction of the bulb could be used as an antioxidant and antidiabetic agent for therapeutic purposes; however, further identification and characterization of bioactive compounds responsible for the antioxidant, antidiabetic, and cytotoxicity is required for further validation.

Keywords: Antidiabetic, Brine shrimp, Bulb, DCM, IC_{50}, LC_{50}

INTRODUCTION

Nepal has a varied physiography ranging from 60m to 8848m above sea level, resulting in a wide range of vegetation belts ranging from tropical to alpine. About 13,067 plant species are described from Nepal (Chaudhary *et al.*, 2020), however only 2,500 species are utilized in traditional medicine to treat various illnesses and disorders (Kuwar *et al.*, 2021). Similarly, the Government of Nepal (GoN) has recognized 148 species of medicinal and aromatic plants (MAPs) in Nepal, which are distributed from lowland Terai to high Himalayan ranges (Bhattarai and Subedi, 2023). Moreover, over 701 species are utilized medicinally, 30 species are prioritized for research and development, and 12 species are prioritized for agrotechnology development in Nepal (DPR, 2017). Therefore, there is a need for modern technology to validate the long-standing use of these significant medicinal plants by analyzing both *in vivo* and *in vitro* bioactivities, as well as their phytochemical analysis for valuable biochemical profiling.

An antioxidant is a molecule that may neutralize radicals that are unstable such as oxygen, nitrogen, and lipidic radicals, and prevents or slows the oxidation of other molecules while also protecting a biological system (Ladaniya, 2023). Strong evidence suggests that the buildup of free radicals contributes to many harmful pathophysiological processes, including cancer, diabetes, cardiovascular, and neurodegenerative illnesses (Gilgun-Sherki *et al.*, 2002; Rahman *et al.*, 2015). The human body, however, contains a sophisticated network of naturally occurring enzymatic and non-enzymatic antioxidant defenses that work to neutralize the negative effects of free radicals and other oxidants. Reactive oxygen species (ROS) in excess can weaken cellular antioxidant defenses, causing oxidative stress, which damages proteins and DNA (Betteridge, 2000; Gilgun-Sherki *et al.*, 2002); cardiac arrest, malignancy, age-related disorders, metabolic disorders, and arterial sclerosis (Ames *et al.*, 1993). By providing electrons to these damaged cells, antioxidants are molecules that stop and stabilize ROS-caused cell damage, minimizing the harmful effects of ROS on human health. It is believed that oxidative stress, which results in oxidative harm to cell constituents such as lipids, proteins, and nucleic acid, is to blame for diabetes long-term complications (Rahimi *et al.*, 2005). A metabolic disorder called diabetes impairs the body's capacity to make, use, or react to insulin. Studies have demonstrated that the polyphenols (flavonoids and phenolic compounds) found in several medicinal plants serve as antioxidants and antidiabetics. Inhibiting carbohydrate hydrolyzing enzymes like α-amylase and α-glucosidase with a drug or diet might delay the synthesis or absorption of glucose, which is one of the therapeutic strategies for lowering postprandial hyperglycemia. Additionally, a workable method for general cytotoxicity testing is crucial as an initial step in the investigation of the bioactive substances found in plants. The Brine shrimp (*Artemia salina* Leach) test provides an assay that can be quick, straightforward, and more significantly, affordable and repeatable (McLaughlin, 1991).

Lilium nepalense D. Don (called Ban lasun or Khiraule in Nepali) is one of the biggest genera of the Liliaceae family, with 110 species scattered throughout the temperate northern hemisphere and into the northern subtropics (Stevens, 2015). In Nepal, *Liium* has ten different species (Press *et al*., 2000; www.Efloras.org). *L. nepalense* can be found in Nepal in the temperate zone between 2200 and 3400 meters above sea level (IUCN, 2004). It is a 0.6 to 1 m tall erect bulbous plant (Fig. 1) that has large, prominent flowers and has been classified as a Data Deficient (DD) plant (Anonymous, 2001). Locals utilize the bulb as a diuretic, antipyretic, tonic, vegetable, and flavoring agent, and to relieve pain in the area around their hearts (IUCN, 2000). The active chemical components, or secondary metabolites, are found in different plant parts and are responsible for the medicinal characteristics of plants (Pant, 2014). More than 180 different chemicals, including steroidal saponins, phenolic glycerides, polysaccharides, alkaloids, and flavonoids have been extracted and ascertained from the genus *Lilium* (Zhou *et al*., 2021). To the best of our information, no research has been done on the phytochemical makeup and bioactivities of *L. nepalense*. Therefore, this research aims to evaluate the antioxidant, antidiabetic, and Brine shrimp lethality bioassay of bulb extracts.

MATERIALS AND METHODS

Plant material collection

Lilium nepalense bulb (Fig. 1) was collected in May 2021 at a height of 2200 meters above sea level from Phulchowki Hill in Lalitpur, Nepal. By tallying the herbarium specimens from the National Herbarium & Plant Laboratories (KATH), the collected plant was identified as *L. nepalense*. It was then deposited to the Tribhuvan University Central Herbarium (TUCH), Tribhuvan University, Kirtipur (voucher no. C35).

Fig. 1. *Lilium nepalense*: (A) Plants with bulbs and roots, (B) Collected bulbs.

Chemicals, reagents, and enzyme

The chemicals and enzymes utilized in this research were of analytical grade. Methanol, ethanol, dichloromethane (DCM), hexane, ascorbic acid, and gallic acid were of HiMedia Laboratories Pvt. Ltd., while α-glucosidase, 4-Nitrophenyl β-D-glucopyranoside (p-NPG), quercetin and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were of Sigma Aldrich Chemicals Pvt. Ltd.

Extract preparation and fractionation of the crude extract

After completely removing all soil particles from the bulb with running water and air drying it, the bulb was ground into powder in an electric grinder. Approximately 350 g of powder was macerated in 90% methanol at room temperature for 48 h before being filtered with the help of filter paper. The filtrate was evaporated in a rotavapor (EYELA Co, Ltd, USA) at a low pressure to produce the crude extract. For fractionation, 50 g of the crude extract was dissolved in 100 mL of distilled water to form a mixture solution. At first, the mixture solution was mixed with hexane in a 1:2 ratio in a separating funnel and shaking well for 3-4 min separated the upper hexane layer from the aqueous layer, and then the aqueous layer was mixed with DCM in a 1:2 ratio to separate the lower DCM layers from the aqueous layer successively. This process was repeated three times in the separating funnel. The hexane and DCM layers were concentrated in a rotary evaporator under low pressure to get hexane and DCM fractions. Moreover, the aqueous layer was concentrated in a rotavapor under reduced pressure to get an aqueous extract. The aqueous extract was dissolved in 100% methanol, which was then concentrated in a rotavapor at a low pressure to get a methanol fraction. They were kept in vials and deposited at 4°C in the freezer for further use.

Antioxidant activity by DPPH assay

The antioxidant activity of the fractions was tested by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay following Blois (1958) and Desmarchelier *et al.* (1997) with some modifications. 100 μL of DPPH solution (0.1 mM of DPPH) was combined with 100 μL of different concentrations of extracts (25 to 400 μg/mL) in a 96-

well plate. A spectrophotometer (Synergy BioTek, Co. Ltd, UK) was used to measure the mixture's absorbance at 517 nm after it had been incubated at ambient room temperature in the dark for 30 minutes. Ascorbic acid-containing reaction mixture and reaction mixtures without the crude extracts served as the corresponding positive and negative controls. The formula below was used to compute the percentage of DPPH radical scavenging activity:

$$\% \text{ DPPH radical scavenging activity} = \frac{Ab_0 - Ab_1}{Ab_1} \times 100 \ldots (1)$$

Ab_1 is the absorbance of the extracts or standard, while Ab_0 is the absorbance of the negative control. After plotting the percentage of inhibition versus concentration, the IC_{50} was determined. At each concentration, the experiment was run in triplicate.

Estimation of total phenolic content (TPC) and total flavonoid content (TFC)

The TPC of the plant extract (1 mg/mL) was evaluated using Folin-Ciocalteau reagent (FCR) comprising gallic acid as standard pursuing Zhang *et al.* (2006) with some changes. The mixture solution containing 20 μL of various plant extract or standard gallic acid, 100 μL FCR (10%) followed by 80 μL Na_2CO_3 (1 M) was incubated in the dark for 15 minutes and absorbance was taken at 765 nm. Using a gallic acid standard curve, the result was reported as milligrams of gallic acid equivalent per gram of dry weight (mg of GAE/g) of the extract.

Similarly, an aluminum chloride complex-forming assay was used to assess the TFC of the fractions comprising quercetin as standard following Chang *et al.* (2002) with some changes. The whole assay mixture containing 130 μL standard quercetin or 20 μL plant extract with 110 μL distilled water, 60 μL ethanol, 5 μL 10% $AlCl_3$, and 5 μL 1 M potassium acetate were incubated in the dark for 30 minutes and absorbance was taken at the 415 nm wavelength. Using the quercetin standard curve, the result was reported as milligrams of quercetin equivalent per gram of dry weight (mg of QE/g) of the extract.

***In vitro* α-glucosidase inhibition assay**

The antidiabetic activity of fractions was determined by measuring the alpha-glucosidase inhibitory activity (van de Laare *et al.*, 2005). A 96-well plate was filled with a mixture of 20 μL of various extract concentrations (50-400 μg/mL), 20 μL of α-glucosidase enzyme (0.5 unit/mL), and 60 μL of phosphate buffer (6.8 pH, 0.1 M). Initial absorbance was taken at 405 nm in a spectrophotometer (Synergy LX) after the mixture solution was pre-incubated at 37°C for 15 min. The mixture solution was then mixed with 40 μL of p-NPG (5 mM), and it was incubated at 37°C for 15 min. By adding 60 μL of Na_2CO_3 (1 M), the reaction process was stopped. In a spectrophotometer, the final absorbance was taken at 405 nm which calculates the volume of p-nitrophenol emitted from p-NPG. Acarbose was used as a positive control and phosphate buffer as a negative control of alpha-glucosidase inhibitors. The average of the three results from each experiment was recorded. The given formula was used to determine the % inhibition of α-glucosidase and the concentration of extract necessary to inhibit 50% of alpha-glucosidase activity (IC_{50}) was calculated using a linear regression equation.

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_1} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where, A_0=Absorbance of enzyme-substrate reaction with phosphate buffer

A_1= Absorbance of enzyme-substrate reaction with plant extracts

Cytotoxicity test by Brine shrimp lethality assay

The cytotoxicity of crude extract was determined on live Brine shrimp nauplii by following Meyer *et al.* (1982) and Fatope *et al.* (1993) with slight modification. To prepare an artificial seawater solution, 3.5 g of NaCl was initially added to 100 mL of distilled water. Brine shrimp (*Artemia salina*) eggs weighing about 10 mg were incubated in seawater for 48 h with the temperature set at 23°C (using an 80-watt bulb) to hatch eggs into larvae, or nauplii. In 5% DMSO, an extract stock solution (10 mg/mL) was prepared. Then, each concentration of plant extracts such as 5.0 mg/mL, 4.0 mg/mL, 3.0 mg/mL, 2.0 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and 0.25 mg/mL with seawater containing 25 nauplii were added in a 2 mL Eppendorf tube separately. Instead of using plant extract, seawater was utilized as a negative control, while potassium dichromate was used as a positive control. Three duplicates of each experiment were carried out. The tubes were exposed to light for 24 hours and then, all living nauplii were counted. The lethal concentration causing death in 50% (LC_{50}) for each plant extract was determined using the given formula and regression line obtained by plotting the concentration against the mortality percentage on a probit scale.

$$\% \text{ mortality} = \frac{\text{No.of dead larvae (Nauplii)}}{\text{Initial No.of live larvae (Nauplii)}} \times 100 \ldots\ldots (3)$$

Data analysis

For each sample, the experiment was done in triplicate, and results were revealed as mean ±S.D. IC_{50} and LC_{50} values were analyzed using a linear regression equation. Microsoft Excel 2010 was used for all calculations.

RESULTS AND DISCUSSION

Yield of crude extract and fractions

The cold and hot maceration of 350 g powder of *L. nepalense* bulb in 90% methanol yielded 14.28% crude extract (Table 1). The three solvents had quite varied yields, and the polarity-based fractionation showed that methanol fraction had highest yield (31.5%), followed by DCM fraction (0.62%), and then hexane fraction (0.56%) (Table 1). It showed that the polar solvent yields were higher than the non-polar solvent yields. Fractionation of crude extract by liquid-liquid partition method relies on the polarity of the used solvent and the polarity of the components. Plants contain a variety of

bioactive chemicals with varying polarities. The polarity of the target compounds must therefore be complemented by the extraction solvent. Hexane, DCM, and methanol were the three solvents used in this study to examine extraction yields.

Table 1. Yield of crude extract and fractions (Hexane, DCM, and Methanol) in the bulb of *L. nepalense.*

Powder/Crude extract taken (g)	Method applied	Solvent used	Amount of Crude extract/fraction (g)	Yield (%)
Powder (350 g)	Cold & hot maceration	90% Methanol	Crude extract (50g)	14.28%
Crude extract (50 g)	Liquid-liquid partition	Hexane	Hexane fraction (0.289 g)	0.56%
Crude extract (50 g)	Liquid-liquid partition	DCM	DCM fraction (0.31 g)	0.63%
Crude extract (50 g)	Liquid-liquid partition	100% Methanol	Methanol fraction (15.75 g)	31.50%

Antioxidant activity by DPPH assay

The DPPH assay was used to assess the free radical scavenging capacity of the methanol, DCM, and hexane fractions in an *in vitro* condition. Antioxidants' ability to donate hydrogen is largely responsible for their influence on the scavenging of DPPH radicals. Comparing the antioxidant value to the positive control ascorbic acid, which had an IC_{50} of 19.81±0.603 μg/mL, the antioxidant value ranged from 134.99±9.75 to 2510.24±96.54 μg/mL (Table 2). This study demonstrated that, compared to other fractions, the DCM fraction exhibited the strongest free radical scavenging activity (IC_{50}=134.99±9.75 μg/mL) (Fig. 2). It might be because DCM is an intermediate polar solvent, allowing the synergistic effects of both polar and nonpolar types of compounds to manifest themselves (Thapa *et al.*, 2023). Similarly, the hexane fraction had the lowest antioxidant activity (IC_{50}=2510.24±96.54 μg/mL), which may be related to the fact that hexane is a nonpolar solvent that dissolves nonpolar compounds having lower antioxidant capacity. The DCM has stronger antioxidant activity than other fractions, according to research done on *Syzygium cumini* leaf (Franco *et al.*, 2020), *Apium graveolens* (Emad *et al.*, 2022), and *Paris polyphylla* rhizome (Thapa *et al.*, 2023). Although the bulbs of the *Lilium* species are frequently used as food and medicine, there have been very few studies examining their antioxidants and other bioactivities. According to Jin *et al.* (2012), six species of *Lilium*, including *L. regale, L. concolor, L. pumilum, L. leucanthum, L. davidii* var. *unicolor*, and *L. lancifolium,* have higher phenolic contents and antioxidant properties. Locals consume the bulbs of *L. nepalense* as food, vegetables, and medicines, therefore thorough investigation into its bioactivities and phytochemical profile may open up new opportunities for the food and drug industries.

Table 2. Antioxidant activity, total phenolic content, total flavonoid content, and α-glucosidase inhibition of various fractions of *L. nepalense.*

Fractions	Antioxidant Activity (IC_{50}: μg/mL)	Total phenolic content (mg of GAE/g dry wt)	Total flavonoid contents (mg of QE/g dry wt)	Antidiabetic activity (IC_{50}: μg/mL)
DCM	134.99±9.75	243.97±33.78	7.68±0.85	182.01±20.50
Hexane	2510.24±96.54	4.58±0.99	0.55±0.48	2767.53±158.72
Methanol	1650±263.91	75.38±10.98	1.03±0.21	1903.85±90.24
Control	19.81±0.603	-	-	125.73±13.54

Total phenolic contents (TPC) and total flavonoid contents (TFC)

Using the gallic acid calibration curve (10-60 μg/mL, Y= 0.018x, R^2 = 0.980), the total phenolic content of the methanol, DCM, and hexane fractions of the *L. nepalense* bulb was calculated by applying the Folin-Ciocalteu Reagent (FCR) concerning gallic acid equivalent (mg of GAE/g of the dry weight of extract). The highest total phenolic content was found in DCM fraction (243.97±33.78 mg GAE/g dry weight), followed by methanol fraction (75.38±10.98 mg GAE/g dry weight) and hexane fraction (4.58±0.99 mg GAE/g dry weight) (Fig. 3).

Similarly, the total flavonoid content of the methanol, DCM, and hexane fractions of *L. nepalense* bulb was determined in terms of mg Quercetin equivalent per gram of dry mass (mg of QE/g dry weight) of extract. The flavonoid content of the various plant extracts was determined by applying the calibration curve that was created using quercetin (10-80 μg/mL) with the line of equation (Y=0.0256x, R^2=0.983). The higher total flavonoid content was reported in the DCM fraction (7.68±0.85 mg of QE/g dry weight), followed by methanol fraction (1.03±0.21 mg of QE/g dry weight) and hexane fraction (0.55±0.48 mg of QE/g dry weight) (Fig. 4).

This study demonstrated a positive relationship between the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant (free radical scavenging) activity of the tested extracts, i.e., the fraction with better antioxidant activity (less IC_{50}) had a higher total phenolic content and total flavonoid contents. Therefore, the total phenolic and total flavonoid contents of the extracts/plants might determine their antioxidant activity.

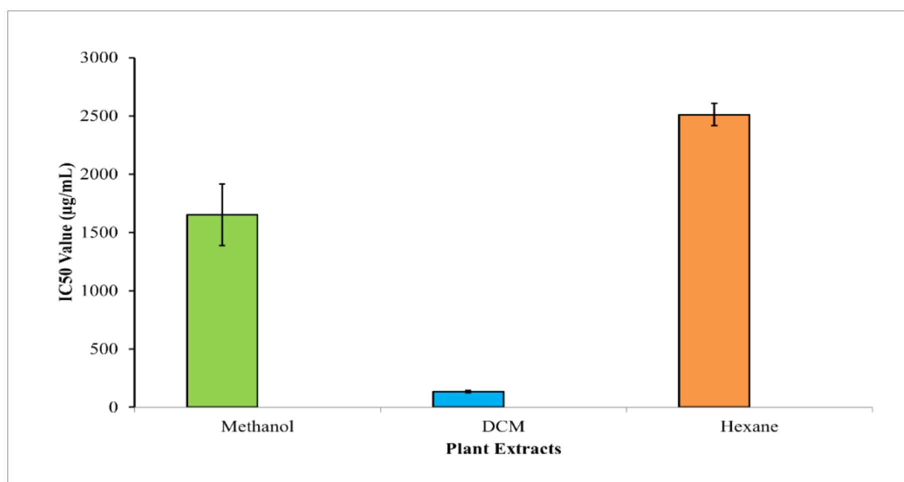

Figure 2. Antioxidant activity (IC_{50}) of various fractions of *L. nepalense* (Note: Error bars represent the standard deviation of three independent measurements).

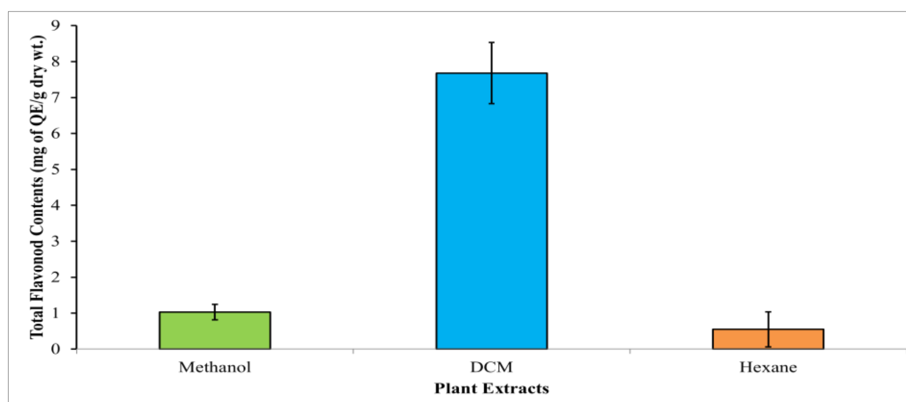

Figure 3. Total phenolic contents (TPC) of various fractions of *L. nepalense* (Note: Error bars represent the standard deviation of three independent measurements).

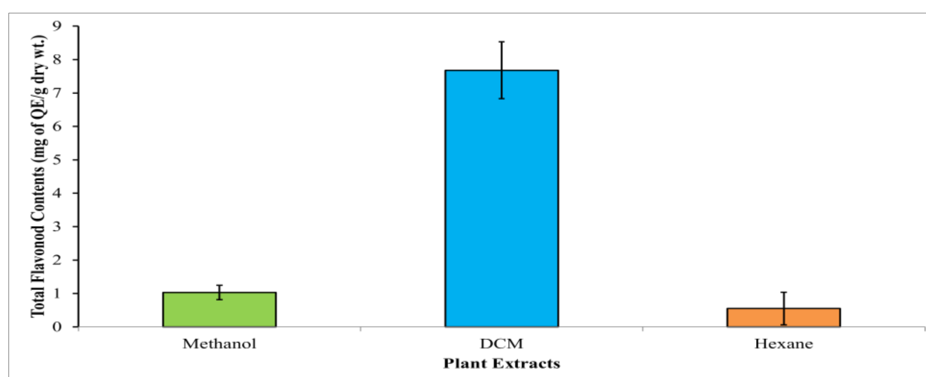

Figure 4. Total flavonoid contents (TFC) of various fractions of *L. nepalense*. (*Note: Error bars represent the standard deviation of three independent measurements*).

In vitro α-glucosidase inhibition assay

The antidiabetic activity of the DCM, methanol, and hexane fractions was assessed by alpha-glucosidase inhibition assay. The highest alpha-glucosidase inhibitory activity was found in the DCM fraction (IC_{50}=182.01±20.50 µg/mL), followed by methanol fraction (IC_{50}=1903.85±90.24 µg/mL) and hexane fraction (IC_{50}=2767.53±158.72 µg/mL) in comparison to the positive control acarbose (IC_{50}=125.73±13.54 µg/mL) (Table 2 & Fig. 5). However, Mir *et al.* (2020) reported that the water extract of *Lilium polyphyllum* had the highest inhibition percentage on α-glucosidase than ethanol and DCM crude extracts. The DCM fraction had stronger antidiabetic activity than other fractions, according to research done on the olive mill plant waste (Mwakalukwa *et al.*, 2020), *Clerodendrum volubile* (Erukainure *et al.*, 2018), and *Syzygium cumini* leaf (Franco *et al.*, 2020). The level of α-glucosidase inhibitory

compounds in the DCM fraction may be higher than in other fractions. Furthermore, there are a few reports on antidiabetic research on the bulb of *Lilium* species. *In vivo* and *in vitro* studies have demonstrated possible hypoglycemic action in extracts of various *Lilium* species, encompassing *L. lancifolium* and *L. brownii* var. *viridulum* (Zhou *et al.*, 2021).

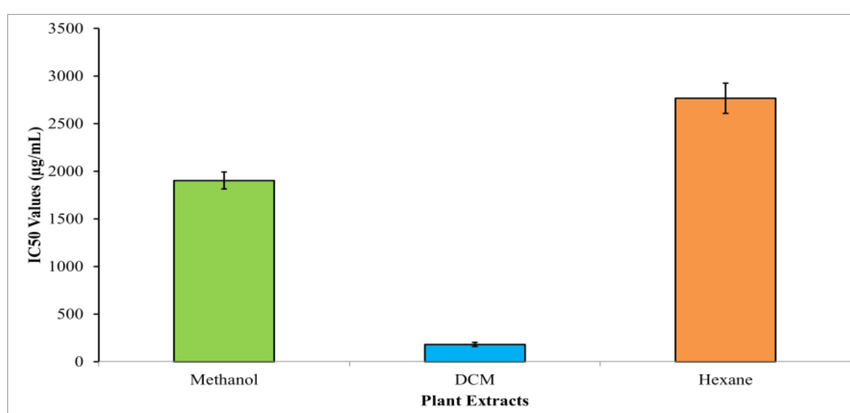

Figure 5. Antidiabetic activity (IC_{50}) of various fractions of *L. nepalense.* *(Note: Error bars represent the standard deviation of three independent measurements).*

The conversion of starch into simple sugars is facilitated by the alpha-glucosidase enzyme. These enzymes help in the breakdown of the carbohydrates and starches that make up our diets into glucose, which is absorbed through the intestines and increases blood sugar levels. In type 2 diabetic patients, α- glucosidase is thought to be one of the potential targets for lowering post-prandial glucose levels. However, α-glucosidase inhibitors prevent the small intestine from absorbing carbohydrates. They block enzymes that change complicated non-absorbable carbohydrates into simple absorbable carbohydrates through competitive inhibition. Numerous phytoconstituents with α-glucosidase inhibitory action have been identified from various plants, including 103 flavonoids, 61 terpenes, 8 steroids, 37 alkaloids, 37 phenols, 49 quinines, 73 phenylpropanoids, 43 additional chemicals (Kumar *et al.*, 2011; Yin *et al.* 2014).

The antioxidant and antidiabetic activities of the tested extracts/plants were found to be positively correlated in this study, meaning that the fraction with stronger antioxidant activity also had better α-glucosidase inhibitory activity. As a result, the extracts' or plants' antioxidant activity may affect how effective they are at preventing diabetes.

Cytotoxicity test by Brine shrimp lethality assay

The Brine shrimp (*Artemia salina*), a simple zoological creature, has been used by many researchers to test the lethality of plant extracts (Fig. 6). It is an easy and affordable bioassay used to assess the preliminary toxicity screenings for plant extracts and for screening different chemical components present in different bioactivities. This study found that no nauplii died below a concentration of 500 μg/mL and that only 4% of nauplii died at a concentration of 1 mg/mL of crude extract after 24 h observation. It can be because the plant extracts have fewer toxic metabolites present in lower concentrations for nauplii. The degree of mortality was discovered to be equivalent to the concentration of the extracts (Table 3).

According to this study, the crude extract of the bulb showed less cytotoxicity on nauplii and had an LC_{50} of 3.83 mg/mL in comparison to the positive control potassium dichromate (LC_{50}=134.34±15.66 μg/mL). Any plant extract with an LC_{50} of less than 1000 μg/mL is regarded as pharmacologically active (Meyer *et al.*, 1982). Moreover, it is a quick and thorough test that uses a large number of organisms for statistical validation, requires no specialized equipment, and only a small amount of material for bioactive compounds of either natural or synthetic origin (Kwon *et al.*, 2007).

Table 3. Brine shrimp lethality test in the crude extract of *L. nepalense.*

Extract concentration (mg/mL)	No. of surviving Nauplii			Average No. of surviving Nauplii	% Mortality	LC_{50} (mg/mL)
	T_1	T_2	T_3			
0.25	25	25	25	25	0	3.83
0.5	25	24	25	25	0	
1.0	25	23	24	24	4	
2.0	20	19	21	20	20	
3.0	14	16	15	15	40	
4.0	13	12	11	12	52	
5.0	9	7	8	8	68	
Negative control	25	25	25	25	0	

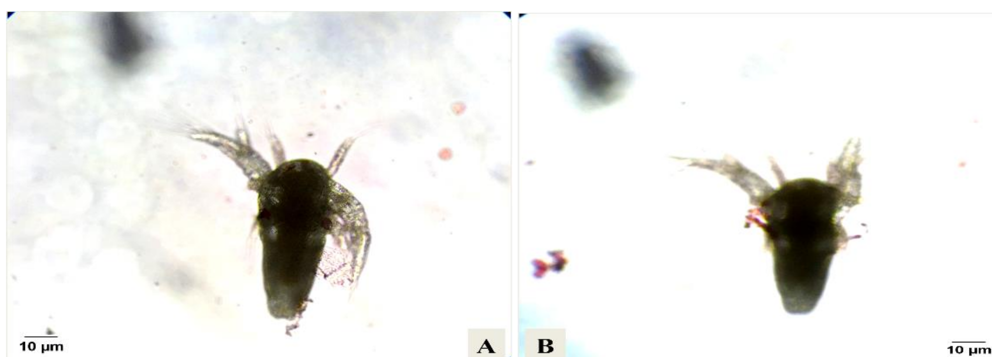

Figure 6. Microscopic images of Brine shrimp larvae (nauplii) after 48 h incubation at 23°C in artificial sea-water solution.

CONCLUSIONS

This study revealed that the DCM fraction of the *L. nepalense* bulb might be used as a source of antioxidants and anti-diabetic agents for therapeutic purposes. Therefore, it might be suitable for consumption and might serve as a source of antioxidant and anti-diabetic agents from the diet. Although, it seems less toxic to Brine shrimp, it might be a good idea to thoroughly explore its anticancer or cytotoxic properties against many types of human cancer cell lines. It is also necessary to explore bioactive compounds responsible for antioxidant, antidiabetic, and cytotoxic activities.

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AUTHORS CONTRIBUTION

Conceptualization: CBT, BP & HDB; Fieldwork: CBT and AB; Laboratory work: CBT & AB; Supervision: HDB & BP; Writing original drafts: CBT; Review and editing: HDB, BP, and KKP.

CONFLICT OF INTEREST

The authors do not have any conflict of interest in this research work.

DATA AVAILABILITY

The raw data used in this work can be obtained upon request from the corresponding author.

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Research Article

Induction, Proliferation and Differentiation of Callus in *Paris polyphylla* Sm. through Leaf Culture

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Abstract

Paris polyphylla Sm. is a vulnerable medicinal plant employed in the treatment of various ailments. This study seeks to establish a protocol for callus induction, proliferation, and differentiation of *P. polyphylla*. Immature leaf explants were cultured on MS medium with varying concentrations of plant growth regulators (PGRs), including 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn), 6-benzylaminopurine (BAP), Thidiazuron (TDZ), α-Naphthalene acetic acid (NAA), and Gibberellic acid (GA_3), along with 10% coconut water. After 12 weeks of primary culture, the optimal callus induction was observed in MS medium supplemented with 0.25 mg/l 2,4-D + 0.5 mg/l Kn. In the secondary culture at 8 weeks, the best callus proliferation, as determined by callus weight or growth index, occurred in MS medium supplemented with 2.0 mg/l BAP alone, 2.0 mg/l Kn alone, 1.0 mg/l TDZ alone, combinations of 2.0 mg/l Kn + 1.0 mg/l BAP + 2.0 mg/l GA_3, and combinations of 0.5 mg/l NAA + 2.0 mg/l BAP + 2.0 mg/l GA_3, as well as 10% coconut water. Furthermore, callus differentiation into mini rhizomes with root primordia was successfully achieved in MS media containing 2.5 mg/l Kn and 10% coconut water. This study reports, for the first time, the formation and differentiation of callus from leaf explants in *P. polyphylla*. Large-scale callus generation from leaf explants has the potential to enhance the production of bioactive secondary metabolites for therapeutic purposes and facilitate the development of plantlets through organogenesis.

Keywords: 2,4-D, Callus, GA_3, Growth index, Primary culture

Introduction

Paris polyphylla Sm., commonly known as Paris root in English, Rhizoma Paridis in Pharmacopoeia, and Satuwa in Nepali, is a vulnerable medicinal plant found in the subtropical to subalpine forests of South Asian countries at altitudes ranging from 1800 to 3500 meters above sea level (IUCN, 2004). This plant is extensively utilized in traditional ethnomedicine, traditional Chinese medicine (TCM), Ayurveda, and Homeopathy for treating a wide array of conditions such as cuts, wounds, burns, fever, anthelmintic, scabies, diarrhoea, dysentery, liver cancer, antipyretic, pain relief, anti-inflammatory, coughing and purgative, breast cancer, fractures, convulsions, and strains, acting as

an antidote, detoxicant, and soothing agent (Long et al., 2003; IUCN, 2004; Li et al., 2012). Steroidal saponin is the primary constituent of *P. polyphylla*, consisting of polyphyllin D, diosgenin, pennogenin, dioscin, and Paris saponin I, II, VI, VII, H, and polyphyllin VII (Lee et al., 2005; Zhang et al., 2014; Chen et al., 2019; Wang et al., 2019; Thapa et al., 2022).

Unfortunately, due to overutilization, habitat loss, and illegal trading for pharmaceutical and conventional medical purposes, the population of *P. polyphylla* is diminishing in its natural habitats. The process of developing callus tissue from plant cells in a nutrient medium with the use of plant growth regulators (PGRs) is known as in-vitro callus induction. The concentration of PGRs in the medium can be altered to guide the callus progression towards root formation, shoot growth, or somatic embryogenesis. Organ primordia corresponding to the callus tissues are then formed after additional cell proliferation and differentiation, contributing to the regeneration of complete plantlets and the production of bioactive compounds using precursors and elicitors. Callus culture is widely employed in both basic research and industrial applications, aiding in the establishment of cell suspensions, protoplast separation, extraction of bioactive compounds, and synthesis of significant pharmaceuticals in sizable quantities (Jhang et al., 1974; Furuya et al., 1983; Pant, 2014).

The induction of callus using various explants in culture is valuable for regenerating complete plants through organogenesis or embryogenesis, as well as for producing essential bioactive compounds. Additionally, the use of precursors and elicitors, along with chemical analysis of callus, contributes to laying the foundation for the production of natural drugs from in vitro-raised callus.

While existing literature discusses plant regeneration from rhizomes and other parts of *P. polyphylla* (Teerawatsakul et al., 2014; Raomai et al., 2014; Raomai et al., 2015; Devi et al., 2017), there is no information regarding callus induction and plant regeneration from the leaf. This study aims to fill this gap by investigating the process of callus induction, proliferation, and differentiation in MS media supplemented with various PGRs at different concentrations and combinations, utilizing leaf explants.

Materials and Methods

Plant material

P. polyphylla plants sourced from the Baglung district in western Nepal were collected in April and May of 2021 and potted. The herbarium specimen was documented, identified, and stored in the Tribhuvan University Central Herbarium (TUCH).

Preparation of explants

Immature leaves were carefully detached from the mother plants and thoroughly cleaned under running tap water for one and a half hours with the addition of a few drops of Tween-20 (Qualigens). This cleaning process was repeated three times using distilled water. The explants underwent surface sterilization with 70% ethanol for 30 seconds, followed by immersion in a solution containing 0.1% mercuric chloride ($HgCl_2$) for 3–4 minutes. To eliminate any $HgCl_2$ residue, the explants were washed three times with sterile distilled water. Using a sterile cork borer on the sterilized leaves, leaf discs (0.8–1.0 cm diameter) were created through gentle pressing.

Preparation of culture medium

Stock solutions were utilized to formulate Murashige and Skoog's (MS) medium (1962) for all leaf explant tissue cultures. The MS media were enriched with varying concentrations of plant growth regulators (PGRs), such as 2,4-dichlorophenoxyacetic acid (2,4-D) (0.25, 0.5, 1.0, 1.5, and 2.0 mg/l) and kinetin (Kn) (0.25, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/l) individually and in combinations. The primary culture included 3% (w/v) sucrose, 10% coconut water (v/v), and 0.8% (w/v) agar. The pH was adjusted to 5.6 before autoclaving. For the secondary culture, Kn, BAP, and TDZ (0.5, 1.0, 1.5, and 2.0 mg/l) were added to MS medium separately, in combination with BAP, NAA, and GA3 (0.5, 1.0, 1.5, and 2.0 mg/l), and in combination with BAP, Kn, and GA_3 (0.5, 1.0, 1.5, and 2.0 mg/l). GA_3 was added at a constant concentration (2.0 mg/l) in MS media. Controls included full, 1/2, and 1/4 strengths of MS media without added PGR supplements and 10% coconut water. Approximately 25 mL of medium were dispensed into 300 mL sterile autoclaved culture jars (78 mm×122 mm) or 15 ml of medium in sterile culture tubes (25 mm×150 mm) for culture

initiation, covered with aluminum foil, and autoclaved at 121°C for 30 minutes under a pressure of 15 lb per square inch.

Culture establishment

Aseptically, small leaf discs (0.8–1.0 cm in diameter) were inoculated onto the MS medium. Following a 12-week primary culture, the regenerated callus was subcultured on MS medium. Calli for subculture were weighed in the airflow chamber to prevent contamination. The culture tubes and dishes were then transferred to the culture room and maintained at 25±2°C with 16-hour photoperiods. The experiment was conducted three times, with each treatment having three replicates. Eight-week-old calli were collected, and their growth index (%) and moisture contents (%) were determined according to Adhikari & Pant (2013) for further analysis.

"The moisture content of callus (%) = (Fresh callus wt. - Dry callus wt.) / Fresh callus wt. × 100"

"(Growth index) Increase in callus wt. (%) = (Fresh callus wt. - Fresh callus explant wt.) / Fresh callus explant wt. × 100"

Statistical analysis

Microsoft Excel 2010 was employed to calculate the percentages of moisture content and callus growth index, along with their average values and standard deviation. Additionally, the two-way ANOVA test was conducted in Microsoft Excel 2010.

Results and Discussion

Callus induction in MS medium

Inoculation of leaf discs onto MS media containing varying concentrations of 2,4-D and Kn (0.25, 0.5, 1.0, 1.5, 2.0, & 3.0 mg/l) along with 10% coconut water resulted in the induction of callus (Figure 1). As stated by Trigiano & Gray (2000), many tissues in plant tissue culture require a specific combination of plant growth regulators (PGRs) to initiate the appropriate growth response. Notably, MS media at full, half, and quarter strength without PGR supplements and 10% coconut water failed to induce callus formation. Similarly, employing different concentrations of Kn alone, 2,4-D alone, and a combination of NAA+Kn in the MS medium did not induce callus in *P. polyphylla*. Soniya and Das (2002) found that kinetin alone in MS medium did not induce callus in *Piper longum* leaf explants, but when enriched with 2,4-D+Kn, callus formation from leaf explants was successful. While kinetin is not always essential, exogenous application of auxin is crucial for initiating callus development (Okazawa et al., 1967). In tissue culture, kinetin is often used to induce callus production (in combination with auxin) and to develop shoots from the callus (with reduced auxin concentrations) (Duszka et al., 2009).

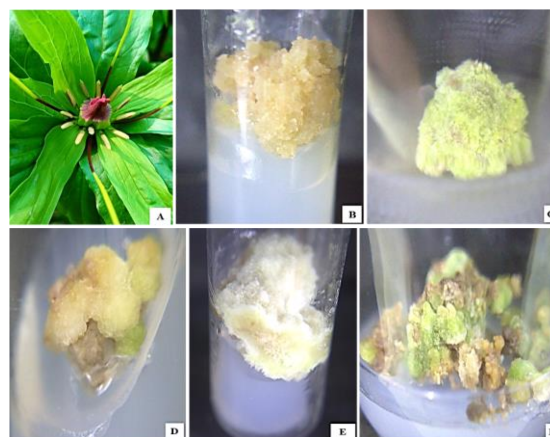

Figure 1: Induction of callus from leaf explant. Plant: *Paris polyphylla* (A), Callus: MS+0.25 mg/l 2,4-D+0.5 mg/l Kn (B), MS +2.0 mg/l Kn (C), MS+1.0 mg/l TDZ (D), MS+2.0 mg/l BAP+0.5 mg/l NAA (E), MS+1.0 mg/l BAP+2.0 mg/l Kn (F).

The most effective callus-inducing media in this study were MS media fortified with 0.25 mg/l 2,4-D+0.5 mg/l Kn (85%), followed by 0.25 mg/l 2,4-D+0.25 mg/l Kn (70%), 0.25 mg/l 2,4-D+1.0 mg/l Kn (60%), 0.25 mg/l 2,4-D+1.5 mg/l Kn (55%), and 0.5 mg/l 2,4-D+0.26 mg/l Kn (50%) after 12 weeks of initial culture (primary culture) (Table 1). The induction of callus was favoured when the concentrations of 2,4-D and Kn were nearly equal or when the Kn concentration exceeded the 2,4-D concentration. In nature, calli form on plants due to wounds, tumour-inducing bacteria (the Ti gene), and genetic tumours (Bhatiya, 2015). However, callus can also be artificially induced using a leaf or another explant and the appropriate nutritive medium. Generally, auxin alone or in combination with cytokinin induces callus formation in various plant species. The ratio of auxin to cytokinin determines callus induction (at an intermediate ratio), root development (at a high auxin to cytokinin ratio), and shoot regeneration (at a high cytokinin to

auxin ratio). In some species, callus induction may occur without auxin or cytokinin, as abscisic acid and brassinosteroid hormones can induce it (Goren et al., 1979; Hu et al., 2000).

This study also revealed that callus induction and growth initially increased, then decreased, as Kn concentration was raised from 0.25 mg/l to 1.0 mg/l in MS media with a constant 0.25 mg/l of 2,4-D (Table 1). Similarly, callus induction and growth decreased as 2,4-D concentrations in MS media increased from 0.25 mg/l to 1.5 mg/l, while Kn concentration remained constant. All calli were compact and light white in the primary culture. The morphology of the callus changed from compact to friable when subcultured in the same concentrations of 2,4-D and Kn. However, when subcultured in cytokinins alone, such as BAP, Kn, and TDZ, the callus morphology shifted from friable to more compact and green. This change may indicate the initiation of organogenesis in the callus. Previous researchers successfully induced callus from leaf segments in MS media fortified with 2,4-D alone, NAA alone, indole-3-butyric acid (IBA) alone, or a combination of 2,4-D+Kn in *Melaleuca alternifolia* (Kiong et al., 2007), a combination of IAA+BAP in *Piper longum* (Sathelly et al., 2016), a combination of 2,4-D+KN in *Piper auritum* (Domínguez et al., 2006), and a combination of NAA+BAP in *Bergenia ciliata* (Shrestha & Pant, 2011). The cumulative data indicate that callus induction from leaf explants in MS media enriched with auxins, either alone or in combination with cytokinins, is genotype-dependent. Additionally, callus induction within a plant species is influenced by factors such as explant type, orientation, plant growth regulators, plant and explant age, medium composition, plant metabolic state, temperature, growing conditions, and source plant type (Klimek-Chodacka et al., 2020).

Table 1: Induction of callus in different concentrations of Kinetin and 2,4-Dichlorophenoxy acetic acid.

Concentration of 2,4-D (mg/l)	**Concentration of Kinetin (mg/l)**					
	0.25	0.5	1.0	1.5	2.0	3.0
0.25	L	L	L	M	M	S
0.5	M	M	M	M	M	-
1.0	M	M	S	S	S	-
1.5	S	S	S	S	-	-
2.0	-	-	-	-	-	-

S=Small quantity (fresh wt. <0.5 g), M=Moderate quantity (fresh wt. 0.5-2.0 g), L=Large quantity (fresh wt. >2.0 g)

Callus proliferation in MS medium

The calli derived from MS medium, containing a combination of 2,4-D+Kn and 10% coconut water, were maintained by subculturing in the same concentration of 2,4-D and Kn. Subsequently, the calli were further subcultured in MS medium supplemented with different concentrations of BAP, Kn and TDZ alone, and in combinations such as NAA+BAP+GA_3, BAP+Kn+GA_3, and 10% coconut water. The proliferation of callus increased with the elevated concentrations of all PGRs, including BAP, Kn and TDZ alone, in the MS medium. Although callus proliferation occurred in all concentrations and combinations of PGRs, the most effective proliferation was observed in MS medium enriched with 2.0 mg/l Kn alone, as evidenced by the calli's fresh weight and dry weight after 8 weeks of subculture (Table 2). At 2.0 mg/l of Kn, there was a remarkable 647.33% increase in callus growth (growth index), with the initial fresh weight of the callus explant at 0.33 g yielding 6.52 g of callus. Similarly, it was observed that callus developed in MS medium in combination with BAP+Kn+GA_3 exhibited a higher growth index compared to callus grown in MS medium in combination with NAA+BAP+GA_3 (Tables 3 and 4). The maximum callus growth index (589.03%) was observed in 1.0 mg/l BAP + 2.0 mg/l Kn + 2.0 mg/l GA_3 from an initial callus explant weight of 0.35 g (Table 4), while the maximum callus growth index (570.58%) was observed in 2.0 mg/l BAP + 0.5 mg/l NAA + 2.0 mg/l GA_3 from an initial callus explant weight of 0.37 (Table 3). This suggests that the synergistic action of two cytokinins, BAP and Kn, played a role in promoting callus proliferation and development.

Furthermore, callus proliferation and development decreased when NAA (auxin) concentrations were increased from 0.5 mg/l to 2.0 mg/l while BAP (cytokinin) concentration remained constant (Table 3). This indicates that a higher concentration of auxins combined with cytokinins is not favourable for callus proliferation. Similarly, when the concentrations of BAP and Kn were increased, the proliferation of calli grown in MS medium supplemented with BAP+Kn+GA_3 increased in terms of fresh weight or growth index but slightly decreased after reaching a concentration of 1.0 mg/l Kn (Table 4). This may be attributed to the saturation concentration of BAP+Kn for callus proliferation.

The callus morphology underwent changes after subcultures, transitioning from slightly compact light white to compact white, compact yellowish, and compact green. Calli produced in combination with cytokinin and auxin (BAP+NAA) exhibited compact light yellow and compact white morphologies, whereas calli in MS media treated with cytokinins, either alone or in combinations, displayed compact light green and compact white morphologies. Genetic variability in the callus, metabolite release, and PGR content in the media could be contributing factors to the alteration of callus morphology following sub-culture. In tissue culture, explants with different genotypes respond diversely to the same growth conditions (Nehara et al., 1989, 1990), and PGRs influence the synthesis of metabolites, organogenesis, and cell development (Liang et al., 1991). The results of the two-way ANOVA test, examining the p-values of plant growth regulators such as BAP, Kn and TDZ alone at different concentrations (0.5, 1.0, 1.5, and 2.0 mg/l) versus callus growth (g) or growth index, demonstrated a significant difference at the <0.005 level ($p = 7.91 \times e{-26}$ and $6.55 \times e{-16}$). This indicates that callus proliferation, in terms of fresh weight (g) or growth index, was dependent on the types of plant growth regulators used and their various concentrations added to the MS medium. The successful induction of callus in leaf explants was achieved with the addition of an organic supplement (10% coconut water) in the MS medium. In MS media treated with 2,4-D+Kn and 10% coconut water, callus induction occurred, while media without 10% coconut water did not induce callus. According to Gnasekaran et al. (2009), coconut water stimulates cell division and growth in culture by acting as a cytokinin-like plant growth regulator and containing various nutritional and hormonal components.

Callus differentiation in MS medium

The callus, initially induced in MS medium enriched with 2,4-D and Kn, underwent subculturing in MS medium containing varying concentrations of Kn (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) along with 10% coconut water. In the presence of 2.5 mg/l Kn+10% coconut water, mini rhizomes with root primordia were successfully formed, and subsequent adventitious roots developed from these mini rhizomes (Figure 2). Kinetin, as a synthetic plant growth regulator, primarily facilitates shoot initiation and development. While rooting in the callus may have been initiated by the application of exogenous auxins (2,4-D) in combination with kinetin and 10% coconut water during the initial callus induction from the leaf explant. In tissue culture, the synthetic auxin 2,4-D is commonly used to stimulate somatic embryogenesis, but it is rarely employed commercially for root induction. Kinetin's role in supporting callus development suggests that the exogenous auxin-induced activation of callus growth is likely assisted by the inclusion of kinetin in the medium. Cytokinins like kinetin can induce the differentiation and regeneration of axillary buds (Le Bris, 2017). For roots to form in culture, the medium must contain a relatively low amount of auxin (Okazawa et al., 1967).

Table 2: Independent impacts of BAP, Kn, & TDZ on the proliferation and growth of callus following secondary culture.

BAP (mg/l)	Kn (mg/l)	TDZ (mg/l)	Callus explant fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5			0.33±0.03	1.19±0.14 CW	0.38±0.04	91.19
1.0			0.30±0.07	2.42±0.23 CW	0.88±0.12	229.65
1.5			0.30±0.01	3.71±0.13 CY	1.54±0.06	362.67
2.0			0.33±0.04	4.57±0.26 CG	2.10±0.16	449.95
	0.5		0.32±0.04	2.56±0.38 CY	0.79±0.09	243.98
	1.0		0.35±0.03	3.76±0.17 CW	1.30±0.06	366.98
	1.5		0.31±0.03	5.24±0.28 CG	2.15±0.08	518.42
	2.0		0.33±0.04	6.52±0.35 CG	2.96±0.15	647.33
		0.5	0.34±0.06	3.55±0.17 CW	1.12±0.04	345.22
		1.0	0.31±0.06	5.88±0.01 CG	2.83±0.09	582.30
		1.5	0.32±0.04	4.33±0.38 CG	1.04±0.16	425.77
		2.0	0.37±0.05	3.44±0.18 CY	0.83±0.07	334.77

CW: compact light white, CG: compact light green, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Table 3: Cumulative impacts of BAP+NAA+GA_3 on the proliferation and growth of callus following secondary culture (2.0 mg/l GA_3).

BAP (mg/l)	NAA (mg/l)	Callus explants fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5	0.5	0.33±0.07	1.96±0.67 CY	0.71±0.26	179.68
1.0	0.5	0.37±0.08	2.53±0.11 CW	0.98±0.01	238.59
1.5	0.5	0.36±0.06	2.85±0.07 CW	1.31±0.01	273.08
2.0	0.5	0.36±0.06	5.76±0.18 CW	2.74±0.05	570.58
0.5	1.0	0.34±0.06	2.69±0.22 CW	0.99±0.10	256.41
1.0	1.0	0.35±0.05	3.42±0.29 CY	1.47±0.02	332.54
1.5	1.0	0.37±0.04	3.73±0.10 CY	1.71±0.01	363.87
2.0	1.0	0.35±0.07	4.66±0.15 CW	2.33±0.10	458.68
0.5	1.5	0.39±0.03	1.30±0.06 CY	0.46±0.03	100.32
1.0	1.5	0.36±0.07	2.54±0.09 CW	1.01±0.01	240.66
1.5	1.5	0.37±0.03	2.79±0.02 CY	1.28±0.03	266.36
2.0	1.5	0.33±0.03	3.28±0.15 CY	1.38±0.27	318.41
0.5	2.0	0.35±0.03	1.01±0.11 CY	0.37±0.03	67.15
1.0	2.0	0.37±0.04	1.44±0.10 CY	0.56±0.05	118.55
1.5	2.0	0.35±0.03	2.43±0.10 CW	1.12±0.06	229.52
2.0	2.0	0.36±0.06	2.74±0.11 CW	1.30±0.08	261.13

CW: compact light white, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Table 4: Cumulative impacts of BAP+Kn+GA_3 on the proliferation and growth of callus following secondary culture (2.0 mg/l GA_3).

BAP (mg/l)	Kn (mg/l)	Callus explants fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5	0.5	0.37±0.04	2.09±0.13 CW	0.75±0.05	191.27
1.0	0.5	0.39±0.04	2.33±0.05 CW	0.93±0.03	216.46
1.5	0.5	0.33±0.05	2.40±0.02 CG	0.98±0.01	226.65
2.0	0.5	0.39±0.03	2.52±0.04 CG	1.26±0.02	236.33
0.5	1.0	0.37±0.08	2.62±0.04 CW	0.94±0.01	247.59
1.0	1.0	0.33±0.04	2.74±0.05 CY	1.13±0.02	262.73
1.5	1.0	0.35±0.05	2.82±0.05 CG	1.21±0.02	270.31
2.0	1.0	0.32±0.07	2.89±0.01 CG	1.33±0.02	278.59
0.5	1.5	0.35±0.07	3.21±0.03 CW	1.14±0.02	310.98
1.0	1.5	0.36±0.06	3.34±0.04 CY	1.35±0.04	323.26
1.5	1.5	0.36±0.06	3.59±0.01 CG	1.67±0.05	348.95
2.0	1.5	0.34±0.03	3.67±0.03 CY	1.81±0.02	358.19
0.5	2.0	0.45±0.20	5.18±0.06 CG	1.79±0.02	509.48
1.0	2.0	0.35±0.08	5.94±0.04 CG	2.84±0.13	589.03
1.5	2.0	0.36±0.05	5.80±0.01 CG	2.61±0.01	573.96
2.0	2.0	0.34±0.05	5.74±0.09 CY	2.74±0.17	568.63

CW: compact light white, CG: compact light green, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Figure 2: Various stages of mini rhizomes and root initiation in MS media supplemented with 2.5 mg/l Kn from callus.

Conclusion

In MS medium supplemented with 2,4-D+Kn and 10% coconut water, callus formation can be initiated from leaf segments. The most effective plant growth regulators for callus proliferation in MS medium are kinetin and the combination of BAP+Kn+GA_3, surpassing BAP and TDZ alone, and the combination of BAP+NAA+GA_3. Kinetin alone also supports the differentiation of callus into mini rhizomes with adventitious roots. While increased concentrations of BAP, TDZ and Kn alone, as well as the combination of BAP+Kn+GA_3 in MS medium, elevate callus proliferation, no distinct pattern of proliferation is observed following subculture in MS medium with NAA+BAP+GA_3. The protocol devised for in-vitro callus induction and growth from leaf segments, utilizing elicitors and precursors, holds promise for the synthesis and enhancement of crucial bioactive compounds. Furthermore, the callus can serve as a source for organogenesis and somatic embryogenesis, facilitating plant regeneration.

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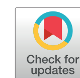
Review article

Bioactive secondary metabolites in *Paris polyphylla* Sm. and their biological activities: A review



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ABSTRACT

Paris polyphylla Sm. is an important medicinal plant used to treat a variety of diseases through traditional medicine systems such as Ayurveda, Tibetan traditional medicines, Chinese traditional medicines, and others around the world. The IUCN red list has designated it as "vulnerable" due to a decline in wild population by over-exploitation, habitat degradation, illegal collection for trade and traditional use. This review paper aims to summarize the bioactive secondary metabolites in *Paris polyphylla*. Paris saponins or steroidal saponins are the main bioactive chemical constituents from this plant that account for more than 80% of the total compounds. For instance, polyphyllin D, diosgenin, paris saponins I, II, VI, VII, and H are steroidal saponins having anticancer activity comparable to synthetic anticancer medicines. Antioxidant, anticancer, anti-leishmaniasis, antibacterial, antifungal, anthelmintic, antityrosinase, and antiviral effects of extracts and pure compounds were also demonstrated *in vivo* and *in vitro*. In conclusion, this review summarizes the bioactive components from the *P. polyphylla* which will be useful to researchers and scientists, and for the development of potential drugs.

1. Introduction

Previously, the genus *Paris* was assigned to the Liliaceae and Trilliaceae families, however in the APG III system, it is assigned to the Melanthiaceae family. *Paris* includes roughly 24 species found across the world, from Europe to Asia (Zhang et al., 2011). Except for the European *P. quadrifolia* and the Caucasian *P. incompleta*, practically all of the 24 species are restricted to East Asia (19 species in China) (Ji et al., 2006). *Paris* has 27 species globally, including 22 species and 12 endemic species in China (Cunningham et al., 2018); 33 species, and 15 varieties in Southwest China (Ding et al., 2021). The World Checklist of Selected Plant Families (WCSP) listed 32 *Paris* species and 8 varieties of *P. polyphylla* in 2020. *P. polyphylla* has four subspecies and one variety in Nepal (www.eFloras.org, 2/4/2021). The Department of Plant Resources, Government of Nepal (DPR, 2017) has classified *P. polyphylla* as a "medicinal plant prioritized for agrotechnology development". It is distributed from sub-tropical to sub-alpine regions in various parts of the world (IUCN, 2004; Cunningham et al., 2018). In Nepal, it is distributed within an altitudinal range of 1500–3500 m from west to east (IUCN, 2004; Kunwar et al., 2020). It is known as ‘Satuwa’ in Nepali, ‘Paris root’ in English, and ‘Haimavati’ in Sanskrit. The rhizomes are used in traditional medicine known as ‘Rhizoma Paridis’ in Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2015).

Paris polyphylla (Figure 1) flourishes on thickets, grassy or rocky slopes of damp, humus, nitrogen and phosphorus rich soil under the canopy of the forest (Paul et al., 2015; K.C. et al., 2010; Deb et al., 2015). It grows in undisturbed areas with a canopy cover of more than 80% (Deb et al., 2015). The wild population of *P. polyphylla* is declining due to habitat destruction, deforestation, over-exploitation, illegal collection and harvesting, and has listed as "vulnerable" in the IUCN Red List of Threatened Species (Chauhan, 2020). Overharvesting mainly during the season earlier than seed maturation may result in infrequent seed formation and germination that appears to be a severe threat to plant regeneration (Negi et al., 2014).

The rhizome and other parts of *P. polyphylla* in the form of infusions, juices, powders and pastes have been used in the traditional medicine to treat cuts, wounds, blisters, scabies, rashes or itching, burns, sprain, headache, fever, anthelmintic, vermifuge, expectorant, antispasmodic,

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Figure 1. Photographs of aerial parts and rhizomes of *P. polyphylla*.

digestive, gastritis, diarrhoea, dysentery, menstruation pain, tonic, antidote of poison (aconite poisoning), antidote of poisonous insects and snake, antiseptic, jaundice, vasoconstriction in the kidney, vasodilation in spleen and limbs (Liang, 2000; Rajbhandari, 2001; Manandhar, 2002; DOA, 2003; IUCN, 2004; Bhattarai et al., 2006; Kunwar et al., 2006; Baral and Kurmi, 2006; Dutta, 2007; K.C. et al., 2010; Acharya, 2012; Jamir et al., 2012; Li et al., 2012; Shah et al., 2012; Luitel and Pathak, 2013; Lamichhane et al., 2014; Deb et al., 2015). *P. polyphylla* is widely used in traditional Chinese medicine (TCM) for the treatment of boils, venomous snake bites, carbuncles, sore throat, and traumatic discomfort (Chinese Pharmacopoeia Commission, 2015). The main raw material for 'Yunnan Baiyao' and 'Gong Xue Ning (GXN) capsule' is a rhizome of this plant. Back discomfort, bleeding, shattered bones, wound healing, pain, fungal illnesses, poisonous snakes or bugs bites, skin allergy, tumours, and a variety of disease conditions are treated with the 'Yunnan Baiyao' (Long et al., 2003). GXN capsules were developed in China using the saponin extract of *P. polyphylla* var. *yunnanensis* to treat abnormal uterine bleeding (Zhao and Shi, 2005; Guo et al., 2008). It is also a source for "Jidesheng Sheyaopian" a Chinese patent medicine. The objective of this review paper is to summarize the biological activities of the components of *P. polyphylla*.

2. Method

Research articles published between 1990 and 2021 on secondary metabolites and their biological activities of *Paris polyphylla* were accessed through Google Scholar, PubMed and ProQuest using phrases "*Paris polyphylla* secondary metabolites", "anticancer activity of *Paris polyphylla*", "antimicrobial activity of *Paris polyphylla*", "antioxidant activity of *Paris polyphylla*" and "anthelmintic activity of *Paris polyphylla*". This review does not include articles from conference proceedings and those written in languages other than English.

3. Bioactive compounds of *Paris polyphylla*

Terpenes, alkaloids, glycosides, phenolics, volatile oils, terpenoids, saponins, steroids and resins are active secondary metabolites found in medicinal and aromatic plants (MAPs) (Dubey, 1993; Ramawat and Goyal, 2004). Secondary metabolites of MAPs are used in drugs, perfumes, agrochemicals, flavouring agents and pigments (Ramawat and Goyal, 2004; Chawla, 2014). The existence of secondary metabolites in MAPs confers therapeutic properties, the majority of which likely originated as chemical defences against predation or infection. Because of the structural diversity of secondary metabolites and the wide spectrum of pharmacological activity, MAPs are regarded as excellent sources of novel pharmaceutical medicines (Pant, 2014).

Various compounds have been isolated and characterized from the rhizomes, roots, aerial stem and leaves of *P. polyphylla* including steroidal saponins (Buckingham, 1994; Wang et al., 2005; Devkota et al., 2007; Xiao et al., 2009; Kang et al., 2012; Wu et al., 2012a; Li et al. 2012, 2013), flavonoid glycosides (Chen et al., 1995; Kang et al., 2012; Wu et al., 2012a), sterols (Chen et al., 1990; Wu et al., 2012a), triterpenoid saponins (Wu et al., 2012b) and polysaccharides (Zhou and Yang, 2003). From 1960 to 2010, more than 90 components were isolated, including steroidal saponins, phytosterols, flavones, and phytoecdysones (Zhang et al., 2011), and about 67 steroidal saponins were isolated from 11 species of the genus *Paris* (Huang et al., 2009). Till 2020, around 320 chemical components have been isolated, including steroidal saponins, C-21 steroids, phytosterols, insect hormones, pentacyclic triterpenes, flavonoids, and other chemical substances (Ding et al., 2021). More than 50 paris saponins have been identified from *P. polyphylla* var. *yunnanensis* (Chinese Pharmacopoeia Commission, 2015), however, only four paris saponins; paris saponins I, II, VI and VII have been officially recognized as quality standard components of the Chinese Pharmacopoeia (Qin et al., 2018). Saponins are a type of glycoside consists of aglycones (water--insoluble) such as steroids or triterpenoids, as well as one or more sugar chains (water-soluble) such as glucose, galactose, pentose, or methyl pentose. Saponins have their aglycons constituents which are mainly diosgenin, pennogenin, 24-hydroxy pennogenin, 27-hydroxy pennogenin, 23, 27-dihydroxy pennogenin, 25S-isonuatigenin, nuatigenin, and C-21 steroidal saponins. Saponins in plants have diverse structures due to the presence of different sugars at different locations and orientations. Antitumor, anti-oxidative characteristics, expectorants, inhibition of platelet aggregation, insecticidal, antidiabetic, antifungal/anti-yeast, antiparasitic, antibacterial, antihyperlipidemic, and anti-inflammatory qualities are just a few of the therapeutic applications of steroidal saponins (Sparg et al., 2004). Structures of some of the main compounds are represented in Figure 2.

Wang et al. (2005) isolated two new and six known compounds from the rhizome of *P. polyphylla*, including falcarindiol, β-ecdysterone, pennogenin-3-O-α-L-arabinofuranosyl (1→4)-β-D-glucopyranoside, pennogenin-3-O-α-L-arabinofuranosyl (1→4)-[α-L-rhamnopyranosyl (1→2)]-β-D-glucopyranoside, diosgenin-3-O-β-D-glucopyranoside, diosgenyl-3-O-α-L- rhamnopyranosyl (1→4)-β-D-glucopyranoside, diosgenin-3-O-α-L-

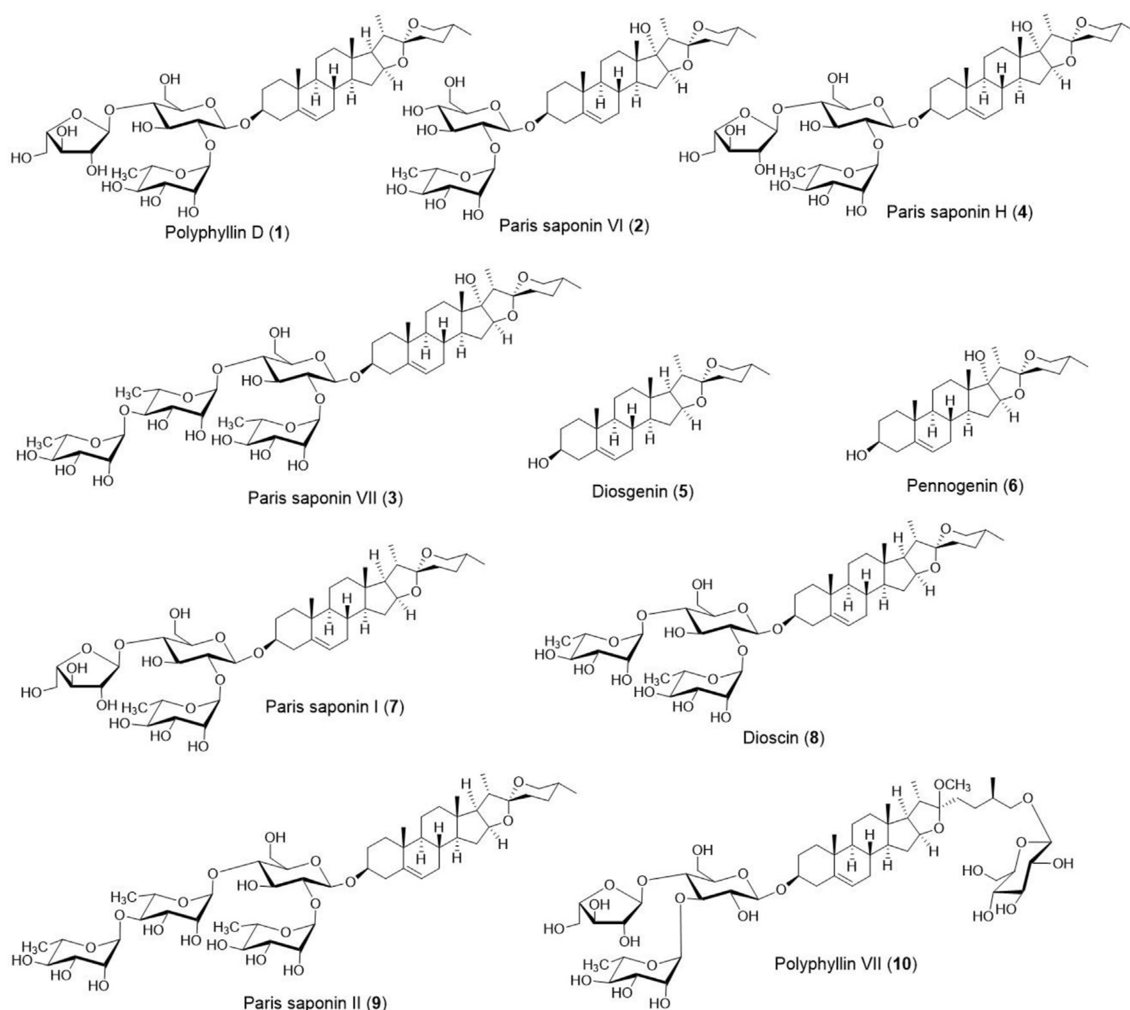

Figure 2. Structure of the major compounds of *Paris polyphylla*.

rhamnopyranosyl (1→2)-β-D-glucopyranoside, & diosgenin-3-O -α-L-rham nopyranosyl (1→4)-[α-L-rhamnopyra nosyl (1→2)]-β-D-glucopyranoside. Devkota et al. (2007) isolated four known compounds from the rhizomes of *P. polyphylla* collected from Parbat district, Nepal, viz: przewalskinone B, polyphyllin C, polyphyllin D and dioscin. Xiao et al. (2009) isolated five paris saponins: paris saponin I (PSI), paris saponin V (PSV), paris saponin VI (PSVI), paris saponin VII (PSVII) and paris saponin H (PSH). The rhizome contains pariphyllin A, pariphyllin B, paristerone, polyphyllin D, and trillin (Buckingham, 1994). Li et al. (2013) also isolated nine steroidal saponins, viz. PSI, PSII, PSV, PSVI, PSVII, PSH, dioscin, gracillin and PGRR from the rhizome of *P. polyphylla*. Kang et al. (2012) isolated three new steroidal saponins; parisyunnanosides G−J, and three known compounds; padelaoside B, pinnatasterone and 20-hydroxyecdyson from the rhizomes of *P. polyphylla* var. *yunnanensis*. According to Li et al. (2012), *P. polyphylla* is an important medicinal plant containing saponin steroids polyphyllin D, dioscin and balanitin-7.

Wu et al. (2012a) isolated eighteen steroidal saponins and sterol from the rhizome of *P. polyphylla*, viz. pariposide A, pariposide B, pariposide C, pariposide D, pariposide E, pariposide F, (3β,25R)-spirost-5-en-3-ol 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyrano side, (3β,25R)-spirost-5-en-3-ol3-O-β-Dglucopyranosyl-(1→6)-glucopyranoside, (3β,25R)-spirost-5-en-3-ol3-O-β-D-glucopyranosyl-(1→6)-[α-L-rhamnopyranosyl-(1 →2)]-β-D-gluco pyranoside, (3β,25R)-spirost-5-en-3-ol-3-O-αyrano syl-(1→4)-α-L-rhamnopy ranosyl-(1→4)-β-D-glucopyranoside, (3β, 25R)-spirost-5-en-3-ol-3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyr anosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, (3β, 17α, 25R)-spirost-5-ene-3,17-diol 3-O-β-D-glucopyranoside, (3β, 17α,25R)-spirost-5-ene-3,17-diol-3-O-α-L-rhamnopyranosyl-(1→2)-β-D glucopyranoside, (3β,17α,25R)-spirost-5-ene-3,17-diol-3-O-α-L-arabino furanosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopy ranoside, (3β, 22E)-stigmasterol-5,22-dien 3-O-β-D-gluco pyranoside, β-daucosterol, 24-epi-pinnatasterone and 20-hydroxyecdyson. Wu et al. (2012b) also isolated six new oleanane-type triterpenoid saponins from the rhizome of *P. polyphylla*; paritrisides A-F along with nine known triterpenoid saponins; paritriside A, paritriside B, paritriside C, paritriside D, paritriside E, paritriside F, 3β-hydroxyoleane-12-en-28-oicacid3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranoside, 3β-hydroxyoleane-12-en-28-oicacid 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-xylopyr anoside, 3β-hydroxyoleane-12-en-28-oic acid 3-*O*-α-L-arabinopyranoside, 3β-hydroxyoleane-12-en-28-oic acid 3-*O*-β-D-xylopyranoside, 3β-hydroxyoleane-12-en-28-oic acid 3-*O*-β-D-glucuronide, 3β-hydroxyoleane-12-en-28-oicacid 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, 3β-hydroxyoleane-12-en-28-oic acid 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, 3β,23-dihydroxyoleane-12-en-28-oic acid 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranoside, and 3β,23-dihydroxyoleane-12-en-28-oic acid3-*O*-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranoside.

4. Biological activities of the secondary metabolites

Chemical components of *P. polyphylla* have anticancer, antioxidant, anti-leishmanial, anthelmintic, antibacterial, antifungal, anti-gynaecological disease, antiviral, and antityrosinase properties (Tables 1 and 2). The biological activity of components was examined

Table 1. Biological activity of some important isolated compounds of *Paris polyphylla*.

Compound name	Biological activity	Reference
Polyphyllin D (**1**)	**Breast cancer:** *In vitro*: Induced apoptosis in estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-231 cells with IC_{50} of 5 μM and μ2.5 M, respectively. *In vivo*: Reduced tumour growth by 50% in nude mice carrying MCF-7 cells at 2.73 mg/kg body weight.	Lee et al. (2005)
	Ovary cancer: *In vitro*: Anti-proliferative effects against SKOV3, A2780CP, A2780S, M41, M41-R, TYKNU, TYKNU-R, OVCAR8, HAYA8, OVCAR5, MCAS, PEO1, IGR-OV1, IMCC5, OVCAR2, OVCA420, OVCA432, OVCA433, TOV-112D cell lines with IC_{50} ranging from 0.2 to 1.4 μM.	AlSawah et al. (2015)
	Leukaemia: *In vitro*: Induced apoptosis in the human erythroleukemia cell line (K562) and peripheral blood mononuclear cells (PBMC) with an IC_{50} of 0.8 ± 0.1 μM.	Yang et al. (2016)
	Anthelmintic activity: *In vivo*: Inhibited the activity of *Dactylogyrus intermedius* (a freshwater fish ectoparasite) with EC_{50} of 0.70 mg/L, which was higher than of the mebendazole (EC_{50} = 1.25 mg/L).	Wang et al. (2010)
	Leukaemia: *In vitro*: Induced apoptosis in drug-resistant K562/A02 human leukaemia cells, with an IC_{50} of 0.9 μM in K562 cells and 0.8 μM in K562/A02 cells, respectively.	Wu et al. (2013)
	Hepatocellular cancer: *In vitro*: Induced apoptosis in the HepG2 and R-HepG2 liver cancer cell lines with the IC_{50} of 7μM and 5μM, respectively, compared to Cisplatin (50μM and 167μM) and Taxol (20μM and 50μM).	Cheung et al. (2005)
	Brain tumour: *In vitro*: Induced apoptosis in U87 human glioma cells with an IC_{50} of 4.94×10^{-5} M.	Yu et al. (2014)
	Antiangiogenesis in the tumour: *In vitro*: Decreased endothelial cell migration and capillary tube formation at 0.3 μM and 0.4 μM in a human microvascular endothelial cell line (HMEC-1HMEC-1 cells). *In vivo*: Induced 70% abnormalities in intersegmental vessel formation (ISV) in zebrafish embryos at doses of 0.156 μM and 0.313 μM.	Chan et al. (2011)
Paris saponin VI (PSVI) (**2**)	**Hepatocellular toxicity:** *In vitro*: Induced apoptosis in HL-7702 and HepaRG cell lines with IC_{50}s of 8.18 μM and 6.65 μM, respectively.	Wang et al. (2019)
	Lung cancer: *In vitro*: PSVI triggered apoptosis in lung cancer cells (A549 and NCI–H1299) with IC_{50} of 4.53 ± 0.56 μM in A549 cells and 5.46± 0.45 in NCI–H1299 cells after 48 h. *In vivo*: In nude mice bearing A549 tumour xenografts, tumour inhibitory rates of PSVI in A549 cells were 25.74%, 34.62.71%, and 40.43% at 2, 3, and 4 mg/kg, respectively.	Lin et al. (2015)
	Brain cancer: *In vitro*: PSVI induced apoptosis in glioma cell lines (U251, U343, LN229, U87, and HEB) with IC_{50} value of 3.65 ± 0.428 μM in LN229 cells, 5.00 ± 0.372 μM in U87 cells, 5.13 ± 0.528 μM in U251 cells, and 3.99 ± 0.397 μM in U343 cells after 24 h. After treatment with PSVI, normal HEB cells showed only minor cytotoxicity.	Liu et al. (2020)
Paris saponin VII (PSVII) (**3**)	**Human cervical cancer:** *In vitro*: Induced apoptosis in human cervical carcinoma Hela cells with an IC_{50} of 2.62 ± 0.11 μM, When cells were exposed to 0.8, 1.6, and 2.4 μM of PSVII for 24 h, the proportion of apoptotic cells was 10.50%, 17.37%, and 38.60%, respectively.	Zhang et al. (2014)
	Ovary cancer: *In vivo*: Inhibited the growth of SKOV3/DDP cells, increased caspase-3 5.71 times and 11.06 times, and reduced Bcl-2 expression 33.3% and 61.1% in 48 and 24-hour groups of PSVII (50 μM/L and 100 μM/L), respectively. Silica nanocomposite also inhibited the growth of SKOV3/DDP cells.	Yang et al., (2015b)
	Hepatocellular toxicity: *In vivo*: Induced apoptosis in HL-7702 and HepaRG cells with IC_{50}s of 0.80 and 2.75 μM, respectively.	Wang et al. (2019)
	Liver cancer: *In vivo*: HepG2/ADR cells and HepG2 cells treated for 48 h with PSVII (0.88, 1.32, 1.98, and 2.97 μM) had a higher apoptosis rate or a lower ADR (a chemotherapy drug) IC_{50}. Those treated with PS VII (≤1.98 μM) and ADR (5 nM) showed increased ADR accumulation, decreased drug-resistant gene expressions, and increased cell apoptosis.	Tang et al. (2019)
Paris saponin H (PSH) (**4**)	**Hepatocellular carcinoma:** *In vitro*: Lowered cell viability in PLC/PRF/5 and Huh7 cells at 1.25–20 μM, increased apoptosis at 1.25 μM, and elevated caspase-3 at 2.5, 5.0, and 10 μM. *In vivo*: Inhibited tumour growth in hepatocellular carcinoma (HCC) xenograft model of nude mice, at doses of 5 mg/kg and 10 mg/kg of PSH	Chen et al. (2019)
Diosgenin (**5**)	**Anthelmintic activity**: *In vivo*: Inhibited the activity of *Dactylogyrus intermedius* with EC_{50} of 0.44 mg/L. It was more efficacious than mebendazole (EC_{50} = 1.25 mg/L).	Wang et al. (2010)
	Lung cancer: *In vitro*: Induced apoptosis in the lung adenocarcinoma cell line (LA795) from mice with an IC_{50} of 149.75 ± 10.43 μM/L after 24 h. *In vivo*: Decreased tumour growth in T739 mice with LA795 lung adenocarcinoma by 33.94% with oral treatment, but Cyclophosphamide (a chemotherapy drug) decreased tumour development by 56.09%.	Yan et al. (2009)
Pennogenin (**6**)	**Hepatocellular cancer:** *In vitro*: Inhibited the growth of HepG2 cells with IC_{50} values from 9.7 μM to 13.5 μM. **Antifungal activity:** *In vitro*: Inhibited the growth of *Saccharomyces cerevisae hansen* with MIC values from 0.6 mg/mL to 2.5 mg/mL. The MIC values of the compound against *Candida albicans* were from 0.6 mg/mL to 1.2 mg/mL.	Zhu et al. (2011)
Paris saponin I (PSI) (**7**)	**Lung cancer:** *In vitro*: PSI combined with hyperthermia at 43 °C induced apoptosis on a non-small cell lung cancer (NSCLC) PC 9 cell line with IC_{50} of 1.21 μg/mL. When compared to the PSI alone, the percentage of cells in the G2/M phase arrest increased from 33.59 to 42.58%.	Zhao et al. (2015)
	Human gastric cancer: *In vitro*: PSI sensitized the human gastric cancer cell line (SGC-7901) to the cisplatin with minimal damage. PSI had an IC_{50} of 1.12 μg/mL in SGC-7901 cell lines after 48 h at 0.2–6.4 μg/mL. Cisplatin had an IC_{50} of 30.4 μM in SGC-7901 cell lines after 48 h at 1–64 μM concentration. The IC_{50} of Cisplatin was reduced to 20.3 μM when it was coupled with PSI (0.3 μg/mL).	Song et al. (2016)
	Ovarian cancer: *In vitro*: PSI induced apoptosis in SKOV3 cells with an IC_{50} of 15 μM/L & in a mouse model of human ovarian cancer. *In vivo*: In a subcutaneous xenograft mouse model, PSI treatment at 15 and 25 mg/kg inhibited the growth of SKOV3 cells by 38 and 66%, respectively.	Xiao et al. (2009)
	Hepatocellular toxicity: *In vitro*: PSI induced apoptosis in HL-7702 and HepaRG cells, with IC_{50}s of 0.84 and 4.66 μM, respectively, at 24 h.	Wang et al. (2019)
	Lung cancer: *In vitro*: PSI triggered apoptosis in the gefitinib-resistant non-small cell lung cancer (NSCLC) cell line PC 9 ZD with IC_{50}s of 2.51, 2.07, and 1.53 μg/mL after 24, 48, and 72 h of incubation.	Jiang et al., (2014a)

(*continued on next page*)

Table 1 (*continued*)

Compound name	Biological activity	Reference
	In vivo: The 18F fludeoxyglucose microPET scan for glucose metabolic activity in tumours in xenograft nude mice revealed a lower tumour SUV in the PSI treatment groups compared to the control group.	
	Lung cancer: *In vitro*: With an IC_{50} of 2.5132 μg/mL, PSI reduced the proliferation of gefitinib-resistant lung cancer cell line (PC9ZD cells) over 24 h.	Jiang et al., (2014b)
	Lung cancer: *In vitro*: PSI reduced the proliferation of three non-small cell lung cancer (NSCLC) cells (H1299, H520, H460) and one small cell lung cancer (SCLC) cell (H446). PSI at 4 mM caused early-stage apoptosis in H1299 and H520 cells, with the latter reaching a high of 73.54 ± 3.44%. However, at 4 mM, the H446 cells went into late-stage apoptosis.	Liu et al. (2016)
	Liver cancer: *In vivo and in vitro*: PSI reduced vasculogenic mimicry (VM) production in hepatocellular carcinoma (HCC) cell lines (SMMC7721, PLC, HepG2, Hep3B, and Bel7402), as well as transplanted hepatocellular carcinoma cells. Patients with HCC who were given PSI before surgery had lower microvessel density (MVD) and VM than those who were not.	Xiao et al. (2018)
	Liver cancer: *In vitro*: PSI (at 0.5–2 μg/mL) sensitized HepG2 cells to cisplatin-induced cytotoxicity after 24 h of treatment with 0.2–100 μM cisplatin.	Han et al. (2015)
	Bone tumour: *In vitro*: PSI induced apoptosis at 0–2.5 μM in MG-63, Saos-2, and U-2 OS human osteosarcoma cells.	Chang et al. (2015)
	Lung cancer: *In vitro*: PSI caused apoptosis in the cisplatin-resistant human non-small cell lung cancer cell line (A549/DDP) with an IC_{50} of 1.54 ± 0.26 μM/mL in the A549 and 1.08 ± 0.20 μM/mL in the A549/DDP cell lines.	Feng et al. (2019)
Dioscin (**8**)	**Anthelmintic activity:** *In vivo*: Dioscin had a substantial EC_{50} of 0.44 mg/L against *Dactylogyrus intermedius* (a freshwater fish ectoparasite), which was higher than the mebendazole (EC_{50} = 1.25 mg/L).	Wang et al. (2010)
Paris saponin II (PSII) (**9**)	**Lung cancer:** *In vitro*: PSII promoted apoptosis in human lung cancer cells (NCI–H460 and A549) as soon as 2 h after 1 μM treatment, but did not affect normal human pulmonary epithelial cells (BEAS-2B). The production of cytoplasmic acidic vesicular organelles (AVOs) was reduced and apoptosis was promoted in NCI–H460 cells treated with 1 μM PSII in the presence or absence of 10 mM CQ over 24 h.	Zhang et al., (2016a,b)
	Hepatocellular toxicity: *In vitro*: PSII induced apoptosis in HL-7702 and HepaRG cells, with IC_{50}s of 1.88 and 3.74 μM, respectively.	Wang et al. (2019)
	Ovary cancer: *In vivo*: PSII induced apoptosis in human ovarian cancer cells (OC SKOV3 and OC HOC-7) with lower IC_{50}s of 7.17 μM and 6.44 μM, respectively, when compared to VP16 (chemotherapy drug) with higher IC_{50}s of 14.67 μM and 6.44 μM, respectively. *In vivo*: PSII inhibited primary human umbilical vascular endothelial cells (HUVEC) proliferation, angiogenesis of rat aortic rings, tumour growth, and angiogenesis in an ovarian cancer tumour xenograft mouse model.	Xiao et al.(2014)
	Ovary cancer: *In vitro*: PSII inhibited more human ovary cancer SKOV3 cell proliferation than VP16-etoposide (a chemotherapy drug) treatment at the same dose and time point, with lower IC_{50}s (20.99, 10.44, 8.83, and 6.98 μM, days 1–4, respectively) than VP16 (82.04, 17.18, 11.80, and 8.01 μM, days 1–4). *In vivo*: In a xenograft mouse model of ovarian cancer, the combination of PSII therapy and constitutive inhibition of IκBα activity inhibited the development of human ovarian cancer cells significantly.	Yang et al., (2015a)
	Colorectal cancer: *In vitro*: PSII induced apoptosis in human colorectal cancer cell lines (HT 29 and HCT 116) with an IC_{50} of 1.89 μM in HT 29 cells and 2.43 μM in HCT 116 cells, respectively. PSII, on the other hand, showed an IC_{50} of 18.96 μM in human colonic epithelial cells (HcoEpiC), about 10 times higher than in colon cancer cells.	Chen et al. (2018)
	Ovarian cancer cells: *In vitro*: PSII had a 90.0% inhibition index after 7 days of therapy at 10 μM, compared to PSI (80.3%) and the etoposide (69.2%) in the human ovarian cancer cell line (SKOV3). On PS II-treated SKOV3 cells, the IC_{50} and total growth-inhibiting concentration (TGI) were 2.4 μM and 6.3 μM, respectively, compared to PSI (3.1 μM and 9.3 μM) and etoposide (3.2 μM and 9.7 μM). *In vivo*: In human SKOV3 ovarian cancer xenografts in athymic mice, intraperitoneal administration of PSII and PSI at 15 mg/kg and 25 mg/kg doses inhibited tumour growth by 46% and 70%, and 40% and 64%, respectively.	Xiao et al. (2012)
Polyphyllin VII (PPVII) (**10**)	**Hepatocellular carcinoma:** *In vitro*: PPVII induced apoptosis in hepatocellular carcinoma HepG2 cells with IC_{50} of 1.32 μM, 0.85 μM, 0.78 μM at 24 h, 48 h, and 72 h. Other hepatocellular carcinoma cell lines (Hep3B, Bel7402, and 7721) also induced cytotoxicity with IC_{50} of 2.61 μM, 2.86 μM, and 2.30 μM, respectively, after 24 h.	Zhang et al., (2016a,b)
	Lung cancer: *In vitro*: PPVII induced apoptosis in A549 human lung cancer cells with an IC_{50} of 0.41 ± 0.10 μM after 24 h.	He et al. (2020)
	Nasopharyngeal carcinoma: *In vitro*: PPVII triggered apoptosis in human nasopharyngeal carcinoma (NPC) cell lines such as HONE-1 and NPC-039 cells with IC_{50}s of 2.33 ± 0.22 μM and 2.30 ± 0.31 μM, respectively. *In vivo*: PPVII inhibited tumour growth in NPC carcinoma xenograft model mice.	Chen et al. (2016)
	Lung cancer: *In vitro*: PPVII induced apoptosis and autophagy in the cisplatin (DDP)-resistant human non-small cell lung cancer (NSCLC) cell line (A549/DDP), with an IC_{50} of 2.26 ± 0.30 μM/mL in the A549 and 1.84 ± 0.23 μM/mL in the A549/DDP cell lines.	Feng et al. (2019)
	Lung cancer: *In vitro*: PPVII triggered apoptosis in lung cancer cells such as A549 and NCI–H1299 cells, with an IC_{50} of 1.59 ± 0.12 μM in A549 cells and 1.87 ± 0.09 in NCI–H1299 cells at 48 h. *In vivo*: In Nude mice bearing A549 tumour xenografts, tumour inhibitory rates of PSVII in A549 cells were 25.63%, 41.71%, and 40.41% at 1, 2, and 3 mg/kg respectively.	Lin et al. (2015)
	Brain cancer: *In vitro*: PPVII triggered apoptosis in glioma cell lines such U87-MG and U251 cells with IC_{50} of 4.24 ± 0.87 μM and 2.17 ± 0.14 μM respectively. PPVII (at 0.4 μM) and TMZ (a chemotherapy drug) boosted cytotoxicity in U251 cells and at 0.8 μM in U87-MG cells, indicating that even low concentrations of PPVII can increase TMZ cytotoxicity.	Pang et al. (2019)

against cancer cell lines, bacteria, enzymes and other parasites in the form of crude extract, a mixture of compounds (steroidal saponins), or pure compounds.

4.1. Anticancer activity

Cancer is a non-communicable disease in which some of the body's cells grow out of control, resulting in malignant tumours that spread to other regions of the body via metastasis. The rate of cell division and cellular attrition determine the proliferation of cancer cells. The rate of cell growth in cancer cells is uncontrolled resulting in tumour invasion. Due to its high mortality rate, cancer is a severe problem in both developed and developing countries. According to the American Cancer Society, there were 1,762,450 new cancer cases and 606,880 cancer deaths in the United States in 2019 (Siegel et al., 2019). As a result, several anticancer drugs must be used to drive cancer cells apoptosis. In the short term, radiotherapy, chemotherapy and immunotherapy are successful for certain individuals, but they come with a slew of side effects, including

toxicity, tumour spread and a high rate of tumour recurrence (Song et al., 2015; Chen et al., 2018). Chemotherapy has several drawbacks including multidrug resistance and significant dose-related toxicity limit its practical application (Han et al., 2015; Feng et al., 2019). There is a pressing need to find more effective and less hazardous anticancer drugs. Many clinically utilized cancer chemotherapy drugs are derived from natural products, which are still hotspots for innovative lead discovery (Newman and Cragg, 2012).

Methanol, ethanol, petroleum ether, water and dichloromethane extracts as well as steroidal saponins obtained from various parts of *P. polyphylla* such as the rhizome, root, leaves, stem and whole plant have shown anticancer activity against lung cancer (Yan et al., 2009; Li et al., 2013; He et al., 2014; Hu et al., 2017; Qin et al., 2018), oesophagal cancer (Li et al., 2012), bone cancer (Ruamrungsri et al., 2016), prostate cancer (Zhang et al., 2018), breast cancer (Qin et al., 2018), bladder cancer (Guo et al., 2018), liver cancer (Qin et al., 2018), colon cancer (Qin et al., 2018) and digestive cell cancer (Sun et al., 2007). Methanol extract had the lowest IC_{50} of <10 μg/mL in both chondrosarcoma cell lines and normal canine primary chondrocyte cells (Ruamrungsri et al., 2016). Similarly, ethanol extract had IC_{50} ranging from 10 μg/mL to 30 μg/mL than the aqueous extracts on the six human digestive tumour cell lines (Sun et al., 2007). Ethanol extracts induced an anti-tumour response *in vivo* in PC3 xenograft development in BALB/c nude mice, in which the highest dose exhibited an effect similar to that of 5-FU (positive control) (Zhang et al., 2018). Saponins can cause cell death in a variety of ways including programmed (apoptosis and autophagy) and non-programmed routes (Escobar-Sá nchez et al., 2015). Total saponins, on the other hand, were found to be cytotoxic against five cancer cell lines (human leukaemia, lung cancer, liver cancer, breast cancer and colon cancer) (Qin et al., 2018). They were utilized as agents to limit cell proliferation and necrotic induction since their effect on tumour cells was assessed with a lower IC_{50}.

Similarly, pure compounds extracted from *P. polyphylla* were found to have anticancer properties against a variety of cancer cells. Polyphyllin D was the most frequently studied steroidal saponin for cancer treatment and it was found to have the activity against breast cancer (Lee et al., 2005), ovary cancer (AlSawah et al., 2015), leukaemia (Yang et al., 2016; Wu et al., 2013), liver cancer (Cheung et al., 2005), brain tumour (Yu et al., 2014) and antiangiogenesis in the tumour (Chan et al., 2011). In cancer cell lines, it works as a strong anticancer agent *in vitro* with a lower IC_{50} ranging from 0.2 to 1.4 μM in ovary cancer cells (AlSawah et al., 2015), 0.8–0.9 μM in leukaemia cells (Yang et al., 2016; Wu et al., 2013). Paris saponin VI showed anticancer activity toward the liver cancer line with IC_{50} of 8.18 μM and 6.65 μM (Wang et al., 2019). Paris saponin VII inhibited the growth of human cervical cancer cells with an IC_{50} of 2.62 ± 0.11 μM (Zhang et al., 2014), liver cancer cells with an IC_{50} of 0.80–2.75 μM (Wang et al., 2019; Tang et al., 2019) and drug-resistant ovarian cancer cell lines (Yang et al., 2015a,b). Similarly, paris saponin H inhibited the growth of liver cancer cells with an IC_{50} of 1.25 μM (Chen et al., 2019), diosgenin lung cancer cells with IC_{50} of 149.75 ± 10.43 μM (Yan et al., 2009), pennogenins liver cancer cells with IC_{50} of 9.7–13.5 μM (Zhu et al., 2011). Paris saponin I inhibited the growth of ovarian cancer cells with IC_{50} of <15 μM (Xiao et al., 2009), liver cancer cells with IC_{50} of 0.84–4.66 μM (Wang et al., 2019), gastric cancer cells with IC_{50} from 30.4 to 20.3 μM (Song et al., 2016) and lung cancer cells with IC_{50} from 1.21 to 2.51 μg/mL (Jiang et al., 2014a, 2014b; Liu et al., 2016; Zhao et al., 2015). Likewise, paris saponin II inhibited the growth of lung cancer cells (Zhang et al., 2016a,b), liver cancer cells (Wang et al., 2019), and ovary cancer cells (Xiao et al., 2012, 2014; & Yang et al., 2015); polyphyllin I inhibited the growth of liver cancer cells (Xiao et al., 2018; Han et al., 2015), bone cancer cells (Chang et al., 2015) and lung cancer cells (Feng et al., 2019); polyphyllin VII inhibited the growth of lung cancer cells (Lin et al., 2015; He et al., 2020; Feng et al., 2019; Lin et al., 2015), liver cancer cells (Zhang et al., 2016a,b), nasopharyngeal cancer cells (Chen et al., 2016) and brain cancer cells (Pang et al., 2019; Liu et al., 2020).

The data reveals that IC_{50} of saponins is comparable to that of synthetic chemotherapeutic drugs, and the same saponin type has anticancer action against multiple types of cancer. Because, drug resistance and clinical relapse are widespread in cancer treatment, the use of *P. polyphylla* steroidal saponins maybe a dependable source. Natural products inhibited the growth of human cancer cells *in vitro* and *in vivo* by triggering apoptosis and cell cycle arrest, with only minor harmful side effects on the host's normal tissues and cells (Hannun, 1997; Zhang et al., 2018). Excessive consumption of paris saponins resulted in nausea, vomiting, diarrhoea, and possibly heart palpitations and seizures (Liu et al., 2012). As a result, natural products extracted from *P. polyphylla* such as steroidal saponins and triterpenoid saponins have fewer negative effects in humans than synthetic drugs, and can thus be developed as natural drugs for cancer treatment. Because, the amount of steroidal saponin generated *in vivo* cannot meet the requirement, the approach for *in vitro* enhancement of these chemicals using tissue culture technology will be advantageous in future.

These compounds have also demonstrated suppression of carcinoma cell proliferation, cell autophagy and cell death occurs on the types of cancer cell lines and the compounds/drugs used via numerous routes based such as mitochondrial dysfunction (Lee et al., 2005; Cheung et al., 2005; Xiao et al., 2009; AlSawah et al., 2015; Wu et al., 2013; Zhang et al., 2014; Jiang et al., 2014a, 2014b; Zhao et al., 2015; Song et al., 2016; Yang et al., 2016; Tang et al., 2019; Wang et al., 2019), cell arrest at G2/M phase (Xiao et al., 2009; Jiang et al., 2014a, 2014b; Zhao et al., 2015; Lin et al., 2015; Song et al., 2016), cell arrest at G1-phase (Chen et al., 2018), cell arrest at G2/S-phase (Wang et al., 2019), ROS-oxidative stress pathway (Wang et al., 2019), mitogen-activated protein kinase (MAPK) pathways (Xiao et al., 2009; Chen et al., 2016), suppress pathological angiogenesis (Xiao et al., 2014; Yang et al., 2015a,b), suppress nuclear factor-κB (NF-κB) pathway (Yang et al., 2015a,b; Han et al., 2015; Chang et al., 2015; Chen et al., 2018; He et al., 2020), suppress vasculogenic mimicry (Xiao et al., 2018), suppress the CIP2A/AKT/mTOR pathway (Feng et al., 2019), suppress PI3K/Akt pathway (He et al., 2020) and suppress ROS induced AKT/mTORC1 activity (Pang et al., 2019).

4.2. Antioxidant activity

Antioxidants are chemicals that prevent proteins, lipids, DNA, and other molecules within cells from free radicals and oxidative stress. Oxidative stress is reported to result in ageing and diseases such as cancer, heart disease, cognitive decline and immune system decline. Water-soluble antioxidants, on the other hand, react with oxidants in the cell cytosol and blood plasma, whereas lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Vertuani et al., 2004). Methanol, ethanol, petroleum ether, water extracts and steroidal saponins derived from the rhizome of *P. polyphylla* showed antioxidant activity (Mayirnao and Bhat, 2017; Devi et al., 2018; Lepcha et al., 2019). Ethanol extract of *P. polyphylla* had a strong antioxidant activity with an IC_{50} value of 68 μg/mL (Devi et al., 2018), but the methanol extract had a very weak antioxidant activity with an IC_{50} value of 1.09 mg/mL (Mayirnao and Bhat, 2017). Antioxidant activity of sample or extract is classified as strong if the IC_{50} value is 50–100 μg/mL, moderate if the IC_{50} value is 100–150 μg/mL, and weak if the IC_{50} is 151–200 μg/mL (Prakash and Okawa, 2001; Diantini et al., 2013).

4.3. Antimicrobial activity

Methanol, ethanol and water extracts from the leaves, rhizome and whole plant of *P. polyphylla* showed antifungal activity (Mayirnao and Bhat, 2017; Deng et al., 2008; Qin et al., 2018; Joshi et al., 2020) and antibacterial activity (Mayirnao and Bhat, 2017; Qin et al., 2018; Joshi et al., 2020). Similarly, pure compounds isolated from *P. polyphylla* also showed antifungal activities *in vitro*. Pennogenins showed antifungal activity with minimal inhibitory concentration (MIC) of 0.6 mg/mL to

Table 2. Biological activity of crude extracts of *Paris polyphylla*.

S.N.	Extract	Source	Biological Activity	Reference
1.	Methanol extract	Rhizome	**Lung cancer:** *In vivo*: The extract (2.5, 5.0, and 7.5 mg/kg) inhibited tumour growth, volume, and weight in Lewis bearing-C57BL/6 mice at a rate of 26.49 ± 17.30%, 40.32 ± 18.91%, and 54.94 ± 16.48%, respectively. *In vitro*: The extract (0.25, 0.50, and 0.75 mg/mL) induced apoptosis in human lung adenocarcinoma A549 cell lines.	Li et al. (2013)
			Antioxidant activity: *In vitro*: Methanol extracts of rhizomes collected from two places Tholung (PPT) and Uttaray (PPU) showed free radical scavenger of DPPH with an IC_{50} of 2.01 μg/mL and 2.55 μg/mL, respectively. PPT had an IC_{50} of 2.22 μg/mL and PPU had an IC_{50} of 2.57 μg/mL, according to the ABTS test. **Cytotoxicity on HeLa, HepG2, and PC3**: *In vitro*: Methanol extracts inhibited HeLa cell (cervical cancer cell) growth >90% at 100 μg/mL. PPT and PPU both had a moderate effect on HepG2 cells (non-tumorigenic hepatic cells) growth up to 30 μg/mL concentration, whereas PPT inhibited growth by 73.47% at 100 μg/mL concentration. Both extracts inhibited PC3 (prostate cancer cell line) cells at a dosage of 100 μg/mL.	Lepcha et al. (2019)
			Antioxidant activity: *In vitro*: Methanol extract has a stronger antioxidant activity with an IC_{50} of 1.09 mg/mL. **Antimicrobial activity:** *In vitro*: At 5 mg/mL, methanol extract inhibited the growth of *Aspergillus niger* (97.74%), *Staphylococcus aureus* (95.58%), *Escherichia coli* (95.58%), and *Trichoderma reesei* (74.41%). The antifungal activity was best against *A. niger*, with a zone of inhibition diameter of 33 mm, and lowest against *T. reesei*, with a zone of inhibition diameter of 31 mm. The antibacterial activity was best against *E. coli*, with a zone of inhibition diameter of >31 mm.	Mayirnao and Bhat (2017)
			Anthelmintic activity: *In vivo*: With an EC_{50} of 18.06 mg/L, methanol extract exhibited substantial efficacy against *Dactylogyrus intermedius* (a freshwater fish ectoparasite).	Wang et al. (2010)
		Leaves	**Antiviral activity:** *In vitro*: With an IC_{50} of 8.74 μg/mL and a SI/selectivity index (CC_{50}/EC_{50}) of 1.75, methanol extract exhibited antiviral activity against Chikungunya virus (CHIKV).	Joshi et al. (2020)
			Antifungal activity: *In vitro*: At 1000 μg/mL, methanol extract inhibited the growth of *Candida albicans* (99 % inhibition).	
			Antibacterial activity: *In vitro*: At 1000 μg/mL, methanol extract inhibited the growth of *Pseudomonas aeruginosa* (100%), *Staphylococcus aureus* (80%), *Listeria innocua* (65%), *Escherichia coli* (57%), *Salmonella enteric* (67%), and *Shigella sonnei* (47%).	
2.	Dichloromethane and methanol extract	Rhizome	**Bone cancer:** Dichloromethane extracts induced apoptosis in SW1353 chondrosarcoma cells with an IC_{50} of 9.74 ± 0.36 μg/mL, but had a less effect on the percentage of viability and necrosis of normal canine primary chondrocyte cells (IC_{50} of 382.70± 8.20 μg/mL). In both primary chondrocytes and SW1353 chondrosarcoma cells, methanol extract showed the lowest IC_{50} of <10 μg/mL.	Ruamrungsri et al. (2016)
3.	Ethanol extract	Roots	**Human oesophagal cancer cells**: *In vitro*: ethanol extract induced apoptosis at 25 mg/mL, 50 mg/mL, 100 mg/mL, and 200 mg/mL concentrations, and increased the expression of the cancer suppressor gene (connexin26) at the mRNA and protein levels in oesophagal cancer ECA109 cells.	Li et al. (2012)
		Rhizome	**Antioxidant activity:** *In vitro*: The total phenol concentration was 0.68 mg/g catechol and 0.47 mg/g catechol with the ethanol and petroleum ether extracts respectively by Folin's Ciocalteu reagent, and the inhibitory concentration value of ethanol extract was 68 μg/mL (ascorbic acid 7.8 μg/mL). It means that the ethanol extract has a larger total phenolic content and, as a result, has more antioxidant activity.	Devi et al. (2018)
			Antifungal activity: *In vitro*: Ethanol extract showed antifungal activity on *Cladosporium cladosporioides.*	Deng et al. (2008)
			Abnormal uterine bleeding (AUB): *In vitro*: Using myometrial strips from estrogen-primed or pregnant rats, ethanol extract increased the frequency and intensity of phasic myometrial contractions with 23.19 ± 0.27% of the potassium response, and the EC_{50} of 19.82 ± 0.42 mg/mL.	Guo et al. (2008)
		Stem	**Digestive cell cancer:** *In vitro*: The six human digestive tumour cell lines (SMMC-7721, HepG-2, BGC-823, SW-116, LoVo, and CaEs-17) demonstrated apoptosis with IC_{50}s ranging from 10 to 30 μg/mL. The two liver cancer cell lines, SMMC-7721 and HepG-2, showed the lowest IC_{50} of 12 μg/mL and 10 μg/mL, respectively.	Sun et al. (2007)
		Leaves	**Lung cancer:** *In vitro*: ethanol extract inhibited the growth of A549 human lung cancer cells, that was 47.76 %, 50.24 %, 53 %, and 64.17 % at 25, 50, 100, and 200 μg/mL, respectively.	Hu et al. (2017)
		Whole plant	**Human prostate cancer:** *In vitro*: PPEE induced apoptosis in PC3 and DU145 prostate cancer cells, with IC_{50} values of 3.98 μg/mL and 8 μg/mL, respectively. Cisplatin (a positive control) inhibited prostate cancer cell viability more effectively than PPEE. *In vivo*: In BALB/c nude mice, PPEE at 100 mg/kg resulted in a tumour volume of 333.01 ± 34.77 mm3, representing a 51.05% inhibition rate in PC3 xenograft development.	Zhang et al. (2018)
			Bladder cancer: *In vitro*: Ethanol extracts induced apoptosis on bladder cancer cells with mutant p53, such as HT1197 and J82 cells, with an IC_{50} of 1.2 μg/mL, comparable to the action of cisplatin (chemotherapy drug).	Guo et al. (2018)
			Colon, lung, liver, leukaemia & breast cancer: *In vitro*: TSSAPs had IC_{50}s ranging from 8.12 to 12.61 μg/mL, while TSSRs had IC_{50}s ranging from 1.75 to 6.62 μg/mL in five tumour cell lines (human leukaemia: HL-60, human lung cancer: A-594, human liver	Qin et al. (2018)

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Table 2 (*continued*)

S.N.	Extract	Source	Biological Activity	Reference
			cancer: SMMC-7721, human breast cancer: MCF-7, and human colon cancer: SW480). With IC_{50}s of 1.75 and 3.49 μg/mL, TSSRs showed high cytotoxicity against A549 and SW480 cells, respectively. **Antimicrobial activity:** *In vitro*: TSSAPs and TSSRs inhibited the growth of *E. coli*, *Candida albicans* (5314), and *Candida albicans* (Y0109) with MIC values of 156, 5.15, and 10.3 g/mL, respectively.	
4.	Chloroform, ethyl acetate, and butanol extracts	Rhizome	**Tyrosinase enzyme:** All the extracts showed mild to moderate inhibitory potentials against the enzyme tyrosinase.	Devkota et al. (2007)
5.	*Paris polyphylla* steroidal saponins (PPSS)	Rhizome and Root	**Lung cancer:** *In vitro*: PPSE at 0, 20, 40, and 80 mg/L induced apoptosis in human lung cancer A549 cells with IC_{50} values of 72.55, 49.96, and 21.01 mg/L at 12, 24, and 48 h, respectively.	He et al. (2014)

2.5 mg/mL (Zhu et al., 2011). Steroidal saponins showed antifungal effects on *Candida albicans* with the minimum inhibitory concentrations (MIC) of 5.15 and 10.3 μg/mL respectively (Qin et al., 2018). Pennogenin steroidal saponins showed 0.6–2.5 mg/mL MIC against *Saccharomyces cerevisae*, and 0.6–1.2 mg/mL MIC against *Candida albicans* (Zhu et al., 2011). It shows that pennogenin steroidal saponins were more effective against *Candida albicans* than others. The steroidal saponins were selective in their activity against different types of bacteria such as *Pseudomonas aeruginosa* (100%), *Staphylococcus aureus* (80%), *Listeria innocua* (65%), *Escherichia coli* (57%), *Salmonella enteric* (67%), and *Shigella sonnei* (47%) (Mayirnao and Bhat, 2017; Qin et al., 2018; Joshi et al., 2020).

4.4. Antiviral activity

Methanol extracts from *P. polyphylla* leaves were found to be active against Chikungunya virus with an IC_{50} of 8.74 μg/mL (Joshi et al., 2020). Polyphylla saponin I derived from *P. polyphylla* was found to have antiviral action against the influenza A virus (Pu et al., 2015). On MDCK cells, polyphylla saponin I at 40 μg/mL inhibited 91.4% of influenza A virus infection, while oseltamivir (positive control) at the same dose inhibited 91.7% of influenza A virus infection (Pu et al., 2015).

4.5. Antileishmanian activity

Leishmaniasis is an intracellular protozoan parasitic disease caused by approximately twenty *Leishmania* species. It is spread through the bite of female phlebotomine sandflies of over 90 different species. Every year, between 700,000 and 1 million new cases of leishmania emerge (WHO, 2022). *In vitro* antileishmanial activity was found in steroidal saponins extracted from the rhizome of *P. polyphylla* (Devkota et al., 2007). Strong (IC_{50} = 0.23 μM), mild (IC_{50} = 0.93–36.87 μM), and moderate (IC_{50} = 1.59–83.72 μg/mL) antileishmanial activity were observed in chloroform, ethyl acetate and butanol extracts of the plant.

4.6. Anthelmintic activity

The anthelmintic activity evaluation of the methanol extract of *P. polyphylla* rhizome showed an EC_{50} value of 18.06 mg/L (Wang et al., 2010). Polyphyllin D (EC_{50} of 0.70 mg/L) and dioscin (EC_{50} of 0.44 mg/L) were extracted from crude methanol extract and showed greater anthelmintic activity than crude methanol extract (Wang et al., 2010).

4.7. Gynaecological disorder

One of the most prevalent illnesses in women is abnormal uterine bleeding (AUB). AUB refers to abnormal uterine bleeding caused by structural issues, pregnancy difficulties (Ely et al., 2006) and contraception (Schrager, 2002). It could also be caused by benign and malignant tumours as well as pregnancy-related diseases and endocrine disorders. *In vitro*, steroidal saponins derived from the rhizome of *P. polyphylla* reduced abnormal uterine bleeding in rats by eliciting phasic myometrial contractions (Guo et al., 2018). Total steroidal saponins (TSSP) produced a response in the rat myometrium that was 23.19 ± 0.27% of the potassium response, and the EC_{50} value of TSSP was 19.82 ± 0.42 μg/mL. Under the same conditions, the highest potassium responses to oxytocin and PGF-2a (labor-inducing drugs) were 51.09 ± 0.03% and 42.00 ± 0.05%, respectively. It shows that TSSP has a stronger effect on rat myometrial contraction than oxytocin or PGF-2a.

4.8. Antityrosinase enzyme activity (Cosmetic value)

Copper-containing tyrosinase enzymes found inside melanosomes in plant and animal tissues catalyze the oxidation of tyrosine to produce melanin (black pigment) and other pigments. Tyrosinase inhibitors have shown to be effective in the treatment of melanin hyperpigmentation-related skin diseases or melanin-biosynthesis-related skin diseases. *P. polyphylla* rhizome extracts in chloroform, ethyl acetate and butanol demonstrated weak to moderate inhibition of the tyrosinase enzyme (Devkota et al., 2007). Similarly, przewalskinone B, isolated from the rhizome of *P. polyphylla*, had an IC_{50} of 0.25 mM against the tyrosinase enzyme (Devkota et al., 2007).

5. Conclusion

Due to the existence of useful secondary metabolites, it has been identified as a potential candidate for the treatment of several types of cancer and other disorders in modern medicine. The rhizome is the most extensively used plant part, and it has more activity against cancer cell lines, pathogens, and parasites as compared to above-ground parts. Based on *in vitro* and *in vivo* experiments, several pure steroidal saponins and crude extracts of *P. polyphylla* showed potent activity against carcinoma cell lines, bacteria, and parasites. As a result, it will be a promising plant for future studies of anticancer medications.

6. Future prospectives

Paris polyphylla is an endangered plant species that have been used as high-valued medicinal herb in traditional medicine. The natural population is decreasing due to over-exploitation and collection to meet the demand in traditional medicine. It is necessary to conserve its natural population through plant tissue culture technique and production of high-valued secondary metabolites in culture for sustainable utilization of such compounds in the production of pharmaceutical drugs.

Declarations

Author contribution statement

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Additional information

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RECEIVED CERTIFICATES

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE & TECHNOLOGY
Kirtipur, Kathmandu

PARTICIPATION CERTIFICATE

AWARDED TO

CHANDRA BAHADUR THAPA

With appreciation and good wishes for participation with a poster presentation in the

Ph.D. Festival 2023

Held on 9-10 October, 2023 (22-23 Asoj 2080) in University Campus, Kirtipur, Nepal.

Prof. Dr. Binil Aryal
Dean, IoST, TU, Nepal

Organized by
Institute of Science & Technology, TU

Prof. Dr. Shiva Lal Bhusal
Rector, TU, Nepal

CERTIFICATE OF PARTICIPATION

CHANDRA BDR. THAPA

has successfully participated in the *workshop on Predatory Academic Practices in South Asia,* virtually

MARCH 25 – 26, 2022
Pokhara University, Kaski, Nepal
(also virtually on Zoom platform)

Dr. Nirina Khadgi
Project Lead
National Young Academy of Nepal

Dr. Namraj Dhami
Executive Director
Pokhara University Research Center

Dr. Dhaka Ram Bhandari
Executive Director
Gandaki Province Academy of Science and Technology

International Collaborative Seminar
Wednesday 18 October 2023

Certificate of participation presented to

Mr. CHANDRA BAHADUR THAPA

Participating universities

Tribuhvan University, Nepal
Jambi University, Central Sumatra, Indonesia
Udayana University, Bali, Indonesia
Malikussaleh University, Ache, Indonesia
Chiang Mai Rajabhat University, Thailand
Universiti Pendidikan Sultan Idris, Malaysia
Universiti Tunku Abdul Rahman, Malaysia
National Chung Cheng University, Taiwan
Meiji University, Japan
University of Canterbury, New Zealand
Edith Cowan University, Australia
Queensland University of Technology, Australia

University Convenor

International Moderator

Professor Emeritus Acram Taji AM
Australia

PHOTO PLATES (EXPERIMENTS)

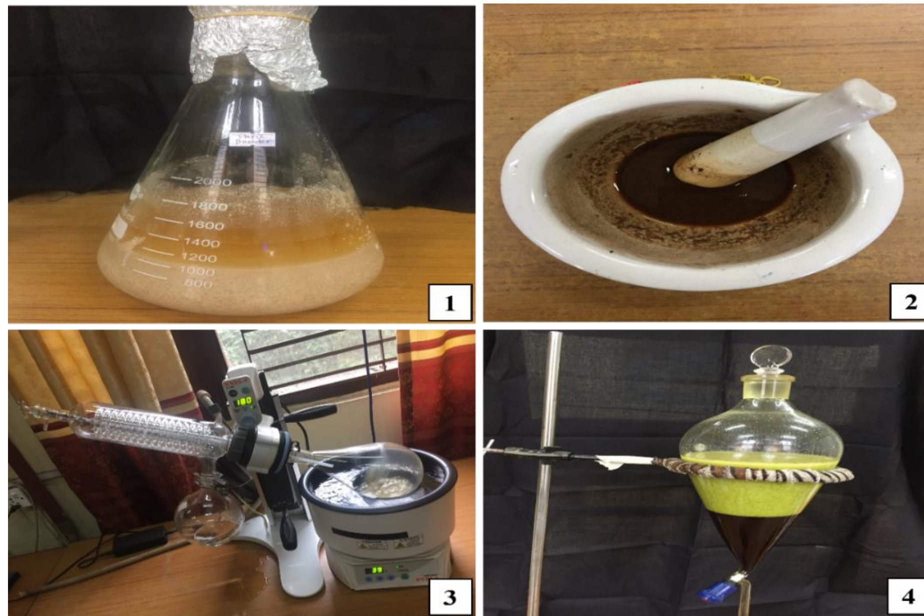


Photo plates: (1) Powder soaked in solvent, (2) Callus extract preparation in mortar and pestle, (3) Rotary evaporator, (4) Fractionation in separating funnel.

Photo plates: (5) Microplate reader, (6) Digital camera for microscopic study, (7) Observation in the binocular microscope, (8) Lux meter for the measurement of light intensity in culture room.

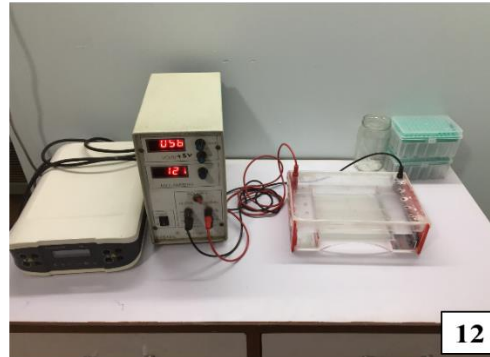
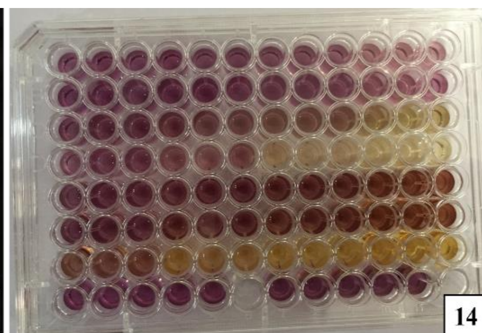
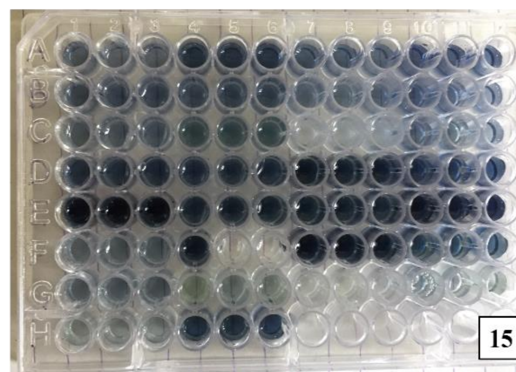


Photo plates: (9) Bacterial pure culture, (10) Bacterial culture in a biosafety cabinet (11) Working in a PCR machine, (12) Agarose gel for DNA band separation.





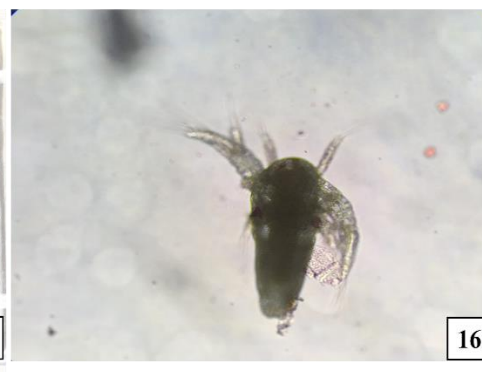


Photo plates: (13) Callus for extract preparation (14) DPPH assay in 96-well plate (15) TFC test in 96-well plate (16) Brine shrimp nauplii.

CONSENT LETTER FOR PLANT COLLECTION FROM DPR, GOVERNMENT OF NEPAL

नेपाल सरकार
वन तथा वातावरण मन्त्रालय
वनस्पति विभाग
जैविक विविधता तथा साईटिस
(.... शाखा)

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फ्याक्स नं.: ४२५११४१

पत्र संख्या:- २०७८/०७९
चलानी नम्बर:- २६२

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वनस्पतिमार्ग, थापाथली
काठमाडौं
मितिः २०७८/०७/१४

विषय: अनुसन्धान अनुमति सम्बन्धमा ।

श्री चन्द्र बहादुर थापा
तिलोत्तमा-१, रुपन्देही।

प्रस्तुत विषयमा यस विभागको मिति २०७८/०७/१० को निर्णयानुसार तपाईंलाई देहाय बमोजिमका शर्तहरुमा रही वनस्पति स्रोतको अनुसन्धान कार्यविधि, २०७०(पहिलो संशोधन,२०७३) बमोजिम अनुसन्धान गर्न निम्नानुसार अनुमति प्रदान गरिएको छ ।

अनुसन्धानकर्ताको नाम	पहिलो	बीचको	थर
	चन्द्र	बहादुर	थापा
अनुसन्धानकर्ताको ठेगाना	स्थायी : तिलोत्तमा-१, रुपन्देही। अस्थायी : कीर्तिपुर, काठमाडौं।	ईमेल :- cbthapa.2009@gmail.com	फोन नं. :९८४७०२९४३५
सम्बद्ध संस्था	संस्थाको नाम :	ठेगाना :	
	श्री वनस्पति शात्र केन्द्रीय विभाग,त्रि.वि. ।	कीर्तिपुर, काठमाडौं ।	
पद	उप-प्राध्यापक ।		
अनुसन्धान तह	व्यक्तिगत : स्नातक तह / स्नातकोत्तर तह / पि.एच.डी. ✓ अन्य :		संस्थागत : राष्ट्रिय ✓ / अन्तर्राष्ट्रिय
अनुसन्धानको शीर्षक	**"In vitro Culture and Comparative Phytochemical Analysis in Some Medicinal Plants"** ।		
अनुसन्धानको क्षेत्र	गुल्मी, पाल्पा, बाग्लुङ, ललितपुर र मकवानपुरका संरक्षण क्षेत्र बाहेकका स्थान।		
अनुसन्धानको विधि		नमुना संकलन (गर्ने ✓)	नमूनाको परीक्षण
		Piper longum L., *Paris polyphylla* Sm. र *Lilium nepalense* D.Don का नमूनाहरु ।	नेपालमा -
अनुसन्धानको समयावधि	सन् २०२१ देखि २०२३ सम्म ।		
अनुसन्धानको शर्तहरु	१. अनुसन्धानकर्ताले विभाग र सम्बन्धित कार्यालयसँग समन्वय गरी अनुसन्धान कार्य गर्नु पर्नेछ । २. अनुसन्धानकर्ताले आफ्नो अनुसन्धानको प्रस्ताव सम्बन्धित कार्यालयमा समेत बुझाउनु पर्नेछ । ३. अनुसन्धानकर्ताले अनुसन्धान समाप्त भएपछि एक प्रति कागजी प्रतिवेदन र एक प्रति विद्युतीय प्रतिवेदन विभागमा बुझाउनु पर्नेछ। ४. अनुसन्धानकर्ताले नतिजाहरु प्रकाशित गर्दा अनुसन्धानमा संलग्न कर्मचारीको योगदानको आधारमा सह-लेखकको रुपमा समावेश गराउनु पर्नेछ । ५. संकलित नमूना नेपाल सरकारको पूर्व स्वीकृति नलिई विदेश लैजान पाईने छैन् । ६. संकलित वनस्पतिका प्रत्येक प्रजातिका मृत नमूनाहरु एक/एक थान श्री राष्ट्रिय हर्बेरियम तथा वनस्पति प्रयोगशाला (KATH) गोदावरी, ललितपुरमा बुझाउनु पर्नेछ। ७. नेपालको प्रतिबन्धित र साइटिस सूचीमा सूचीकृत वनस्पति बाहेकका वनस्पति नमूनाहरु मात्र संकलन गर्नु पर्नेछ । ८. अनुसन्धानकर्ताले नेपाल सरकारको प्रचलित ऐन, कानून तथा स्थानीय नियम, मुल्य र मान्याता विपरित कुनै पनि कार्य गर्न पाउने छैन् ।		

....
मधु देवी घिमिरे
उपसचिव (प्रा.)

"स्वच्छ र सक्षम निजामती प्रशासन: सम्वृद्धि र सुशासन"