Biological Activities and Micropropagation of *Coelogyne nitida* (Wall. ex D.Don) Lindl.

A Dissertation Submitted for the Partial Fulfilment of the Requirement of a Master's Degree in Botany

By

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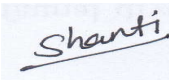
Declaration

The dissertation entitled "**Biological Activities and Micropropagation of *Coelogyne nitida* (Wall. ex D.Don) Lindl."** which is being submitted to the Central Department of Botany, Institute of Science and Technology, Tribhuvan University, Nepal for the partial fulfilment of the requirement of Master's Degree in Botany has been carried out by me under the supervision of **Asst. Prof. Dr. Mukti Ram Paudel**. This work has not been submitted to any other institution for any academic degree.

.....  .....

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This is to recommend that **Shanti Ranabhat** carry out the dissertation entitled **"Biological Activities and Micropropagation of *Coelogyne nitida* (Wall. ex D.Don) Lindl."** for the partial fulfilment of the requirement of a Master's Degree in Botany under my/our supervision. To my/our knowledge, this work has not been submitted to any other institution for any academic degree. She has fulfilled all the requirements laid down by the Central Department of Botany, Institute of Science and Technology, Tribhuvan University, Kirtipur for the submission of the dissertation for the Master's Degree in Botany.

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The dissertation work entitled **"Biological Activities and Micropropagation of *Coelogyne nitida* (Wall. ex D.Don) Lindl."** submitted at the Central Department of Botany, Institute of Science and Technology, Tribhuvan University by **Shanti Ranabhat** has been accepted for the partial fulfilment of Master's Degree of Science (M.Sc.) in Botany.

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Abstract

Coelogyne nitida (Wall. ex D.Don) Lindl. commonly known as "Shining Coelogyne" is famous for its idyllic and glorious flower and has been used in traditional medicine in South Asian countries. The objective of the study was to determine the total phenolic content, total flavonoid content, antioxidant activities, toxicity and antimicrobial activities of the flower, leaf and pseudobulb extract of *C. nitida*. Micropropagation of *C. nitida* using seed was also the aim of this study as orchids are under conservation of CITES. The FC-reagent and aluminum chloride method were used to determine the total phenol and total flavonoid content. The DPPH scavenging assay was employed to determine the antioxidant activity of flower, leaf, and pseudobulb extracts. DPPH of 0.2 mM was used. The DCM fraction of pseudobulb was found to have the highest total phenol and flavonoid content i.e., 272.12 mg GAE/g and 10.23±0.30 mg QE/g respectively. This fraction of pseudobulb exhibited the highest antioxidant activity with an IC_{50} value of 49.96±2.36 µg/ml and also showed antibacterial activity against gram-positive bacterial strains *B. subtalis* (ATCC 6059) and *S. aureus* (ATCC 6538P) with ZOI of 11.75 mm and 11.48 mm respectively. *C. nitida* was found non-toxic from the brine shrimp lethality test with LC_{50} value >1000 µg/mL. Micropropagation is one of the most effective approaches for the long-term conservation and sustainable utilization of this orchid. The highest germination of seed (95%) was recorded in Murashige and Skoog (MS) medium fortified with 2 mg/L BAP and 0.1 mg/L NAA. The FMS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA produced the healthiest shoot and root with maximum shoot number (10.00±0.58), shoot length (6.50±0.11 cm), root number (26±0.57) and root length (2.80±0.05 cm). Successful acclimatization of the *in vitro* developed plantlets was done in substrate containing soil, coco peat, wood powder and *Sphagnum* moss in equal proportion with an 85% survival rate. The finding of the research helps for the sustainable utilization of the horticulturally and medicinally important *C. nitida*. Besides, the DCM fraction of the pseudobulb might have an important bioactive compound which can be used in the drug discovery.

सारंशः

Coelogyne nitida (Wall. ex D.Don) Lindl. सामान्यतया "शाइनिंग शिलोगाएने" भनेर चिनिन्छ। यो वनस्पति सुन्दर र आकर्षक फूलको लागि प्रसिद्ध छ र दक्षिण एसियाली देशहरूमा परम्परागत औषधिमा प्रयोग गरिन्छ। यस अध्ययनको उद्देश्य *C. nitida* को फूल, पात र स्यूडोबल्बबाट बनाइएको Hexane, DCM, Methanol र Aqueous एक्स्ट्रयाक्टको कुल फिनोलिक कनटेन, कुल फ्लाभोनोइड कनटेन, एन्टिअक्सिडेन्ट, विषाक्तता र एन्टिमाइक्रोबियल गतिविधिहरू निर्धारण गर्नु थियो। अर्किडहरू CITES को संरक्षणमा रहेकाले यस वनस्पतिको बीउ प्रयोग गरेर माइक्रोप्रोपगेसन गर्ने पनि अध्ययनको उद्देश्य थियो। कुल फिनोल र कुल फ्लेभोनोइड कनटेन निर्धारण गर्न Folin-Ciocalteu reagent र एल्युमिनियम क्लोराइड विधि प्रयोग गरियो भने एन्टिअक्सिडेन्ट गतिविधि निर्धारण गर्न DPPH स्क्याभेन्जिङ विधि प्रयोग गरिएको थियो। स्यूडोबल्बको DCM अंशमा उच्चतम कुल फिनोल र फ्लेभोनोइड कनटेन क्रमशः 272.12 mg GAE/g र 10.23 mg QE/g रहेको पाइयो। स्यूडोबल्बले IC_{50} मान 49.96±2.36 µg/mL को साथ उच्चतम एन्टिअक्सिडेन्ट गतिविधि प्रदर्शन गर्यो साथै gram-positive ब्याक्टेरियल स्ट्रेन *Bacillus subtilis* (ATCC 6059) र *Staphylococcus aureus* (ATPCC 6538P) विरुद्ध क्रमशः 11.75 र 11.48 मिलिमिटर Zone of Inhibition का साथ जीवाणुरोधी गतिविधि देखाएको थियो। ब्राइन झिंगा मार्ने क्षमता परीक्षणबाट LC_{50} 1000 µg/mL भन्दा धेरै मानको साथ यस बिरुवा गैर-विषाक्त रहेको पाइयो। *C. nitida* को बीउलाई Murashige and Skoog (MS) माध्यमसँग पूरकको रुपमा विरुवा वृद्धि हर्मोन BAP र NAA को प्रयोग गरी बीउ अङ्कुरण र अङ्कुर विकास अनुगमन गरियो। यसैगरी MS माध्यममा NAA, IAA र IBA एकल सथै BAP को संयोजन प्रयोग गरी जरा विकासको तथाङ्कको रेकर्ड गरियो। यस वनस्पतिको बीउ अङ्कुरण भने MS माध्यमसँग पूरकको रुपमा 2 mg/L BAP र 0.1 mg/L NAA मा 95% भएको थियो। MS माध्यमसँग पूरकको रुपमा 0.5 mg/L BAP र 0.5 mg/L NAA माध्यमले अधिकतम अङ्कुर सङ्ख्या (10.00±0.58), अङ्कुर लम्बाइ (6.50±0.11 सेमी), जरा सङ्ख्या (26±0.57) र जरा लम्बाइ (2.80±0.05 सेमी) का साथ सबैभन्दा स्वस्थ अङ्कुर र जरा उत्पादन गर्यो। यसरी इन भिट्रो विकसित *C. nitida* को बीरुवाहरू माटो, कोकोपीट, काठको धूलो र *Sphagnum moss* को बराबर अनुपातमा समावेश गरिएको सब्सट्रेटमा सफल अनुकूलन गरियो र विरुवाहरू बाँच्ने दर 85% भेटियो। यस अनुसन्धानको निष्कर्षले बागवानी र औषधीय रूपमा महत्त्वपूर्ण अर्किडको दिगो उपयोगको लागि मद्दत गर्दछ। यसबाहेक, स्यूडोबल्बको DCM अंशमा एक महत्त्वपूर्ण बायोएक्टिभ कम्पाउन्ड हुन सक्छ जुन औषधि खोजमा प्रयोग गर्न सकिन्छ।

List of Abbreviations and Acronyms

AF	Aqueous Fraction of Flower
AL	Aqueous Fraction of Leaf
AP	Aqueous Fraction of Pseudobulb
2,4-D	2,4-Dichlorophenoxy Acetic Acid
ATCC	American Type Culture Collection
BAP	6-Benzyl Aminopurine
$CHCl_3$	Chloroform
CITIES	Convention of International Trade in Endangered Species of Wild Fauna and Flora
CW	Coconut Water
DCM	Dichloromethane
DF	DCM Fraction of flower
DL	DCM Fraction of Leaf
DP	DCM Fraction of Pseudobulb
DMSO	Dimethyl Sulphoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
EDTA	Ethyldiaminetetraacetic Acid
$FeCl_3$	Ferric Chloride
GAE	Gallic Acid Equivalent
HF	Hexane Fraction of Flower
HL	Hexane Fraction of Leaf
HP	Hexane Fraction of Pseudobulb
H_2SO_4	Sulphuric Acid

IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
IC_{50}	Half Maximal (50%) Inhibition
IUCN	International Union for Conservation of Nature and Natural Resources
LC_{50}	Half Lethal Concentration
MF	Methanolic Fraction of Flower
ML	Methanolic Fraction of Leaf
MP	Methanolic Fraction of Pseudobulb
MHA	Mueller Hinton Agar
MS	Murashige and Skoog
NA	Nutrient Agar
NAA	1-Naphthalene Acetic Acid
NPRL	Natural Product Research Laboaratory
PGRs	Plant Growth Regulators
QE	Quercetin Equivalent
TPC	Total Flavonoid Content
TFC	Total Phenolic Content
TUCH	Tribhuvan University Central Herbarium
WFO	World Flora Online
ZOI	Zone of Inhibition

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1. Introduction

1.1 Background

1.1.1 Orchids

Orchids are a diverse plant group with about 28,000 species categorized into 763 genera (Christenhusz and Byng, 2016). In Nepal, the distribution of orchids ranges from tropical to alpine regions (Acharya *et al.,* 2011). About 506 orchid species are found in Nepal including 18 endemic species (Rokaya *et al*., 2013). Locally, these are recognized as "sunakhari", "sungava". "chandigava," and "jivanti". These are generally collected for food, decoration, and traditional medicine (Hinsley *et al.,* 2017). It has been reported that about 100 species of orchids have been used for medicinal purposes in Nepal (Rajbhandari, 2014).

Orchids have been one of the most availed groups of plants for medicine for a long time (Bulpit *et al.,* 2007). Ayurvedic, Unani and Chinese traditional systems of medicine used numerous orchids to cure human diseases. In the Indian traditional system of medicine, “Ashtavarga” known as the herb of immortality contains the chemicals of *Chrepidium acuminatum*, *Habenaria intermedia, Malaxis muscifera* and *Platanthera edgeworthii* (Singh & Duggal, 2009). The important Chinese medicine “Shi-Hu*”* is made from different *Dendrobium* species such as *Dendrobium moniliforme* and *Dendrobium nobile.* Research has shown that this is used to treat stomach and lung cancer (Ping *et al.,* 2003). Another Chinese herbal medicine known as “Tian-Ma” is also derived from *Gastrodia elata* (an orchid listed in the Chinese *Materia Medica*).The scientific analysis shows that gastrolin which is the main compound of “Tian-Ma” has an anti-delirium, an anti-convulsive effect and also acts as a vasodilator (Sun *et al.,* 2004; Zhang 2003).

Besides traditional medicine orchids are used for various purposes. Vanilla, the second most expensive flavor after saffron is obtained from the pod of climbing orchid *Vanilla planifolia* (Frenkel *et al*., 2004). Nature has bestowed orchids to enhance the beauty of the vegetation. Orchids contribute about 10% of the total flowering species (Tsai *et al.*,2013).These are the best imitators as they appear in the form of different insects, birds, butterflies and canines. Most of the orchids have a sweet scent to lure the pollinators. This feature of orchids has contributed to the cosmetic and perfume industry (Pant, 2013). Flowers of almost all orchids are bright

and soothing in colour with longer shelf life, smooth texture and unique structure. These features have imparted orchids as a significant contributor to the global horticulture and cut flower industry (De *et al.,* 2015; Murthy *et al.,* 2018).

The multifarious uses of orchids have increased their demand both in national and international markets. This situation has encouraged the illegal collection and trade of orchids. The open border with the neighboring country also aids in the illegal trade of unprocessed orchids (Subedi *et al.,* 2013). Besides habitat degradation and fragmentation, climate change and indiscriminate harvesting are the prime causes of the decrease in orchid numbers. In addition to this, the natural factors within orchids also result in a low germination rate. Most of the orchids are habitat-specific (Linder, 1995); need special pollinators (Darwin, 1862) and mycorrhizal association for germination as they lack endosperm (Alghamdi, 2019; Chand *et al.,* 2020; Mc Cormick *et al.,* 2004). The mycorrhizal association may be species-specific (McCormick & Jacquemyn, 2013), generalist (Dighton, 1986) or variable among the species (Muir, 2009).

As a greater step for orchid conservation, the whole Orchidaceae family with 144 species is included in Appendix – II of the Convention of International Trade in Endangered Species of Fauna and Flora (CITES) (Joshi *et al.,* 2017). In Nepal, 302 species of orchids are reported to be threatened (Raskoti, 2009). Realizing this scenario of orchid status Government of Nepal has allocated the protected areas for conservation but a negative correlation is reported between the number of protected areas and a number of medicinal orchid species (Acharya & Rokaya 2010). So, plant tissue culture can be the most effective strategy for the long-term conservation of orchids (Pant, 2013). This technique produces a significant number of plants in a short duration and maximum germination can also be achieved with a suitable culture medium.

Coelogyne is a large genus of orchid comprising 209 accepted species with a broad range of distribution including India, Nepal, China and across Southeast Asia to the Pacific (WFO, 2023).The genus *Coelogyne* was first described by Lindy in 1821 as '*Coelogyne*' which is a Greek word, meaning '*kilos*'= 'hollow' and '*gene*'= 'female'). Later in 1825, he corrected the spelling to *Coelogyne.* In Nepal there are 14 species of *Coelogyne.* Ethno-botanical studies have documented the use of many *Coelogyne* species as medicine by different ethnic communities across Southeast Asia. The paste

of pseudobulb of *C. Corymbosa, C. fimbriation, C. flaccid, C. nitida, C. prolifera* and *C. stricta* is used to relieve headache and fever (Manandhar, 2002; Lawer 1994; Pyakural and Gurung, 2008; Subedi *et al.,* 2013; Thakur *et al.,* 2010 and Vaidya *et al.,* 2002). Similarly, the juice of pseudobulb of *C. cristata, C. fimbriata, C. fuscescens, C. prolifera and C. punctulata* is applied over burns, boils and wounds (Subedi *et al.,* 2013). *C. cristata and C. ovalis* are reported to be aphrodisiac. The whole plant of *Coelogyne ovalis* Lindl. is used against cough, urinary infection and eye disorders. Likewise, the entire plant of *C. barbata* Lindl. ex Griff. is used in the treatment of sore throat, traumatic injuries and 'lung-heat' (Teoh, 2016). Pseudbulb of *Coelogyne nitida* (Wall. ex.D. Don) Lindl. is also used to overcome headaches, fever and burns. Tubers of *C. trinervis* is used to treat fractures and sprains (Teoh, 2016). The use of *Coelogyne* species in the traditional medicinal system is backed by the photochemical study of such plant which shows the presence of secondary metabolites like alkaloids, phenolic, flavonoid and tannins (Natta *et al*., 2022).

1.1.2 Bioactivities of *Coelogyne* species

Coelogyne species have been extensively studied for their secondary metabolites and are found to have a wide range of phytochemicals that have shown promising medicinal properties. These variations in the presence of phytochemicals indicate the presence of powerful free radical scavenging compounds which attributes to the biological activities including antioxidant, anti-inflammatory, antimicrobial and antidiabetic (Pietta *et al*., 2000; Vasanthi *et al*., 2012). Twenty-eight bioactive compounds such as coelogin, coeloginin, coeloginanthridin, coeloginanthrin, flavidin, flavidinin, etc. belonging to 12 different compound classes have been isolated from six species of *Coelogyne* (Majumder *et al.*,1982; Sachdev and Kulshrestha, 1986).

Coelogyne species have been reported to show bioactivities such as antioxidant activity and antimicrobial activity. Antioxidants are the compound that can inhibit the free radicals which can cause different diseases in human bodies. Only four *Coelogyne* species have been documented to have free radical scavenging activities. *C. breviscapa* has an IC_{50} value of 71.91 mg/mL, *C. nervosa* has an IC_{50} value of 126 mg/mL, *C. nitida* has an IC_{50} value of 281.7 mg/mL and *C. ovalis* has 65% scavenging activity (Natta *et al*., 2022; Rashmi *et al*., 2015; Sahaya *et al*., 2013; Singh & Kumaria, 2019).

In this present scenario, antibiotic resistance have been a great threat for the public health around the globe (Peterson and Kaur, 2018). Besides, the chemically synthesised antibiotics have side effect on human health. As a suitable alternative, the researchers are searching for the noble bioactive compounds from the traditionally used medicinal plant. Many species of *Coelogyne* have also been documented for the antimicrobial activities. The bioactive compounds present in *Coelogyne* species are active against both the gram-positive and gram-negative bacteria and also against different fungal species. Some of the species of *Coelogyne* reported for antimicrobial activity are *C. cristata* (Marasini & Joshi, 2013), *C. nervosa* (Shibu *et al*., 2013), *C. berviscapa* (Rashmi *et al*., 2015), *C. speciosa* (Buyun *et al*., 2017) and *C. brachyptera* (Buyun *et al*., 2021). Despite of the documentation of the antimicrobial activities of different *Coelogyne* species the identification of the bioactive compounds responsible for the antimicrobial activities are still unidentified. Besides *Coelogyne nitida* still lacks the documentation for the antimicrobial activity and also parts based study for antioxidant activity. Thus, this study aims for the study of antioxidant and antimicrobial activities of flower, leaf and pseudobulb of *Coelogyne nitida* in different solvent fraction.

1.1.3 Micropropagation of *Coelogyne* species

Micropropagation is a technique used to rapidly propagate plants in controlled laboratory condition. The cellular totipotency hypothesis by Gottlieb Haberland (1902) was the womb of micropropagation. Plant tissue culture is a step of micropropagation in which a tiny portion of a plant called an explant is placed in an artificial nutrient solution under an aseptic condition allowing the optimum condition to function and grow (Bridgen *et al.,* 2018). Tissue culture procedures differ depending on the plant material employed. Some examples of tissue culture are seed culture, embryo culture, callus culture and organ culture.

The asymbiotic germination of orchid seeds began in 1992 with the discovery of the Knudson C medium by Lewis Knudson. *In vitro* multiplication of the plant was aided by the discovery of a root-forming substance (Went, 1929) and of kinetin for shoot induction (Skoog and Tsui, 1948). A subsequent study by Toshio Murashige in 1974 demonstrated the importance of the ratio of auxin to cytokinin in causing shoot initiation, root initiation, and the formation of calluses. Similarly, the Murashige and

Skoog (MS) medium was discovered as an optimal medium for the production of callus in tobacco (Murashige and Skoog, 1962). Currently, the MS medium is the most prevalent medium used in the culture of plant tissue.

Many species of *Coelogyne* have been experimented for the *in vitro* propagation and different protocols have been established based on the research. The MS medium with the addition of coconut water gave maximum seed germination in *Coelogyne nervosa* (Abraham *et al*., 2012). Studies showed the presence of substances like cytokinin and zeatin in coconut water (Letham, 1968) which are the reason for its activity (Skoog, 1994). Half MS medium fortified with phytohormones 1-Naphthalene Acetic Acid (NAA) and 6-Benzyl Aminopurine (BAP) is effective for the roots and shoots development of *Coelogyne stricta* (Basker & Bai, 2006). MS medium in combination of 2,4-Dichlorophenoxy Acetic Acid (2-4,D) and/or 6-Benzyl Aminopurine (BA) increased direct embryo development.

Callus formation is possible with the use of IAA and BA (Devi *et al*., 2012). The healthy seedlings of *Coelogyne flaccida* can be produced from the culture of pseudobulb segments (Kaur & Bhutani, 2013) and seed germination (Parmar & Pant, 2015) in MS medium fortified with NAA and BAP. Among the many inoculums types, medium, and growth regulators investigated, seedling culture on MS medium with 1.0 mg/L NAA and BA combinations is the most potential medium composition for *in vitro* production of *Coelogyne cristata* (Naing *et al*., 2011). During the propagation of immature *Coelogyne fuscescens* seeds on MS, the impact of phytohormones BAP (1 mg/L) and NAA (0.5 mg/L) on early symbiotic seed germination was recorded to be synergistically balanced but NAA (0.5 mg/L) alone enhanced differentiation and plantlet development in MS (Koirala *et al*., 2013).

Acclimatization of the *in vitro* grown plantlets is the process of the adaption to ex vitro environment through gradual weaning to relative humidity and low facilities as compared to the *in vitro* controlled condition. The substrate for the acclimatization also varies on the intra-generic species. The *in vitro* developed *Coelogyne stricta* was successfully acclimatized using substrate containing coconut husk, charcoal. Tile, and brick fragments in equal proportion (Basker and Bai, 2006). Parmer and Pant, 2015 reported 80% survival rate of acclimatized *Coelogyne flaccida* Lindl., in substrate containing tree fern powder and *Sphagnum* moss in the ratio 2:1 and 70% survival rate in cocopeat, *Sphagnum* moss and sand in the ratio 2:1:1. Paudel and Pant, 2012

reported 85% survival in *Esmeralda clarkei* in potting mixture consisting of soil, sand and sawdust (1:1:1). From this background study, the potting mixture of Sphagnum moss, cocopeat, sawdust and soil in equal proportion are selected for the acclimatization of the *in vitro* developed *Coelogyne nitida*. 1.1.3 *Coelogyne nitida* (Wall. ex D.Don) Lindl

Figure 1: Habit photo of *Coelogyne nitida* at Panchase Kaski (a) and (b)

Coelogyne nitida is an epiphytic herb sometimes it is a lithophytes. It is commonly known as "Shining Coelogyne". Locally, it is called "Chandigava". The rhizome is rigid and stout. The pseudobulb is oblong or narrowly ovoid oblong and is covered with leathery sheaths figure (1). Generally, two leaves are present at the apex of the pseudobulb. The leaf blade is oblong to elliptic. The leaf surface is densely textured and petiolated at the base. The flower is white with two eyes like coloured blotches. Sepal is oblong with an acuminate apex and the petal is linear or narrowly oblong. Capsule is obovoid to oblong. The inflorescence is proteranthous (eFlora of China, 2009). *Coelogyne nitida* is widely distributed in West, Central and East Nepal at the elevation of 1300-2400 m above sea level.

Traditionally, the paste is taken against headache and fever, paste is applied externally on burns (Joshi *et al.,* 2017). Similarly, the juice of the pseudobulb is used to get the

relief of stomach ache. Regarding the phytochemistry of *Coelogyne nitida*, Ochrolide (phenanthropyrone), Ochrone A (9,10-dihydro-1,4-phenanthraquinone) and coelonin have been isolated (Bhaskar *et al.* 1989, 1991). Similarly, chromic acid (monomer phenanthrene derivative) has been isolated from *C. nitida* (Anuradha *et al.* 1994).

Based on the previous literature, the micropropagation and biological activities of *Coelogyne nitida* are still obscure and it is needed for its conservation and medicinal usage. Thus, this research explores its biological activities and micropropagation.

1.2 Hypothesis

- The presence of secondary metabolites may vary in flower, leaf and pseudobulb of *Coelogyne nitida*.
- The quantitative measurement of total phenolic content, total flavonoid content, and antioxidant activity of *Coelogyne nitida* may differ depending on the plant part (flower, leaf, and pseudobulb) and the fractionation solvents used for the study.
- The germination rate, shoot development and root development of *Coelogyne nitida* seed in MS medium will be different based on the concentration of plant growth regulators.

1.3 Objective

1.3.1 General objective

The general objective of the present research is to propagate the plant through seed culture and to determine the biological activities of the solvent fractions of different parts of wild *Coelogyne nitida.*

1.3.2 Specific objectives

1. To screen secondary metabolites present in *Coelogyne nitida.*
2. To quantify the total phenolic and flavonoid contents in the different fraction of flower, leaf and pseudobulb.
3. To determine the antioxidant and antimicrobial activities of the extract fractions of flower, leaf and pseudobulb.
4. To propagate a large number of plantlets through tissue culture technique and to acclimatize *in vitro* developed plants.

1.4 Rationale

Coelogyne nitida holds a long history of being used in traditional medicinal systems. However, the scientific validation of having medicinal properties is still lacking. This study is useful for unearthing the phytochemically present in the plants and their biological activities. Besides diverse morphology, attractive color and longer life of flowers (a month) have made it a significant contributor to horticulture. But the matter of dismay is that the whole Orchidaceae family is included in Appendix – II of the CITES. The main contributing factors for being endangered might be the low germination rate in natural habitats and illegal collection and trade. Micropropagation can be a suitable alternative for the commercial production and consumption of this orchid. The micro propagation of the *C. nitida* has not been done yet so the plant tissue culture is important for the conservation of this CITES-listed plant.

2. Materials and Methods

2.1 Materials

2.1.1 Plant Materials

Pods, leaves, flowers and pseudobulb of *Coelogyne nitida* were collected from the wild habitat of Panchase forest, Kaski district of Nepal (plant collection permission was taken from the Department of Forest and Soil Conservation, Kathmandu, Nepal). The orchid collected during the field was identified by Prof. Dr. Bijaya Pant and Dr. Mukti Ram Paudel. The herbarium of the collected orchid was submitted to Tribhuvan University Central Herbarium (TUCH) (Voucher specimen no.: 102-2023) and was verified by Prof. Dr. Suresh Kumar Ghimire.

2.1.2 Test organisms

Zoological organism: *Artemia salina*.

Bacterial strain:

Gram-positive bacteria: *Bacillus subtilis* (ATCC 6059), *Staphylococcus aureus* (ATCC 6538P)

Gram-negative bacteria: *Klebsiella pneumonia* (ATCC 8739), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027).

2.1.3. Chemicals

Methanol, dichloromethane, hexane and water of analytical grade were used in the extraction of plant materials. Chemicals such as 2, 2 - Diphenyl - 1 - picrylhydrazyl (DPPH), quercetin, gallic acid, Folin-Ciocalteu (FC) reagent, aluminium chloride ($AlCl_3$), distilled water and sodium chloride are used for the determination of TPC, TFC and antioxidant activity. The chemicals used in this research were the product of Qualigens Fine Chemicals India, HiMedia Lab. Pvt. Ltd., India. Phytohormones such as auxin (IAA, IBA and NAA), cytokinin (BAP) are used for the micropropagation. MS medium was used for micropropagation. Nutrient agar and Mueller-Hinton agar

(MHA) are used for antimicrobial assay. Antibiotics: ciprofloxacin and amoxicillin are used as a positive control for the antibacterial assay.

2.1.4 Equipments and Instruments

A variety of instruments were used during the experiment, including beakers, conical flasks, measuring cylinders, test tubes, funnels, petri plates, vials, separation funnels, micropipettes, eppendrof tubes, culture tubes, scalpels, forceps, and sterile blades. Various instruments were used in the research including a rotator evaporator, water bath (Eyela, Japan), microplate reader, grinder (Baltra, India), refrigerator, laminar air flow cabinet, autoclave, hot air oven and bunsen burner.

2.2. Methods

2.2.1 Phytochemical Extraction

The collected flowers, leaves and pseudobulb of the *Coelogyne nitida* were shade-dried and powdered using a grinder. Dried powder of about 5 g was soaked in 80 mL of methanol and water (7:3 v/v) and retained for 48 hours in the dark at room temperature with continuous shaking. The solution was then filtered using Whatman No.1 filter paper. This extraction procedure was repeated for three times in order to extract all the potential compounds from the soaked powder. The residue was again drenched in water to make the aqueous fraction. The filtrate was then dried under reduced pressure in a rotary evaporator at 150 rpm and 37 °C. The dried crude extract was stored at 4 °C for further solvent-solvent fractionation.

2.2.2 Solvent-Solvent Fractionation

The dried crude extract was diluted in 100 mL distilled water. The aqueous fraction thus formed was then mixed with 200 ml hexane in a separating funnel and shaken vigorously. The separating funnel was left undisturbed until two separate layers appeared. The lower denser aqueous layer was collected separately. The above-mentioned process for hexane was repeated three times. The hexane layer was collected in a separate conical flask. After the hexane fraction, the aqueous fraction was again mixed with 200 mL DCM and shaken vigorously. After the separation of the layers, the lower DCM layer was taken separately in a conical flask and the process was repeated three times. The final aqueous fraction was dried using a rotary

evaporator and mixed with 50% methanol and only the dissolved fraction was taken as methanol fraction. All the hexane, DCM, methanol and aqueous fractions were dried under reduced pressure at 150 rpm and 37 °C. The fractions obtained were then stored at 4°C for future use. The overall extraction process is shown in the figure 2.

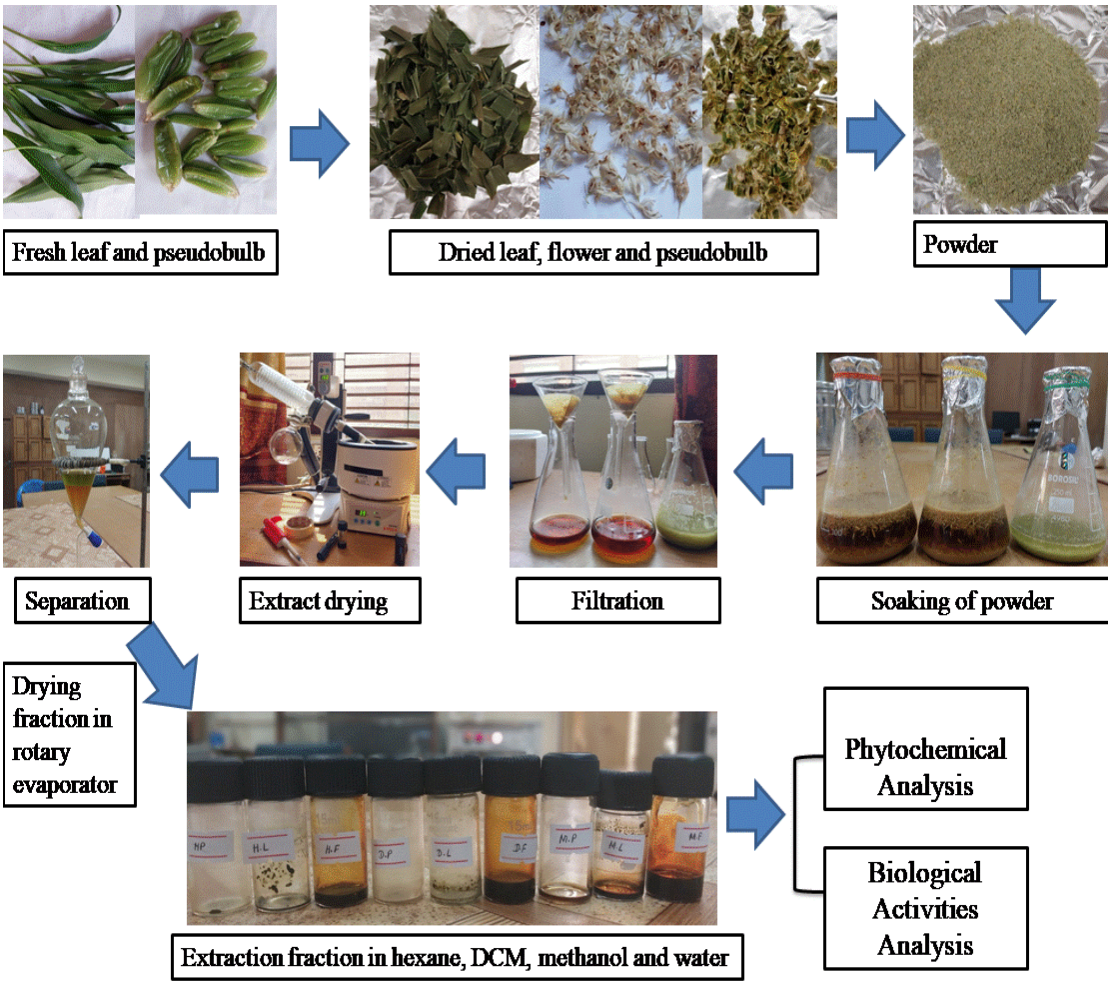

Figure 2: Overall phytochemical extraction process

2.2.3 Extract Yield Estimation

Extract yield is calculated using the following formula;

$$\text{Extract yield (\%)} = \left(\frac{W1}{W0}\right) * 100$$

Where, w_1 = crude extract weight, and w_0 = the initial weight of the dried sample.

2.2.4 Determination of Total Phenolic Content (TPC)

TPC of extract fractions was evaluated using Folin-Ciocalteu reagent as described previously (Ainsworth & Gillespie, 2007; Zang *et al.*, 2006).

2.2.4.1 Preparation of reagents for TPC

FC reagent was prepared by dissolving 1 ml FC reagent in 10 mL distilled water.

Sodium carbonate: 2.12 g of Na_2CO_3 dissolved in 20 mL water to prepare 1M sodium carbonate solution.

2.2.4.2 Gallic acid preparation

The stock solution of gallic acid of concentration 1mg/mL was prepared in ethanol. Six different working concentrations of gallic acid (250, 125, 62.5, 31.25, 15.625, 7.812 µg/mL) was prepared from stock solution.

2.2.4.3 Preparation of plant sample

The stock solution (1 mg/mL) of plant extract was prepared in methanol.

2.2.4.4 Procedure of TPC

Each concentration of serially diluted Gallic acid was loaded in triplicate as standard control. Each extract (20 µL) of 1000 µg/mL concentration was loaded in triplicate. Then 100 µL FC reagents were added to each well containing the plant samples and standard control. Initial absorbance was taken at 765 nm using a microplate reader. Then 80 µL of sodium carbonate was added and incubated for 90 minutes. After 90 min the final absorbance was taken at 765 nm in (Epoch2, BioTek) microplate reader.

2.2.4.5. Calculation of TPC

The TPC of the extract was expressed as milligrams of gallic acid equivalent per gram of dry weight i.e., mg GAE/g. A calibration curve was obtained by plotting the absorbance at different concentrations.

TPC was calculated by using the formula:

$$C = \frac{c * V}{M}$$

C = total phenolic content in milligrams per gram (mg/g) of dry plant material in gallic acid equivalent (GAE).

c = gallic acid concentration calculated from the standard curve in milligrams per millilitres (mg/mL).

V = extract volume in millilitre (mL).

M = extract weight in gram (g)

2.2.5 Determination of Total Flavonoid Content (TFC)

The TFC of *Coelogyne nitida* was determined *via* the Aluminium chloride ($AlCl_3$) colourimetric method by following Chang *et al.* (2002) with slight modifications.

2.2.5.1 Preparation of reagent for TFC

Aluminium chloride (1 g) was dissolved in 10 mL distilled water to make 1M $AlCl_3$ solution. Similarly, 1M potassium acetate was made by dissolving 0.9815 gm in 10 mL distilled water.

2.2.5.2 Preparation of standard quercetin

A stock solution of standard quercetin (80µg/mL) was prepared in methanol. The stock solution was then diluted to different concentrations (80, 70, 60, 50, 40, 30, 20 and 10) µg/mL.

2.2.5.3 Preparation of plant sample

Stock solutions (1 mg/mL) of plant extracts were prepared in methanol. The stock solution was diluted to different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.812) µg/mL.

2.2.5.4 Procedure of TFC

Plant extract (20 µL) of concentration 1000 µg/mL and 110 µL of distilled water was loaded in triplicate to each well. 130 µL different concentration of quercetin was loaded as a standard positive control. Then each well of the plate was loaded with 60 µL of ethanol. The initial absorbance was measured at 415 nm with a microplate reader. Subsequently, five µL 10% $AlCl_3$ and equal volume of potassium acetate were added to each well. The plate is then subjected to incubation for half an hour under dark condition before taking final absorbance at 415 nm.

2.2.5.5 Calculation of TFC

The TFC of the plant fraction extract was expressed as milligrams of quercetin equivalent per gram of dry weight i.e., mg Q/G. A calibration curve was obtained by plotting the absorbance at different concentrations.

TFC was calculated by using the formula:

$$C = \frac{c \times V}{M}$$

C = TFC in milligram per gram (mg/g) of dry plant material in quercetin equivalent (Q).

c = quercetin concentration calculated from the standard curve in milligrams per millilitres (mg/mL).

V = Extract solution volume in millilitre (mL).

M = extract weight in gram (g)

2.2.6 Determination of Antioxidant Activity

The free radical scavenging activity of *Coelogyne nitida* was determined by following the protocol of Blois (1958) and Brand-Williams *et al*. (1995) with slight modification.

2.2.6.1 Preparation of the DPPH solution

2, 2-diphenyl-1-picrylhydrazyl solution (0.2 mM) was made by dissolving 7.88 mg of DPPH in 100 mL ethanol. It was kept in the dark for the antioxidant activity assay.

2.2.6.2 Preparation of standard quercetin

A standard quercetin solution of 1 mg/mL was prepared by dissolving 1 mg of quercetin in 1mL ethanol. The serial dilution was done to get solutions of 100, 80, 60, 40, 20, 10, 5, 2.5, 1.25 µg/mL.

2.2.6.3 Preparation of plant sample

The stock solution (1 mg/mL) of plant extracts was prepared in methanol containing 2.5% DMSO. The working solutions of various concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.812) µg/mL were prepared.

2.2.6.4 Procedure

The blank plate reading of the 96-well plate was measured to check whether the plate was clean or not. Then, 100 µg/mL of plant extract of different concentrations was added in 96-well plate in triplicate and an equal volume of freshly prepared DPPH was added to each well. Negative control was prepared by replacing the plant extract with the same volume of methanol. Quercetin of different concentrations was used as

a positive control. The 96-well plate was kept undisturbed for 30 min in the dark at 37 °C. Absorbance was measured at 517 nm using microplate reader.

2.2.6.5 Calculation of Antioxidant Activity

The antioxidant activities of the samples were calculated using the following formula;

$$\text{Inhibition \%} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The obtained inhibition % values on the Y-axis and concentration on the X–axis was plotted and a standard graph was obtained. Thus, the obtained graphs of linear equations were used to calculate the IC_{50} values.

$$Y = mX + C$$

Where, X = concentration, Y = % inhibition, m = slope of the line and C = Y-intercept of the line.

2.2.7 Brine Shrimp Lethality Test

The lethality test was done using *Artemia salina* following the method of Meyer et al. (1982). The saltwater of 40g/L concentration was used to hatch brine shrimp. Shrimp eggs were added to saline water and left undisturbed at 25ºC temperature for 24 hrs under bright illumination. About 20 alive larvae were treated with plant extract concentrations ranging from 1000 µg/ml to 30 µg/ml. Saltwater was taken as negative control and potassium dichromate was taken as positive control. Observation of the test was done after 24 hours and mortality rate was calculated.

$$\text{Mortality rate (\%)} = \left(1 - \frac{\text{Number of dead larva}}{\text{Number of larva taken}}\right) \times 100$$

The mortality rate of larvae shows the toxicity of the plant extract.

LC_{50} value was determined from the curve of percentage mortality versus log inhibitor concentration. The experiment was done in triplicate and data were expressed as mean ± standard deviation (SD).

2.2.8 Determination of Antimicrobial Activities

The agar well diffusion method was employed to determine the antibacterial activities of *Coelogyne nitida* following the protocol of Ahmad *et al*. (1998) with slight modifications. Two gram-positive and three gram-negative bacterial strains were used

for the study. The diameter of the inhibition zone was measured to calculate the antibacterial activity.

2.2.8.1 Bacterial strains

Five ATCC strains were selected for the determination of the antimicrobial activity. Bacterial strains were provided by the microbiological section of the Natural Product Research Laboratory (NPRL), Thapathali, Kathmandu, Nepal.

Gram positive bacterial strains: *Bacillus subtilis* (ATCC 6059) and *Staphylococcus aureus* (ATCC 6538P).

Gram negative bacterial strains: *Klebsiella pneumonia* (ATCC 8739), *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027).

2.2.8.2 Preparation of medium

a. Nutrient agar plates

Nutrient agar was prepared by adding 28 g of NA powder in 1000 mL distilled water with continuous shaking. Then it was boiled and autoclaved at 121 °C for 15 minutes. The medium thus prepared was allowed to cool up to 50 °C. The medium was aseptically distributed in the sterile petri plate in the ratio of 20 mL per plate and labelled properly.

b. Nutrient broth solution

A nutrient broth solution of 13 g/L was prepared in a conical flask. The flask was then plugged with a cotton plug and sealed with aluminium foil. The broth solution was then distributed in screwed-capped bottles and autoclaved at 15 lb pressure and 121 °C temperature for 15 minutes.

c. Muller Hinton Agar (MHA) plates

MHA medium was prepared by adding 9.5 g of medium in 250 ml distilled water and boiled. The medium was sterilized by autoclaving at 15 lb pressure and 121 °C temperature. The medium was aseptically distributed in petri plates in the ratio of 20 mL per plate and labelled properly. Plates were left undisturbed for solidification.

2.2.8.3 Preparation of plant extract

Plant extract (50 mg) was dissolved in 1 mL water with 50% DMSO.

2.2.8.4 Preparation of the standard culture inoculums

a. Broth culture

The first step was broth culture. Broth culture was done by using the quadrant streaking method. A sterile inoculation loop was used to take bacteria from the stock or specimen and gently streaked on the nutrient agar medium. The plate was then incubated for 24 hours at 35±2 °C. The quadrant streaking method dilutes the bacterial sample or inoculums on nutrient medium by successive streaking and hence forming a discrete colony on the NA surface as shown in Figure 3.

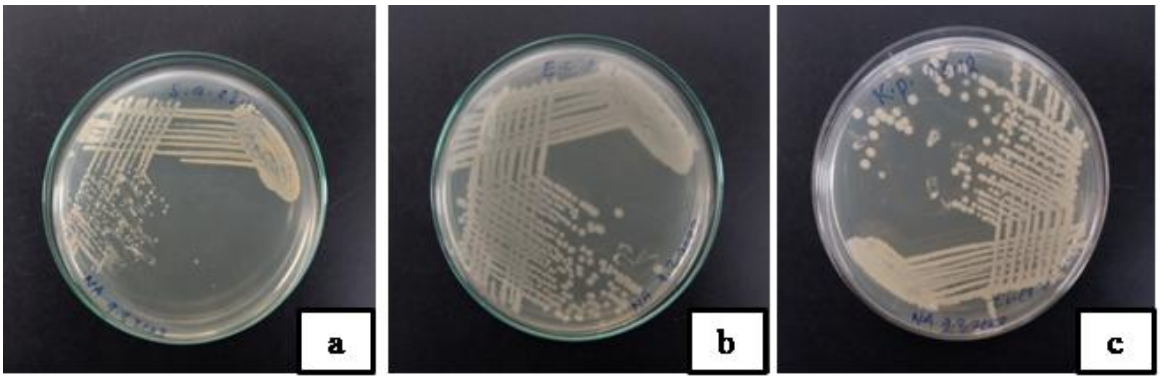

Figure 3: Quadrate streaking method for inoculum dilution

2.2.8.5 Bacterial suspension preparation

After 24 hrs bacterial suspension was made in normal saline (85% NaCl). The turbidity of the solution was kept consistent at 0.5 McFarland standards.

2.2.8.6 Carpet culture

Carpet culture was done in MHA agar plates. A sterile cotton swab dipped in the suspension was swabbed carefully all over the plate. The plate was rotated at 60° after each final swabbing. At last, the swabbing was done along the edge of the plate. The inoculated plates with the half lid open were left to dry for a few minutes inside the cabinet.

2.2.8.7 Preparation of the test plates and screening of antimicrobial activity

After the plates were dried, 3 wells were made in each plate using a sterile 6 mm cork-borer and labelled properly. Out of three wells, one was loaded with 20 µL plant extract; the second one with DMSO as negative control (N) and the third one with positive control (P). Amoxycillin 10 µg for *S. aureus* and ciprofloxacin 5 µg for all

other bacterial strains was used as positive control. The plates were incubated at 35±2 °C for 24 hours. After 24 hrs, a clear zone was observed around the wells with plant extract and positive control. The clear zone was the inhibition zone. The antimicrobial activity was defined in terms of the average diameter of the zone of inhibition in millimetres. The zone of inhibition was measured with a vernier calliper. The average mean of the data was recorded as the antimicrobial activity of the respective plant extract.

2.2.9 Micropropagation

2.2.9.1 Medium preparation

The Murashinge and Skoog medium (1962) was highly used for the *in vitro* propagation of many orchids and different species of the *Coelogyne* genus. Thus, MS medium in combination with different phytohormones was used for the culture of *Coelogyne nitida*. MS medium of one litre was prepared in a conical flask of two litres capacity by adding 100 mL macronutrients (Stock A), 1 mL micronutrients (Stock B), 10 mL iron EDTA (Stock C), 1 mL vitamins (Stock D), 30 g sucrose and 0.1 g myo-inositol. The solution was homogenized using a magnetic stirrer and the final volume was maintained at 1000 mL by adding distilled water. The medium was distributed equally in 10 glass jars and each jar was added with 0.8 g agar and necessary phytohormones. Each jar was covered with aluminium foil and made air-tight with rubber. The jars were kept in a micro-oven for 4-5 minutes for melting agar. MS medium of one jar was equally distributed in 6 culture tubes. The opening of the culture tube was covered with aluminium foil, labelled and made air-tight with rubbers. The medium containing culture tubes and jars was sterilized in an autoclave for 15 min at 121 ℃ and 15 Ib/sq. inch pressure. Afterwards, the tubes and jars were shifted to the culture chamber and culture tubes were kept in a slanting orientation.

2.2.9.2 Hormones and their preparation

Auxin (IAA, IBA and NAA) and cytokinin (BAP) were used for the shoot development and root development in the *in vitro* culture of *Coelogyne nitida*. For the preparation of hormones 5.71 mg of IAA, 4.90 mg of IBA, 5.37 mg of NAA and 4.44 mg were in dissolved a few drops of 1N NaOH in different falcon tubes and volume was maintained at 50 mL by adding sterile water. Thus, prepared hormones were stored in refrigerator for further use.

2.2.9.3 Sterilization of glass-wares and metal instruments

The glass jars, culture tubes, petri plates, beakers and conical flask were dipped into the detergent water for 24 hours and washed with tap water. The glass wares and other metal instruments (forceps and scalpels) were wrapped in aluminium foil and then autoclaved at 121°C for 15 minutes at 15 Ib/Sq. inch pressure. All instruments were oven-dried at 150°C for one hour before using them.

2.2.9.4 Surface sterilization of seed pods

The collected seed pods were cleansed with tween-20 and left in running tap water for 45 minutes. Then the pods were dipped in 0.1% sodium hypo-chloride for 10-15 minutes and washed with distilled water 5 times. The pods were subsequently transferred to the cabinet with laminar airflow then were dipped in 70% ethanol for 1 minute and washed with distilled water.

2.2.9.5 Inoculation and maintenance of culture

The laminar airflow cabinet was sterilized with 70% ethanol and all the necessary material including media containing tubes, forceps, scalpels, Petri plates, tissue paper, spray bottle, sterile water, aluminium foils and rubbers were kept inside. The UV light was turned on for 45 minutes. After 45 minutes air blower was turned on and the inoculation process was started. The forceps and scalpels were sterilized by dipping into the spirit and burning up to red hot condition. The surface sterilized pods were kept in petri plates containing filter paper to absorb moisture and then flame sterilized. The filter paper of the petri plate was replaced and the seed pod was cut longitudinally with the sterile surgical blade. The seeds were scooped out and cultured in different mediums. Each type of medium contains six replicates. The cultures were maintained at 25±2 ℃ and 16/8 hours (light/dark) photoperiods using white fluorescent tubes. The cultures were regularly monitored for recording the germination rate. The protocorms were further sub-cultured for shoot and root development.

2.2.9.6 Shoot and Root Induction

MS medium supplemented with BAP, CW, NAA and in combination was used for the shoot proliferation. Similarly, MS medium supplemented with IAA, IBA, NAA and in combination was used for root induction. Each medium combination contains six replicates. The culture condition was maintained at 25±2 ℃ and 16/8 hours

(light/dark) photoperiods using white fluorescent tubes. The cultures were regularly monitored and data were taken every month.

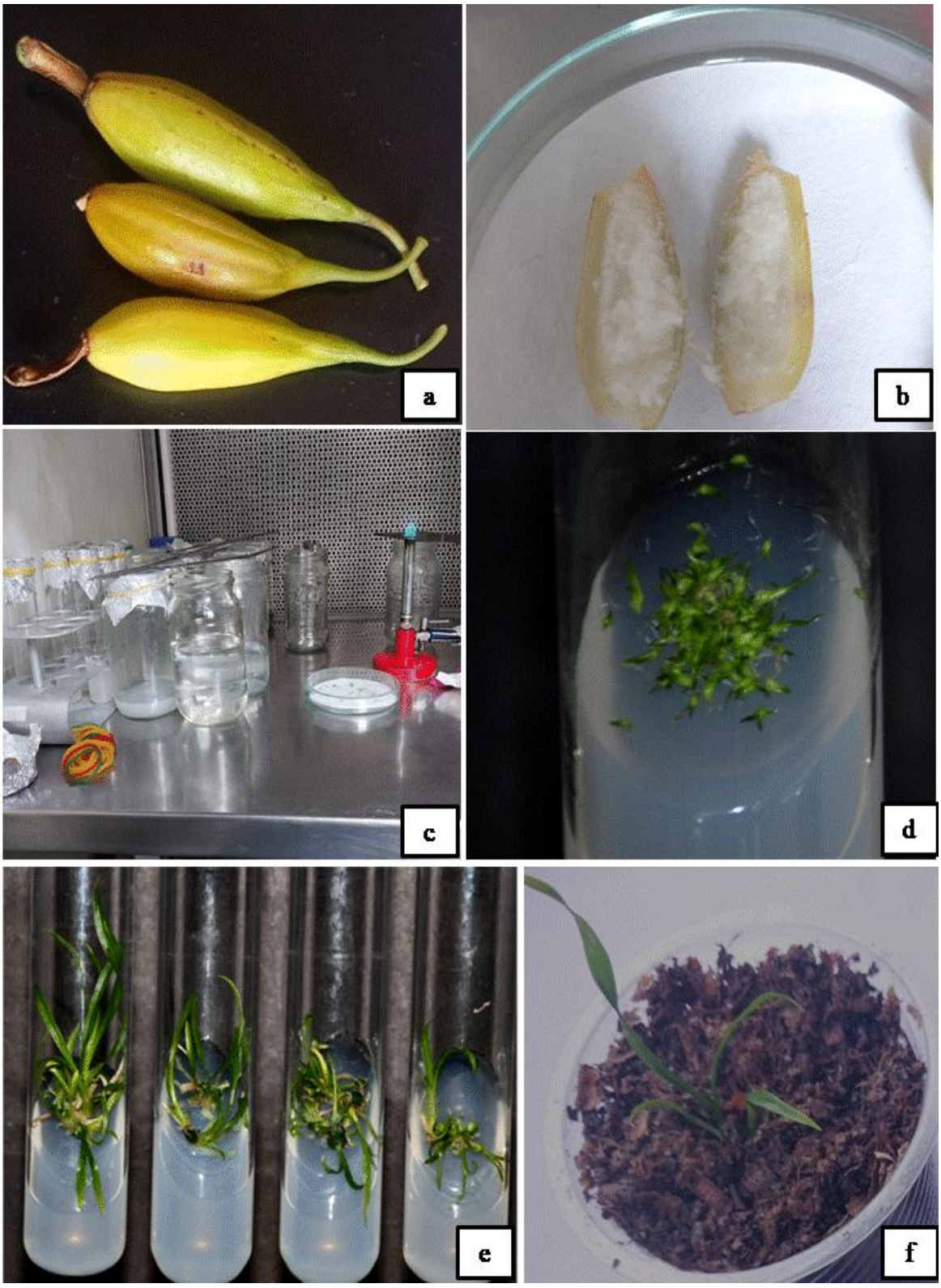

Figure 4: Micropropagation of *Coelogyne nitida* through seed culture

(a. Seed pod b. longitudinal section seed pod c. laminar air flow cabinet for seed inoculation d. Seed germination e. Shoot and root development f. Acclimatization)

2.2.9.7 Acclimatization

Acclimatization of well-grown plantlets was done in pots containing soil, coco peat, sawdust and *Sphagnum* moss in equal proportion. At first, the test tubes containing well-developed plantlets of 4-6 cm height were kept in normal room for few days. Then plantlets were taken out carefully from the culture tubes and washed with water to remove the traces of the medium present in the root. Then the plantlets were treated with fungicide (Bavistin) and washed carefully with distilled water. The *in vitro* developed roots were removed and basal parts of the plantlets were dipped in the rooting hormone rotex. The plantlets were then planted in the pots containing soil, coco peat, sawdust and *Sphagnum* moss in a ratio of 1:1:1:1. Pots were placed in shaded condition at a temperature of 26-27°C with humidity of (70-80%) and indirect sunlight. The whole micropropagation of *Coelogyne nitida* is shown in Figure 4.

2.2.10 Statistical Analysis

A conventional linear equation was used to estimate the total polyphenol and flavonoid contents. The Duncan multiple-range test was employed at $p \leq 0.05$ to determine the significance of differences in total phenol, flavonoid content and mean IC_{50} values of antioxidant activity. Standard equations for gallic acid and quercetin were obtained using a linear regression model. The LC_{50} value of toxicity test was determined using the linear regression equation and probit value. The root and shoot development based on the number and length was recorded and the monthly data was analysed using the Duncan test at $p \leq 0.05$ significance. All measurements were conducted in triplicate, and the data were collected in MS Excel 2019 and analysed using SPSS software.

3. Results

3.1 Extract Yield

Flower of *Coelogyne nitida* had the highest extract yield (33.09%) followed by leaf (24.52%) and pseudobulb (6.52%). In fractionation, pseudobulb and flower had the highest extract yield in methanol i.e. 2.64% and 14.33% respectively whereas leaf had the highest extract yield in DCM fraction (11.90%). The lowest extract yield of the flower was found in aqueous extraction (2.20%). Similarly, the DCM fraction of pseudobulb (0.45%) and hexane fraction of leaf (2.08%) were reported to have the lowest extract yield. The extract yield of the leaf, pseudobulb and flower in different solvents is tabulated below in Table 1.

Table 1: Extract yield of flower, leaf and pseudobulb after fractionation

Parts	Hexane	DCM	Methanol	Aqueous	Total
Flower	5.39%	11.17%	**14.33%**	2.20%	33.09%
Leaf	2.08%	**11.90%**	8.31%	2.23%	24.52%
Pseudobulb	1.29%	0.45%	**2.64%**	2.14%	6.52%

3.2 Phytochemical Screening of *Coelogyne nitida*

The phytochemical screening of crude extract of flower, leaf and pseudobulb of *Coelogyne nitida* revealed the presence of flavonoid, steroids, glycosides, terpenoids, saponins and carbohydrates. Alkaloid and protein were absent in all three parts. Volatile oil was found in the flower and fixed oil in the leaf and pseudobulb. Anthocyanin was slightly present (Table 2).

Table 2: Phytochemical screening of flower, leaves and pseudobulb

S.N	Phytochemical test	Flower	Leaf	Pseudobulb
1.	Fixed oil	_	+	+
2.	Volatile oil	+	_	_
3.	Alkaloid	_	_	_
4.	Flavonoid	+	+	+
5.	Steroids	+	+	+
6.	Glycosides	+	+	+
7.	Anthocynin	Slightly	Slightly	Slightly
8.	Terpenoids	+	+	+
9.	Saponins	+	+	+
10.	Tannins/phenolics	+	+	_
11.	Carbohydrate	+	+	+
12.	Proteins	_	_	_

Legend: "+*"* indicates *Presence* and*"-"* indicates *Absence*

3.3 Total Phenolic Content (TPC)

The TPC of different fractions of flower, leaf and pseudobulb of *Coelogyne nitida* was calculated in terms of gallic acid equivalence (mg GAE/g). The TPC of the extract is calculated using a linear regression equation (Y= 0.008x+0.265, R^2 = 0.998) obtained from the calibration curve of gallic acid (Figure 5).

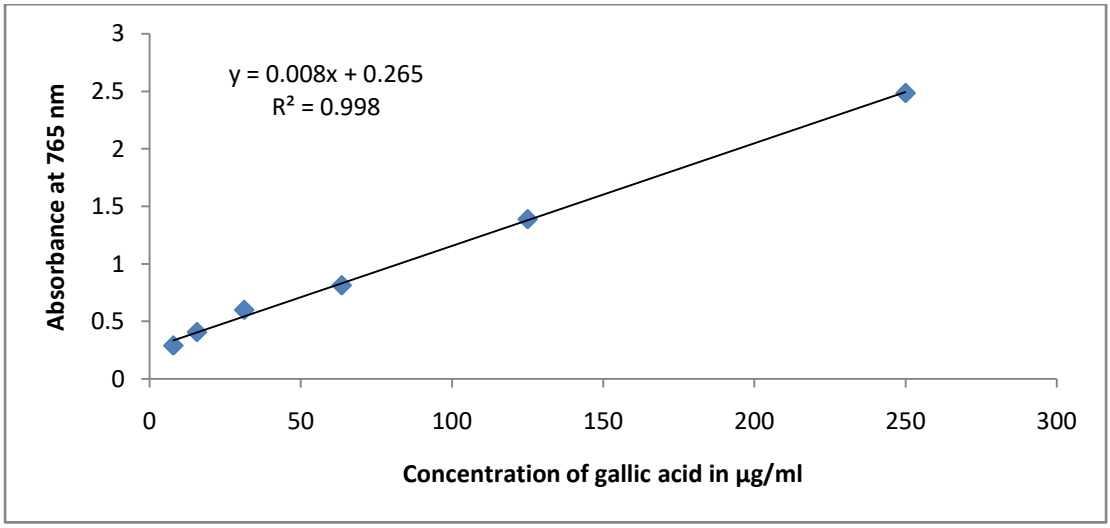

Figure 5: Calibration curve of gallic acid

The highest TPC was found in DP (DCM fraction of pseudobulb) 272.12 mg GAE/g followed by MP (methanol fraction of pseudobulb) 255.25 mg GAE/g and ML (methanolic fraction of leaves) 219.34 mg GAE/g. The lowest TPC was found in AF (aqueous fraction of flower) 163.34 mg GAE/g and MF (methanolic fraction of flower) 169.87 mg GAE/g. The total phenolic content of different parts of *Coelogye nitida* in different solvents is shown in figure 6 and is tabulated below in Table 3.

Table 3. TPC of flower, leaf and pseudobulb of *Coelogyne nitida* in different solvent.

Extract	Total phenolic content (mg QE/g) (Mean±SE)
DCM Flower (DF)	191.58± 6.4^{c}
Hexane Flower (HF)	197.79± 5.05^{d}
Methanol Flower (MF)	169.87± 0.21^{a}
Aqueous Flower (AF)	163.34± 0.72^{a}
DCM Pseudobulb (DP)	**272.12**± 6.0^{g}
Hexane Pseudobulb (HP)	181.50± 0.28^{b}
Methanol Pseudobulb (MP)	255.25± 0.40^{f}
Aqueous Pseudobulb (AP)	181.75± 3.97^{b}
DCM leaf (DL)	200.87± 2.27^{d}
Hexane leaf HL)	180.67± 4.62^{b}
Methanol leaf (ML)	219.34± 5.24^{e}
Aqueous leaf (AL)	193.42± 7.16^{cd}

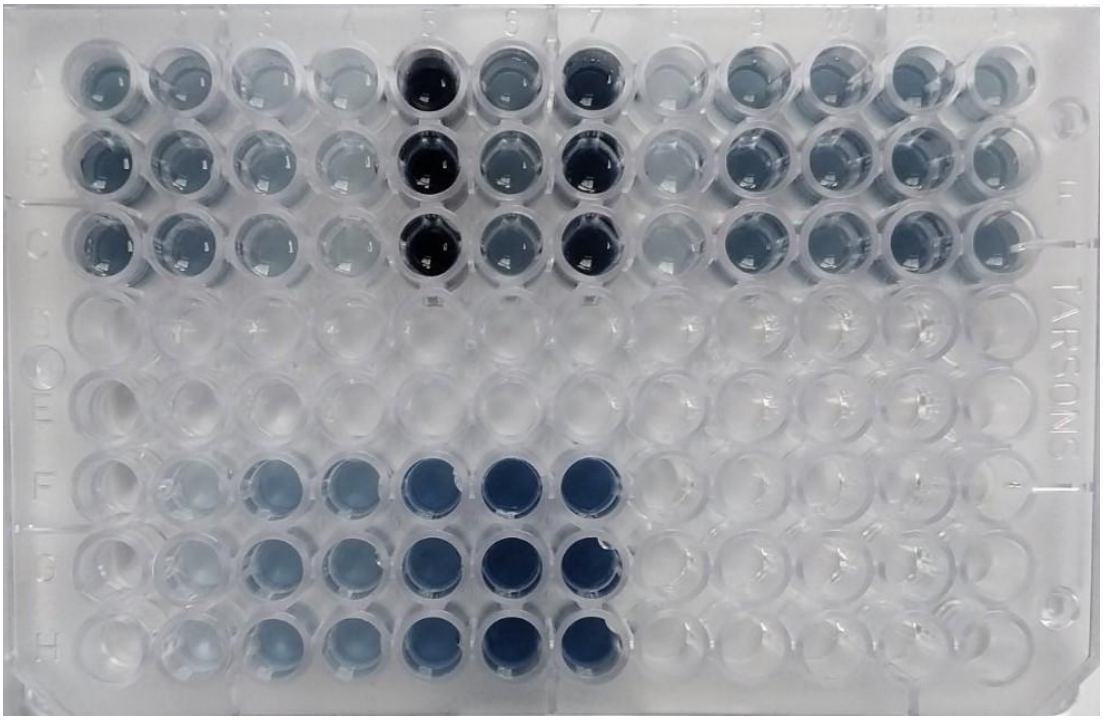

Figure 6: Total phenolic content determination in different extracts.

(1. DF; 2. HF; 3. MF; 4. AF; 5. DP; 6. HP; 7. MP; 8. AP; 9. DL; 10. HL; 11. ML and 12. AL in triplicate (A-C); positive control F-H.)

3.4 Total Flavonoid Content (TFC)

The TFC of different fractions of flower, leaf and pseudobulb of *Coelogyne nitida* was calculated in terms of quercetin equivalence (mg Q/g). The TFC of the fractions is calculated using a linear regression equation (Y= 0.019x+0.033, R^2 = 0.994) obtained from the calibration curve of quercetin (Figure 7).

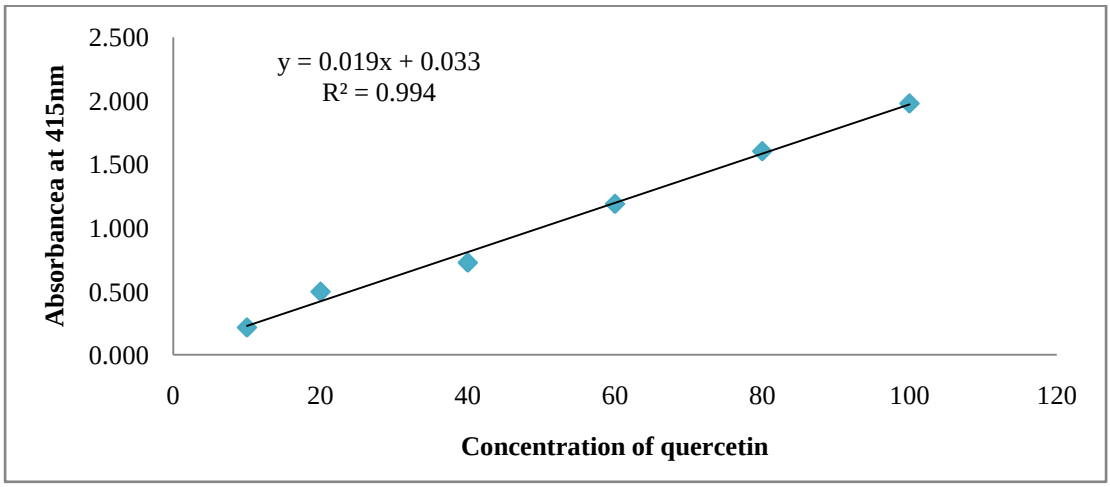

Figure 7: Calibration curve of quercetin

The highest total flavonoid content was found in the DP (DCM fraction of pseudobulb) 10.23mg QE/g followed by MP (methanol fraction of pseudobulb) 8.45mg QE/g and DF (DCM fraction of flower) 6.20mg QE/g. The total flavonoid content of *Coelogyne nitida* in different solvents is shown in Figure 8 and data is tabulated below in Table 4.

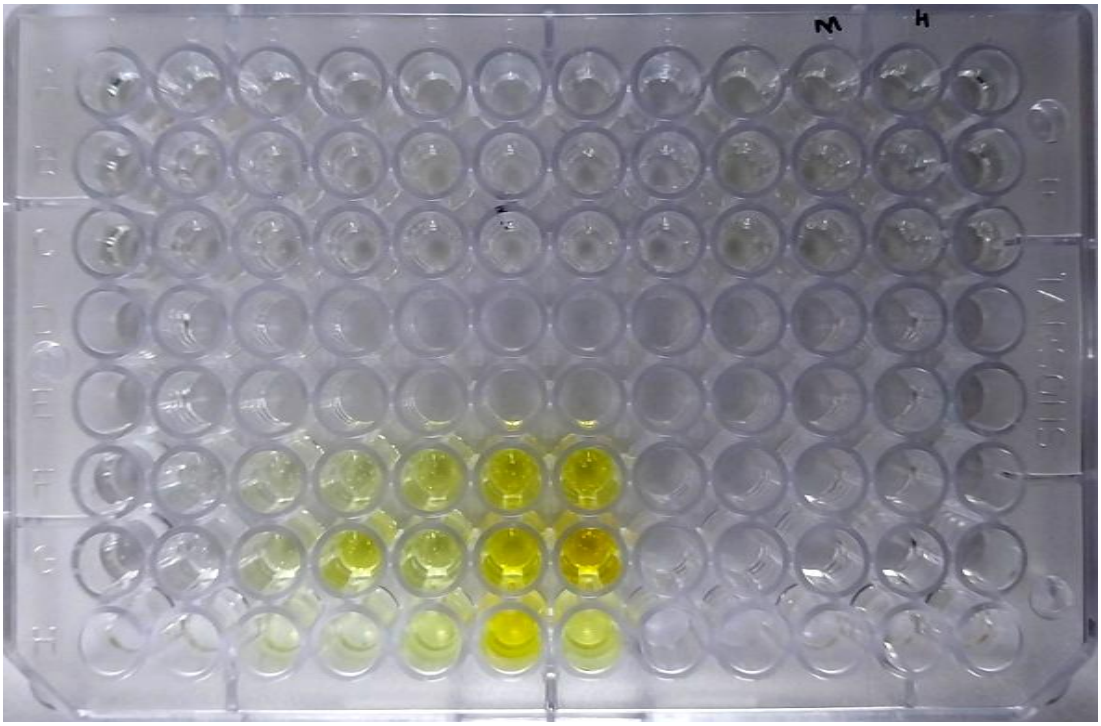

Figure 8: TFC 96 well-plate after taking absorbance

The highest TFC is in the pseudobulb followed by the flower and the least in the leaf.

Table 4: TFC of flower, leaf and pseudobulb of *Coelogyne nitida* in different solvents

Extract	Total flavonoid content (mg QE/g) (Mean±SE)
DCM Flower (DF)	6.20 ± 0.20^{f}
Hexane Flower (HF)	3.60 ± 0.23^{b}
Methanol Flower (MF)	3.35 ± 0.05^{b}
Aqueous Flower (AF)	1.26 ± 0.34^{a}
DCM Pseudobulb (DP)	$\mathbf{10.23\pm0.30^{h}}$
Hexane Pseudobulb (HP)	5.04 ± 0.26^{e}
Methanol Pseudobulb (MP)	8.45 ± 0.16^{g}
Aqueous Pseudobulb (AP)	1.75 ± 0.15^{a}
DCM leaf (DL)	4.54 ± 0.06^{d}
Hexane leaf HL)	3.96 ± 0.2^{c}
Methanol leaf (ML)	4.79 ± 0.18^{e}
Aqueous leaf (AL)	4.34 ± 0.05^{d}

3.5 Antioxidant Activity

The antioxidant activities of flower, leaf and pseudobulb of *Coelogyne nitida* was determined by using DPPH free radical scavenging activity. DPPH is a very stable free radical with a solid purple colour. DPPH loses the intense purple colour on receiving the electron from the antioxidant compound and this transition is measured by absorbance at 517 nm. The antioxidant activity was calculated by plotting a linear graph of the concentration and inhibition percentage and expressed as IC_{50}. The linear curve of quercetin was taken as standard. The IC_{50} of the extract and standard quercetin was calculated using MS Excel 2019.

Among the flower, leaf and pseudobulb, the DCM fraction of the pseudobulb shows the highest antioxidant activity with an IC_{50} value 51.98 µg/mL followed by the DCM fraction of the flower (DF) and methanol fraction of the pseudobulb (MP) with an IC_{50} value of 62.16 and 63.45 µg/mL respectively. The lowest antioxidant activity was shown by the aqueous fraction of the pseudobulb (AP) with an (IC_{50}) value of 764.65 µg/mL.

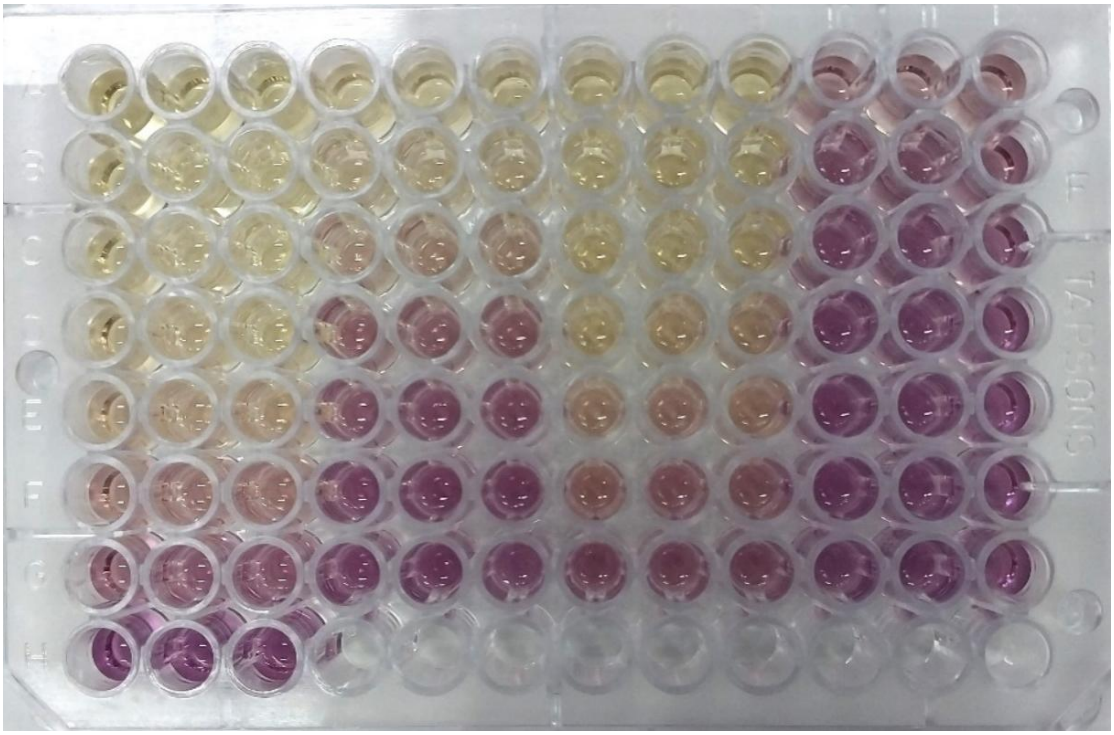

Figure 9: Antioxidant activity 96 well-plate after 30 min incubation (Pseudobulb)

In the leaf, the lowest IC_{50} value was recorded in the DCM fraction with an IC_{50} value 215.57±0.68 µg/mL. The highest IC_{50} value was in aqueous fraction i.e., 422.27 µg/mlL. The figure 9 shows the colorimetric change in different concentrations of

pseudobulb extracts andetail antioxidant activity of different fractions of *Coelogyne nitida* is tabulated in Table 5.

Table 5 Antioxidant activity IC_{50} of flower, leaf and pseudobulb in different solvents

Extract	Antioxidant activity IC_{50} value (Mean±SE)
DCM Flower (DF)	62.16 ± 1.90^{b}
Hexane Flower (HF)	174.32 ± 1.61^{c}
Methanol Flower (MF)	218.34 ± 1.78^{e}
Aqueous Flower (AF)	384.61 ± 0.82^{h}
DCM Pseudobulb (DP)	**49.96 ± 2.36^{a}**
Hexane Pseudobulb (HP)	209.64 ± 0.86^{d}
Methanol Pseudobulb (MP)	63.45 ± 0.36^{b}
Aqueous Pseudobulb (AP)	764.65 ± 1.15^{j}
DCM leaf (DL)	215.57 ± 0.68^{e}
Hexane leaf HL)	299.30 ± 1.64^{f}
Methanol leaf (ML)	356.74 ± 1.67^{g}
Aqueous leaf (AL)	422.27 ± 1.67^{i}

*N =3; Values with different alphabets differ significantly at P<0.05 (Duncan test)

3.6 Brine Shrimp Lethality Test

A lethality test was done in order to determine the toxicity of all fractions of leaf, flowers and pseudobulb of *Coelogyne nitida*. The LC_{50} value of all the fractions was found >1000 mg/mL except that of the hexane fraction of pseudobulb (HP) which had LC_{50} value of 397.48µg/mL

3.7 Antimicrobial Activity

The antimicrobial potential of *Coelogyne nitida* was determined by screening extract fraction of flower, leaf and pseudobulb against two gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and three gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*). The DCM fraction of pseudobulb (DP) shows strong antibacterial activity against gram-positive bacteria *B. subtilis* with 11.75 mm ZOI and moderate against *Staphylococcus aureus* with 11.48 mm ZOI. Similarly, the hexane fraction of pseudobulbs (HP) also shows moderate antibacterial activity against *Bacillus subtilis.* All other fractions of flower, leaves and pseudobulb do not show any inhibition response against the bacterial growth.

Table 6: Antibacterial activity of flower, leaf and pseudobulb extracts in different bacteria.

Bacteria	Zone of Inhibition (mm)												
	Flower				Leaf				Pseudobulb				Positive Control
	D_F	H_F	M_F	A_F	D_L	H_L	M_L	A_L	D_P	H_P	M_P	A_P	
Bacillus subtilis	-	-	-	-	-	-	-	-	11.75	7.5	-	-	33.03±0.8
Staphylococcus aureus	-	-	-	-	-	-	-	-	11.48	-	-	-	32.28±0.66
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-	29.41±0.87
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	31.97±0.43
Klebsiella pneumonia	-	-	-	-	-	-	-	-	-	-	-	-	22.08±0.75

3.8 Plant Tissue Culture

The seed culture of *Coelogyne nitida* was done with the aim to record data about the seed germination, protocorm development, shoot and root formation and acclimatization. After the inoculation of the seed, the white colour of the seed gradually changes its colour to light yellow in the first three weeks and finally to green in the fourth and fifth weeks. The first seed germination was seen in MS media enriched with BAP (1 mg/L) and NAA (0.1 mg/L). The protocorm formation takes place from the seventh week of inoculation. There was no response in MS medium supplemented with 2.5 mg/L BAP and 0.1 NAA (**BN_5**). The late response was recorded in the MS medium supplemented with 10% coconut water (**CW_1**) (Table 7).

Table 7: Germination rate of *Coelogyne nitida*

S.N.	Medium + PGRs			Medium code	Germination rate by surface coverage
	BAP	NAA	C.W (%)		
1.	0.5	0.1	-	BN-1	35%
2.	1.0	0.1	-	BN-2	85%
3.	1.5	0.1	-	BN-3	60%
4.	2	0.1	-	BN-4	95%
5.	2.5	0.1	-	BN-5	-
6.	0.5	0.5	-	BN-6	85%
7.	1.0	0.5	-	BN-7	70%
8.	1.5	0.5	-	BN-8	60%
9.	2	0.5	-	BN-9	30%
10.	-	-	10	CW-1	10%
11.	-	-	15	CW-2	50%
12.	-	-	20	CW-3	75%
13.	-	-	-	F	30%

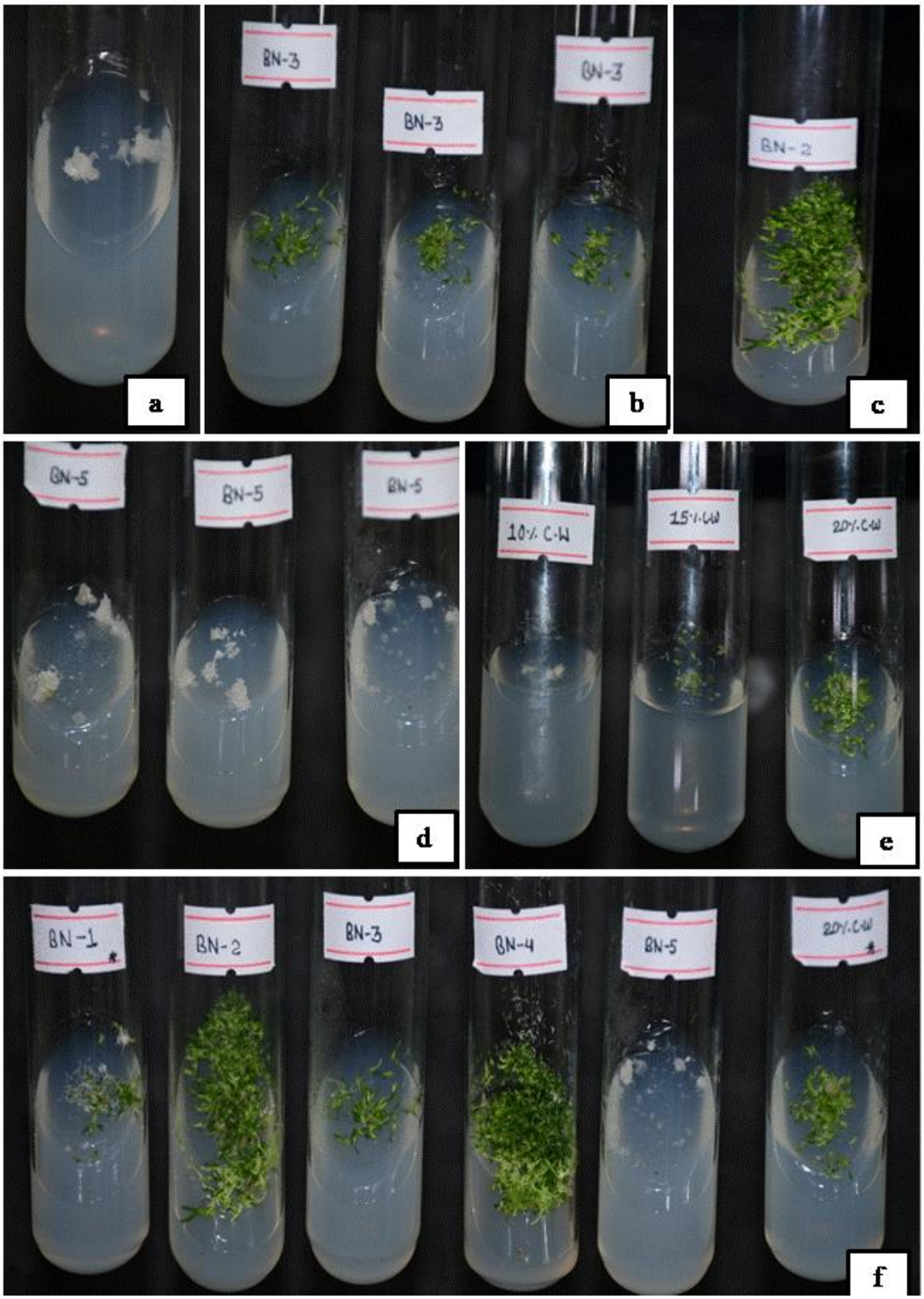

Figure 10: Germination of *Coelogyne nitida* seed in MS medium with different concentrations of PGRs and C.W

3.8.1 Shoot Development

Shoot formation takes was observed after 25 weeks of inoculation. The shoot formation first occurred in MS medium enriched with 2 mg/L BAP and 0.1 mg/L

NAA (**BN_4**) followed by MS medium fortified with 1 mg/L BAP and 0.1 mg/L NAA (**BN_2**) and MS medium containing 0.5 mg/L BAP and 0.5 mg/L NAA (**BN_6**) (Figure 11 & 12). The shoot formation was observed late in MS medium supplemented with coconut water.

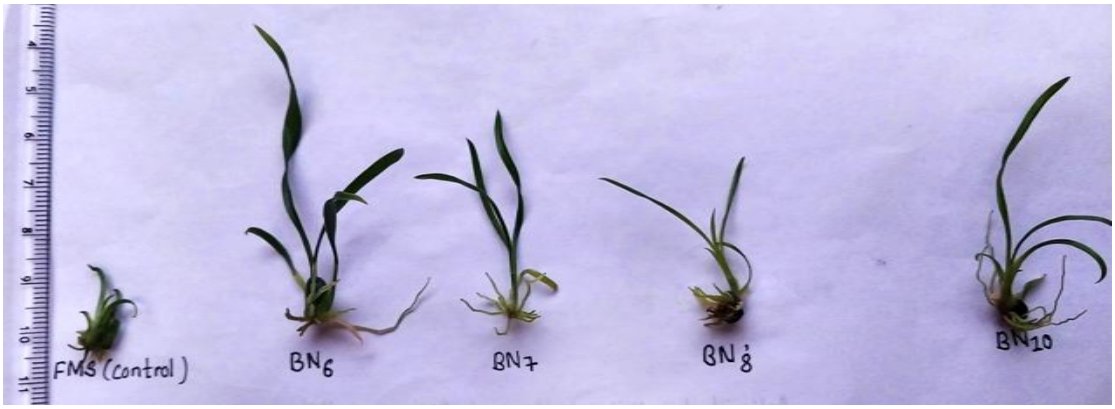

Figure 11: Best shoot development in MS medium with BAP and NAA

Figure 12: Comparison of best shoot development with control

The shoot development of *Coleogyne nitida* in MS medium in combination with different concentrations of BAP, BAP + NAA and organic additive (coconut water) and their combination is tabulated below in Table 8.

Table 8: Shoot development in different phytohormones

Medium Used	Medium code	Shoot Number (Mean±SE)	Shoot Length (Mean±SE) cm
FMS	**F**	**2.67±0.34^{a}**	**1.97±0.9^{b}**
FMS + 0.1 BAP	B_0	3.00±0.58^{b}	2.33±0.9^{d}
FMS + 0.5 BAP	B_1	5.00±1.00^{d}	2.40±0.06^{d}
FMS + 1 BAP	**B_2**	**6.00±0.58^{e}**	**3.23±0.08^{g}**
FMS + 1.5 BAP	B_3	3.00±0.58^{b}	2.46±0.09^{d}
FMS + 2 BAP	B_4	8.00±0.58^{g}	3.66±0.09^{h}
FMS + 0.5 BAP + 0.5 NAA	**BN_6**	**10.00±0.58^{h}**	**6.50±0.11^{j}**
FMS + 1 BAP + 0.5 NAA	BN_7	7.33±0.34^{f}	4.36±0.12^{i}
FMS + 1.5 BAP + 0.5 NAA	BN_8	6.66±0.34^{f}	3.50±0.15^{g}
FMS + 2 BAP + 0.5 NAA	BN_9	5.00±0.58^{d}	2.30±0.06^{c}
FMS + 0.5 BAP + 1 NAA	**BN_{10}**	**7.00±0.58^{f}**	**4.50±0.11^{i}**
FMS + 0.5 BAP + 1.5 NAA	BN_{11}	4.66±0.34^{c}	2.13±0.14^{b}
FMS + 0.5 BAP + 2 NAA	BN_{12}	3.66±0.34^{b}	1.90±0.06^{a}
FMS + 5% CW	C_1	2.66±0.34^{a}	2.23±0.06^{c}
FMS + 10% CW	C_2	4.33±0.34^{c}	2.36±0.20^{d}
FMS + 20% CW	**C_3**	**5.33±0.34^{d}**	**2.63±0.09^{f}**
FMS + 0.5 BAP + 0.5 NAA+ 5% CW	BNC_1	3.66±0.34^{b}	1.80±0.06^{a}
FMS + 0.5 BAP + 0.5 NAA+ 10% CW	BNC_2	4.66±0.34^{c}	2.60±0.06^{f}
FMS + 0.5 BAP + 0.5 NAA+ 20% CW	**BNC_3**	**6.33±0.34^{e}**	**2.80±0.06^{f}**

*N =3; Values with different alphabets differ significantly at P<0.05 (Duncan test)

Among different concentrations of BAP in MS medium tested for the shoot development 1 mg/L BAP gave better results with 8.00±0.58 shoot number and 3.66±0.00 cm shoot length. The maximum shoot number (10.00±0.58) and shoot length (6.50±0.11 cm) were recorded in the 0.5 mg/L BAP and 0.5 mg/L NAA followed by 0.5 mg/L BAP and 1 mg/L NAA with shoot number (7.00±0.58) and shoot length (4.50±0.11 cm**)** In this study, the coconut water was used as the organic additive and 20% coconut in MS medium water gave better result (shoot number 5.33±0.34 and shoot length 2.63±0.09 cm) in comparison with the other concentration of the of coconut water. The effect of coconut water in addition to the same concentration of BAP and NAA i.e., 0.5 mg/L was also tested and the best result was recorded in 20% coconut water with shoot number (5.33±0.34**)** and shoot length (6.33±0.34 cm)among the tested medium.

3.8.2 Root Development

First root formation was observed in MS medium containing 0.5 mg/L BAP and 0.5 mg/L NAA (**BN_6**) followed by MS medium with 1mg/L IBA (**IB_2**) and MS medium with 1 mg/L IBA (**IA_2**) after 25 weeks of inoculation (Figure 13).

Figure 13: Root development in IBA and IAA

The exact picture of the root development is tabulated below in Table 9.

Table 9: Root development in different phytohormones

Table edium Used	Medium code	Root Number (Mean±SE)	Root Length (Mean±SE)
FMS	**F**	**2.67±0.34**	**0.40±0.05^{a}**
FMS + 0.1 NAA	N_0	4.00±0.57^{b}	0.44±0.03^{a}
FMS + 0.5 NAA	N_1	5.34±0.34^{c}	0.50±0.05^{a}
FMS + 1 NAA	**N_2**	**6.00±0.57^{e}**	**1.00±0.05^{e}**
FMS + 1.5 NAA	N_3	3.34±0.67^{a}	0.60±0.05^{b}
FMS + 2 NAA	N_4	3.00±0.57^{a}	0.40±0.05^{a}
FMS + 0.5 BAP + 0.5 NAA	**BN_6**	**26.00±0.57^{p}**	**2.80±0.05^{i}**
FMS + 1 BAP + 0.5 NAA	**BN_7**	**18.00±0.57^{o}**	**2.27±0.08^{h}**
FMS + 1.5 BAP + 0.5 NAA	BN_8	8.67±0.34^{m}	1.13±0.08^{f}
FMS + 2 BAP + 0.5 NAA	BN_9	6.67±0.34^{g}	1.53±0.08^{g}
FMS + 0.5 BAP + 1 NAA	**BN_{10}**	**15.00±0.15^{n}**	**2.57±0.08^{i}**
FMS + 0.5 BAP + 1.5 NAA	BN_{11}	7.00±0.57^{h}	0.90±0.05^{d}
FMS + 0.5 BAP + 2 NAA	BN_{12}	5.67±0.34^{d}	0.60±0.05^{b}
FMS + 0.1 IAA	IA_0	4.34±0.34^{b}	0.70±0.11^{c}
FMS + 0.5 IAA	IA_1	5.00±0.57^{bc}	0.60±0.17^{b}
FMS + 1 IAA	**IA_2**	**6.00±0.57^{e}**	**1.03±0.17^{f}**
FMS + 1.5 IAA	IA_3	5.34±0.88^{c}	0.53±0.08^{a}
FMS + 2 IAA	IA_4	4.67±0.88^{b}	0.67±0.09^{c}
FMS + 0.5 BAP + 0.5 IAA	IA_5	6.67±0.20^{g}	1.00±0.012^{f}
FMS + 0.5 BAP + 1IAA	**IA_6**	**8.00±0.57^{l}**	**1.80±0.06^{g}**
FMS + 0.5 BAP + 1.5 IAA	IA_7	6.00±0.57^{e}	1.10±0.012^{f}
FMS + 0.5 BAP + 2 IAA	IA_8	6.34±0.88^{f}	1.067±0.07^{f}
FMS + 0.1 IBA	IB_0	6.34±0.88^{f}	1.03±0.09^{f}
FMS + 0.5 IBA	IB_1	4.00±0.57^{b}	0.87±0.22^{d}
FMS + 1 IBA	**IB_2**	**7.00±0.15^{h}**	**1.77±0.08^{g}**
FMS + 1.5 IBA	IB_3	3.67±0.34^{a}	0.97±0.012^{d}
FMS + 2 IBA	IB_4	6.00±0.57^{e}	1.57±0.09^{g}
FMS + 0.5 BAP + 0.5 IBA	IB_5	7.34±0.88^{j}	1.00±0.06^{f}
FMS + 0.5 BAP + 1 IBA	**IB_6**	6.67±0.88^{g}	**1.70±0.05^{g}**
FMS + 0.5 BAP + 1.5 IBA	IB_7	6.67±0.34^{g}	1.03±0.08^{f}
FMS + 0.5 BAP + 2 IBA	**IB_8**	**7.67±0.34^{k}**	1.00±0.15^{f}

The three different auxin i.e Indole-3-Acetic Acid (IAA), (Indole-3-Butyric Acid) IBA and NAA were used for the study of the *in vitro* root development of *Coelogyne nitida*. In the medium combination of MS with different concentration of NAA, the 1 mg/L NAA showed better root development with 6.00±0.57 root number and 1±0.58 cm root length. On the MS medium tested with the combination of NAA and BAP for root development, 0.5 mg/L BAP and 0.5 mg/L NAA gave the highest root number 26.00±0.57 with root length 2.80±0.58 cm. Similarly, in MS medium fortified with different concentrations of IAA, 1 mg/L IAA gave the best result with root number 6.00±0.57 with root length 1.03±0.58 cm. In a MS medium combination with different concentrations of IAA and BAP, the best result was seen in 0.5 mg/L BAP and 1 mg/L IAA with root number 8.00±0.57 and root length 1.80±0.058 cm. Likewise, among different concentrations of IBA with MS medium, 1mg/L IBA was found better with root number root number 7.00±1.15 and root length 1.77±0.088 cm. In the case of a combination of IBA and BAP in MS medium, the best response of root development was recorded in 0.5 mg/L BAP and 2 mg/L IBA.

3.8.3 Acclimatization

The *in vitro* developed plantlets of *Coelogyne nitida* of height more than 2.5 cm height were acclimatized at controlled conditions. The seedlings were transferred to pots containing soil, coco peat, wood powder and *Sphagnum* moss in a ratio of 1:1:1:1 (Figure 14). The survival rate of the seedlings was 85%.

Figure 14: Acclimatization of *in vitro* developed *Coelogyne nitida*

4. Discussion

4.1 Phytochemical Extraction

Marceration was the extraction method applied during the extraction process. It is the simplest and effective method for the extraction of phenolic and thermolabile compounds (Cujic *et al.,* 2016). Polar solvents like water, ethanol and methanol are used for the extraction of hydrophilic compounds while non-polar solvents like hexane, DCM and chloroform are used for the extraction of lithophilic compounds (Cos *et al.,* 2006). Most bioactive molecules such as phenolic compounds and polyphenols are hydrophilic. So, in the present study, methanol and water in a ratio of 7:3 was used as solvent for the extraction. The highest extract yield was of flower (33.09%) followed by leaf (24.32%) and the least extract yield was found in pseudobulb (6.52%). This finding suggests the presence of polar compounds in flower and pseudobulb and non-polar compounds in leaf.

In solvent–solvent fractionation of crude extract of flower, the highest extract yield was of methanol fraction (14.33%) and the least extract yield was of aqueous fraction (2.20%). Similarly, in leaf, the highest extract yield was of DCM fraction (11.90%) and the least yield was in hexane fraction (2.08%). Likewise, in pseudobulb extract, the highest yield was in methanol fraction (2.64%) and the lowest yield was in DCM fraction (0.45%). The flower and pseudobulb had the highest extract yield in methanol. This generally indicates the presence of non-polar compounds in both flower and pseudobulb and moderately polar compounds in the leaves since methanol is a more polar solvent than DCM (Abubakar and Haque, 2020; Cos *et al.,* 2006). The DCM fraction of leaves had the highest yield suggesting the presence of a high amount of lithophilic compounds. However, it is worth noting that the extract yield in the methanol and DCM fractions of flower and leaf was almost equivalent, suggesting the proportionate distribution of both extremely polar and moderately polar compounds in these two extract fractions.

4.2 Phytochemical Screening of *Coelogyne nitida*

Orchids are well known for their several bioactive compounds with anti-inflammatory, anti-carcinogenic, anti-rheumatic, hypoglycemic, and antimicrobial activity belonging to diverse compound classes such as polyphenols, phenanthrene, alkaloids, steroids, etc. (Mérillon and Kodja, 2022). In this study, the phytochemical

screening of the crude extract from all three plant parts of *Coelogyne nitida* showed the occurrence of flavonoids, steroids, glycosides, terpenoids, saponins, and carbohydrates, which is in agreement with the previous study of Natta *et al.*,2022. Natta *et al*. (2022) detected the presence of alkaloids and proteins in the phytochemical screening of *C. nitida*. However, alkaloids and proteins were not found in the present phytochemical screening. The use of different screening methods for the alkaloids and proteins might be the reason for this observation. A slight presence of anthocyanin in all three parts of *Coelogyne nitida* was reported. Anthocyanin is produced in plants in response to exposure to sunlight (Zhu *et al*., 2016). The *C. nitida* used for the study was collected from the shaded region with little or no direct sunlight.

4.3 Total Phenolic Content

Phenolic compounds are the most diverse group of secondary metabolites found in plants ultimately produced from the phenylpropanoid pathway (Metha & Gaur, 1999). Phenolic compounds include simple phenols, flavonoids, tannins, lignins, coumarin, anthocyanin, phenolic acids and stilbenes (Das & Gezici, 2018). These compounds are documented to have a wide range of functions within the plants including protection against environmental stress, such as UV radiation and pathogens, attracting pollinators, acting as allopathic agents to inhibit the growth of nearby plants and are also known for their beneficial properties such as antioxidant, anti-inflammatory, antimicrobial and cardio-protective effect (Zhang *et al*., 2006).

The TPC of the plant sample is a simple indicator of its antioxidant activity because phenolic compounds can readily reduce the reactive oxygen intermediates as the electron reduction potential of the phenolic radicals is less than that of the oxygen radical and hence serves as an efficient radical scavenger (Smirnoff, 2005). From the results obtained in this study for total phenolic content, we can observe that all the fractions from flower, leaves and pseudobulb of *C. nitida* had a high amount of phenolic content (>163.34 mg GAE/g dry weight of extract). DCM fraction of pseudobulb of *C. nitida* showed the highest phenolic content (272.12 mg GAE/g) among all the measured fractions. Among the fractions of pseudobulb, the highest phenolic content after DCM fraction (272.12 mg GAE/g) was in methanol (255.25 mg GAE/g) followed by aqueous (181.75 mg GAE/g), and hexane (181.50 mg GAE/g).

This might suggest that the phenolic compounds in the pseudobulb have moderate to high polarity due to the high phenolic content in DCM and methanol fraction. Interestingly, although the extract yield of the pseudobulb was lowest in the DCM fraction, this fraction showed the most significant total phenolic content per gram dry weight of the extract. This surprising discovery suggested that less polar and highly methoxylated aglycone versions of phenolic chemicals might be present in higher quantities in the DCM fractions because they can be easily extracted using less polar solvents. (Dorta *et al.,* 2012; Lafka *et al.,* 2007). Leaves also showed a similar pattern of highest phenolic content in methanol (219.34 mg GAE/g) and DCM fraction (200.87 mg GAE/g) compared to aqueous (193.42 mg GAE/g) and hexane fraction (180.67 mg GAE/g). Similarly, flowers showed the highest phenolic content in hexane (197.79 mg GAE/g) followed by DCM fraction (191.58 mg GAE/g) in comparison to methanol (169.87 mg GAE/g) and aqueous fraction (163.34 mg GAE/g) indicating the presence of moderately to non-polar phenolic compound abundance in the leaves of *C. nitida*. On this premise, it can be inferred that highly hydroxylated aglycone forms of phenolic compounds are more abundant in the methanol fraction since hydroxylated aglycone forms of phenolic compounds are soluble in polar solvents such as methanol, ethanol, and water. etc (Dorta *et al.,* 2012; Lafka *et al.,* 2007).

4.4 Total Flavonoid Content

Flavonoids are a group of bioactive secondary metabolites belonging to the polyphenols class that provide flavour, colour, protection against UV light protection and preservation of plant cell membranes (Nabavi *et al.,*2020; Scarano *et al.,*2018). Flavonoids are also known for various bioactivities such as anti-inflammatory, anti-viral, anti-tumor and antioxidant activities. The result obtained from the TFC assay indicated less flavonoid content in *C. nitida* compared to the TPC. The flavonoid content of the pseudobulb, flower, and leaf of *C. nitida* is about 4% of the total phenolic content in their respective fractions. The low flavonoid content in *C. nitida* indicates that phenolic compounds other than flavonoids (such as phenolic acids and lignans) might be in greater quantity in *C. nitida*. The phytochemical analysis of *the Coelogyne* genus also confirms this observation, as there is not any published scientific report on the isolation of flavonoid from the *Coelogyne* species, while several compounds belonging to phenanthrene and stilbenoid compound class

have been chiefly isolated. (Majumder *et al.,* 1982, 1995, 2002, 2012; Majumder & Maiti, 1988). DCM fraction of the pseudobulb of *C. nitida* showed the highest flavonoid content (10.23 mg QE/g) followed by methanol fraction of the pseudobulb (8.45 mg QE/g) and DCM fraction of flower (6.20 mg QE/g) indicating the greater presence of slightly polar nature of the flavonoid in pseudobulb and flower. All other fractions have a total flavonoid content of less than 5.04 mg QE/g.

4.5 Antioxidant Activity

The present experiment on *in vitro* antioxidant assay of *Coelogyne nitida* indicated potent radical scavenging activity from the DCM fraction. This implies that the compounds responsible for the antioxidant activity are moderately polar(Abubakar & Haque, 2020). Among the three plant parts (pseudobulb, flower, and leaf), the pseudobulb showed the best DPPH activity than the flower and leaf. DCM fraction of pseudobulb (DP) of *C. nitida* had the highest DPPH activity with an IC_{50} value of 49.96 μg/mL. Interestingly, the highest amount of total phenolic (272.12 mg GAE/g) and total flavonoid content (10.23 mg QE/g) was also obtained in the DCM fraction of pseudobulb of *C. nitida*, which signifies that the polyphenols might be contributing to the excellent radical scavenging activity of DP fraction of *C. nitida*. Polyphenols such as flavonoids, phenolic acids, and lignans are well known for their antioxidant activity (Pandey and Rizvi, 2009).

The antioxidant activity (IC_{50} = 49.96 μg/mL) shown by *C. nitida* from our study is far greater than the activity (IC_{50} = 281.7 μg/mL) by *C. nitida* from Natta *et al.,* (2022). In the same article by Natta *et al.,*2022, the total phenolic content *of C. nitida* (10.5 mg GAE/g) was far lower, whereas the flavonoid content of *C. nitida* (132 mg QE/g) was far greater than what we have measured in our study. This discrepancy in the phenolic and flavonoid content of *C. nitida* might be due to various factors such as environments, age of the collected sample, timing of collection, etc. Similarly, the DCM fraction of flower (DF) and methanolic fraction of pseudobulb (MP) also showed potent DPPH scavenging activity with an IC_{50} value of 62.16±1.90 and 63.45±0.36 μg/mL. Both of these fractions also showed significant total phenolic (DF = 191.58 mg GAE/g and MP = 255.25 mg GAE/g) as well as total flavonoid content (DF = 6.20 mg QE/g and MP = 8.45 mg QE/g). This study is in agreement with the study of Rashmi *et al.,* 2015 where the crude ethanolic extract of *Coelogyne*

breviscapa showed potent antioxidant activity with an IC_{50} value of 71.91 µg/mL. All the other fractions have IC_{50}>174 µg/mL. These observations propose that the DCM fraction of pseudobulb and flower of *C. nitida* contains active molecules in greater quantity responsible for the antioxidant activity in *C. nitida*.

4.6 Brine Shrimp Lethality Test

Brine shrimp lethality test is basically used to predict the toxicity of the plant extract. A According to Meyer *et al*. (1982), plant extract with $LC_{50} \leq 30$ µg/mL as very toxic, $LC_{50} \leq 1000$ µg/mL as toxic and $LC_{50} \geq 1000$ µg/mL as non-toxic. The LC_{50} value of all fractions of leaf, flower and pseudobulb in this study was above 1000 µg/mL except that of the hexane fraction of pseudobulb (HP) which had LC_{50} value of 397.48µg/mL. According to Parra (2001), the LC_{50} value of extract above 250 µg/mL is referred as slightly toxic. This result is in agreement with the traditional use of *Coelogyne nitida* as it proves to be less toxic.

4.7 Antimicrobial Activity

Many species of *Coelogyne* have been reported to have strong antimicrobial activity against different bacterial strains. In this study also the DCM fraction of pseudobulb shows moderate antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* with ZOI 11.75 and 11.48 mm respectively. This result was supported by the work of Buyun *et al.* (2016), on *Coelogyne cristata* which shows the moderate anti-bacterial activity of the methanolic extract from the leaves and pseudobulb with 9 mm zone of inhibition (ZOI) against *Staphylococcus aureus*. Similar result was reported by Marasini and Joshi, (2012) in *Coelogyne cristata* and *Coelogyne stricta* against *S. aureus* with the ZOI of 14 mm and 12 mm respectively. The result of this study is not in agreement with the study of Ranjtha *et al.* (2016) which reported strong antibacterial activity of *Coelogyne nervosa* against *S. aureus* and *Pseudomonas aeruginosa* with ZOI 2.16 cm and 1.80 cm respectively.

The hexane fraction of the pseudobulb also shows low antibacterial activity against *Staphylococcus aureus* with ZOI 7.5 mm. The methanolic and aqueous fractions of pseudobulb are not susceptible to the sample bacterial strains of this study. The reason for the difference in the antibacterial activity of four fractions of pseudobulb might be the different solvent system for the extraction. This logic is in agreement with the

result of Buyun *et al.* (2016) where ethyl acetate, hexane and dichloromethane fraction from leaf and pseudobulb of *Coelogyne cristata* do not show any antimicrobial activity. A similar pattern of antimicrobial activity was reported on *Coleogyne cristata* by Mitra *et al.* (2015) where the petroleum ether and chloroform extract from *C. cristata* of 100 mg/mL exhibited antimicrobial activity with ZOI 13 and 14 mm respectively. But the ethanolic extract shows better inhibition against *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 27853) and *Pseudonomas aeruginosa* (ATCC 27853) with ZOI of 17.50 mm, 17.16 mm and 15.52 mm respectively. Another study in the pseudobulb of *Coelogyne speciosa* against different strains of *S. aureus* showed a difference in antimicrobial activity according to the solvent used for extraction. This study reported that the ethanolic extract shows strong antibacterial activity against *S. aureus* with a ZOI diameter of 19 mm. The methanolic extract also shows mild activity against *S. aureus* but acetyl acid, hexane and dichloromethane do not show antibacterial activity (Buyun *et al.,* 2017). This variation in this result of anti-microbial activity might be due to the different degree of susceptibility of the tested bacterium to plant extract, bacterial growth rate, the solvent system used for the extraction of the plant extract and the diffusion rate of the extract in the MHA medium (Mthethwa, 2009).

The hexane, DCM, methanol and aqueous fractions of *Coelogyne nitida* were not susceptible to the gram-negative bacterial strains used in this experiment *Klebsiella pneumonia* (ATCC 8739), *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027).This result is not in agreement with the finding of *Ranjtha et al.*(2016) which reported strong antibacterial activity of the whole plant of *Coelogyne nervosa* against *P. aeruginosa* and *E.coli* with ZOI of 1.80±0.00 cm. Buyun *et al.* (2021) also reported the antimicrobial activity of the ethanolic leaf extract of *Coelogyne brachyptera* against *E. coli* (ATCC® 25922™), *E. coli* (ATCC® 35218™) and *P. aeruginosa* (ATCC® 27583™) strains. The 80% ethanolic extract of *Coelogyne leucantha* also exhibited antibacterial activity against *S. aureus*, *P. aeruginosa* and *E. coli* with ZOI of 15 mm, 13 mm and 8 mm (Yang *et al.,* 2012). In most of the research with positive results for the antimicrobial activity of *Coelogyne species* the extraction solvent was found to be ethanol. But in this present study ethanol is not used for the extraction. In this regard, it can be assumed that the ethanol extract contains more bioactive compounds with antimicrobial activity against gram

negative bacteria. The lack of activity on gram negative bacterial strains can also be assumed due to the cell wall structure. The cell wall of gram negative bacteria has permeability barrier which reduces the active penetration of amphipathic substances (Nikaido & Vaara, 1985).

All four fractions of flower and leaf were not susceptible to the five bacterial strains. This might be due to the presence of less amount of phenolic compound in all fractions of flower and leaves as compared to the pseudobulb of *C. nitida*. The finding of a study by Buyun *et al.* (2021) in ethanolic leaf extract of *Coelogyne brachyptera* is not in agreement with the present result in which the ZOI ranges from 11-15 mm. The study of Mitra *et al.* (2015) in *C. cristata* revealed that the ZOI increases with the increase in extract concentration. The present study on *Coelogyne nitida* was done only on one concentration i.e. 50 mg/mL. So, the exact picture of the anti-microbial activity cannot be ascertained.

4.8 Micropropagation

The MS medium supplemented with 0.5 mg/mL BAP and 0.5 mg/mL NAA was best composition for the *in vitro* production of *Coelogyne nitida* based on the germination rate, shooting and rooting time. In this research, immature capsules of *C. nitida* were chosen because they demonstrated superior germination response. (Pant, 2006). The effective germination response was seen in MS medium enriched with 2 BAP and 0.1 NAA (BN-2). The germination highly depends upon the quality and nature of plant growth regulators (Arditti, 1992).

The first seed germination was observed during the fourth and fifth weeks of culture. The findings of Reddy *et al*. (1992) on the seed germination and seedling growth of four orchid species validated this result. Pardhan and Pant (2009) reported a similar result on *Dendrobium densiflorium*. Protocorm formation was observed from the seventh week of seed inoculation. Basker and Bai, (2010) also reported the same finding in the seed germination of *Eriabam basifolia*. A similar result was reported in *Cymbidium aloifolium* by Nongdam and Chongtham, (2011) and also in *Dendrobium densiflorium* by Pradhan and Pant, (2009) which took six weeks for the protocorm formation.

The first shoot formation was observed after eight weeks of culture. The shoot formation period of the present study is in agreement with the study in *Dendrobium*

densiflorium (Pardhan and Pant, 2009). The first root initiation occurred after 25 weeks of inoculation. A similar result was recorded in *Coelogyne falccida* by Parmar and Pant, (2015). The complete plantlets of *Coelogyne nitida* were prepared after 25 weeks of culture. This result is in agreement with the work of Paudel *et al.*, 2012 on *Esmeralda clarkei* and also by Pant *et al.* (2011) in which the *Phaius tancarvilleae* took 24 weeks for the *in vitro* production of complete plantlets.

Phytohormones IAA, IBA and NAA promote root formation and development. The different phytohormones show different results in the root development. The genetic constituents of the explants and the endogenous growth regulators found in them may be the cause of this phytohormone impact. In addition to this, the germinating seed has different nutritional requirements based on the physiological state of the seed (Yam *et al.*, 1989) and it may vary among species (Arditti and Ernst, 1984). This study revealed that the phytohormone-mediated MS medium is suitable for the *in vitro* production of *Coelogyne nitida*. The best medium combination was found to be the MS medium fortified with 0.5 mg/l BAP and 0.5 mg/L NAA. This finding was supported by the work of Parmar and Pant 2015, on *Coelogyne flaccida*. In this study, better growth and development of the seedling was reported on the medium supplemented with two phytohormones in combination than with an individual. This might be due to the synergistic effect of both the hormones.

The successful acclimatization of the *in vitro* developed plantlets of *Coelogyne nitida* was done in soil, coco peat, wood powder and *Sphagnum* moss in a 1:1:1:1 ratio with an 85% survival rate. The main reason for the use of *Sphagnum* moss for acclimatization is that it has high absorbent capacity which keeps the root of orchids moist and hydrated. Besides, moss also helps for good air circulation around the root. This helps the orchid root to prevent from rot and encourages healthy root growth. Cocopeat, sawdust and soil are used for the supply of necessary nutrient. The successful acclimatization of *C. mossiae* was done in substrate containing brick, charcoal, sphagnum moss and vermiculite in the ratio 1:1;1:1 (Bai. *et al*., 2005). Similarly, Sebastianrai *et al*. (2006) used garden soil, vermicompost and brick gravels in the ratio of 1:1:1 for hardening of *in vitro* grown *C. mossiae*.

Finally, first research hypothesis was found true from the phytochemical screening of the secondary metabolites. Similarly, the second and third research hypotheses were proved from the Duncan test at $p \leq 0.05$ significance in SPSS software.

5. Conclusion and Recommendation

5.1 Conclusion

Coelogyne nitida, a traditionally used plant for fever and headache is found to have flavonoids, steroids, glycosides, terpenoids, saponins, phenolic and carbohydrates in the qualitative phytochemical screening. The quantitative analysis of the TPC and TFC shows a significant amount of the total phenolics and flavonoids in this plant. Among the flower, leaf and pseudobulb of *C. nitida*, the DCM fraction of pseudobulb is found most active followed by the DCM fraction of flower. The pseudobulb (DP) had the highest total phenol and flavonoid content. The presence of the high amount of total phenol and total flavonoid content in the DCM fraction of pseudobulb might be the contributing factor for showing the better response in biological activities such as antioxidant activity and antimicrobial activity. All the extract fractions of flower, leaves and pseudobulb are found non-toxic from the brine shrimp lethality test. This study shows that MS medium fortified with 0.5 mg/L BAP and 0.5 mg/L NAA is the best composition for the *in vitro* production of the plantlets of *Coelogyne nitida*. A substrate integrating soil, coco peat, sawdust, and *Sphagnum* moss in equal proportion was found to be suitable for acclimatisation of the *in vitro* developed plantlets the *C. nitida* with an 85% survival rate.

5.2 Recommendation

Despite the limited research on the bioactivities, *Coelogyne nitida* holds immense potential for future exploration.

- The future study should focus on unravelling the bioactive compound responsible for the therapeutic properties such as antioxidant activity and antimicrobial activity of this plant.
- The use of a broad range of bacteria and fungi can unearth the exact picture of the anti-microbial activity of this plant.
- Identification of the novel molecules and understanding their genetic basis of medicinal properties can be studied using advanced analytical technique such as metabolomics and genomics
- Commercialization of the tissue culture of *Coelogyne nitida* for the horticulture industry and also for mass production for pharmaceutical study.

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Appendices

Appendix-1

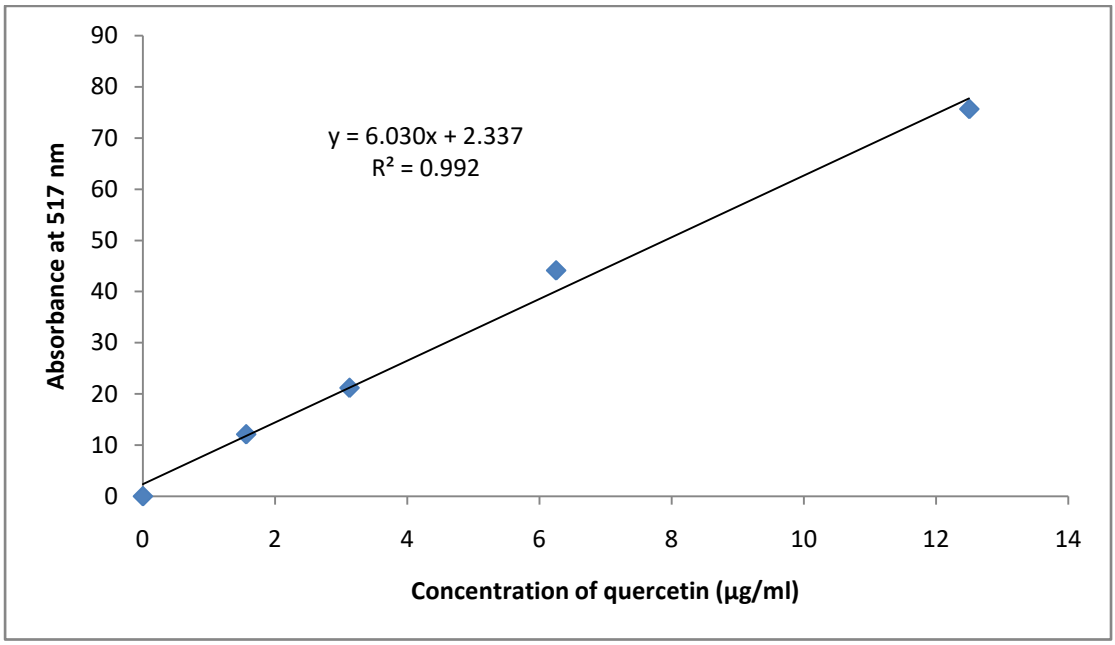
Phytochemical screening

S.N.	Experiment	Test	Colour	Result
1.	Volatile oils	2 ml solution in Petridis is evaporated in water bath till dryness	If the dried residue gives pleasant aromatic smell.	Presence of volatile oil.
	Fixed oil	**Spot Te/Residue test:** 2ml solution was evaporated to get residue and it was mixed with 0.5 ml methanol. The solution was shaken vigorously and filtered. Few drops of filtrate were put on a filter paper by means of a capillary tube.	Transparent filter paper with yellow colour persists	Presence of fixed oil.
2.	Alkaloids	6 ml extract solution in beaker is evaporated till dryness and thus obtained residue is dissolved in 1.5 ml of 2N HCl, filtered and divided into three parts: **Mayers Reagent Test:** 2-3 drops of potassium mercuric iodide (Mayer's reagent) give white yellowish precipitate **Dragendorff's Test:** Few drops of Dragendorff's reagent was added to 2^{nd} part. **Hager's test**: Few drops of Hager's regent (Saturated picric acid solution).	White yellowish ppt. Orange red precipitate Yellow colour precipitate	Presence of alkaloids
3.	Flavonoid	**Shinoda test:** Take 1 ml of alcoholic extract of the medicinal plant and concentrate. The resulting residue is taken in 1.5 ml of methanol and warmed at 50˚c and add a small piece of magnesium metal or powder and 5 – 6 drops of Conc. HCl were mixed slowly to resulting solution.	Pink Scarlet	Appearance of solution becomes red for flavonoid, orange for flavones and violet for Flavonones.
4.	Steroids	**Steroid test:** About 2 ml conc. H_2SO_4 was added to the 1 ml of extract solution.	Appearance of wine-red colour	Indicates the presence of steroids.
5.	Terpenoids	**Terpenoids test:** To the 1 ml extract solution, add 2 ml of $CHCl_3$ and 3 ml conc. H_2SO_4. The resulting solution heat for 2 minutes.	Reddish-brown coloration	The presence of terpenoids.
6.	Tannins	**0.1% $FeCl_3$ Test:** To 0.5 ml of alcoholic extract add 1 ml water and 2-3 drops of 2% $FeCl_3$ solution. If bluish green or bluish black ppt. is formed means presence of and if greenish black means presence of	i. Bluish black ii. Greenish black	i. gallic tannins ii. catecholic tannins. Or it indicates the presence of phenolic compounds

7.	Glycosides	**Salkowski's test:**The extract was mixed with 2 ml of chloroform. Then 2 ml of concentrated H_2SO_4 was added carefully and shaken gently.	Reddish brown	The presence of steroidal ring.
8.	Phenols	Phenolic test	Blue green	
9.	Saponins	**Foam Test:** 0.5 gm of extracts was shaken with 2 ml of water.	If foam produced persists for ten minutes	The presence of saponins.
10.	Protein	**Ninhydrin test**: To the 1ml of extract solution in a test tube, add 2-3 drops of freshly prepared 0.5% ninhydrin solution and heat to boil the test tube.	Violet	Presence of proteins
11.	Carbohydrate	**Molisch'sTest**: Treat the crude extract treat with1 mL Molisch's reagent and add conc. H_2SO_4drop by drop from the side of the test tube without disturbing the solution.	Violet ring at junction	Presence of carbohydrate

(NPRL, Department of Plant Resource)

Standard curve of quercetin for antioxidant activity by DPPH assay



Appendix-2

Figure: Phytochemical screening of leaves of *Coelogyne nitida*

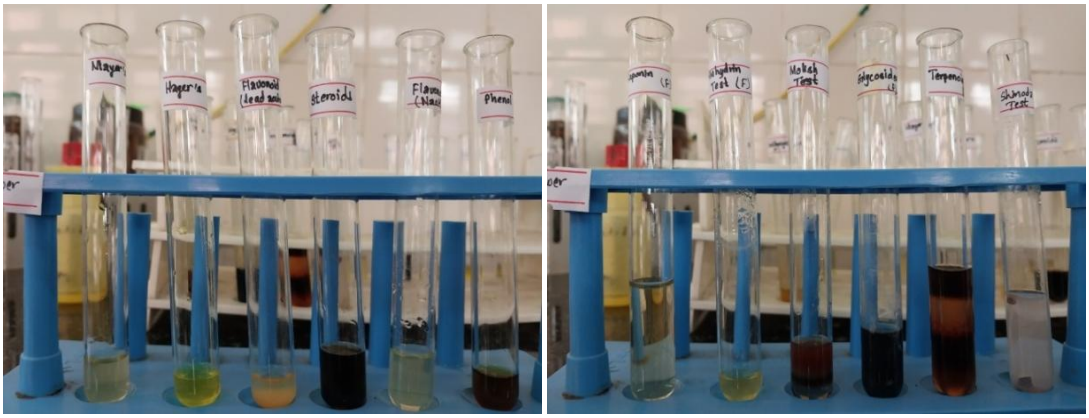


Figure: Phytochemical screening of flower of *Coelogyne nitida*

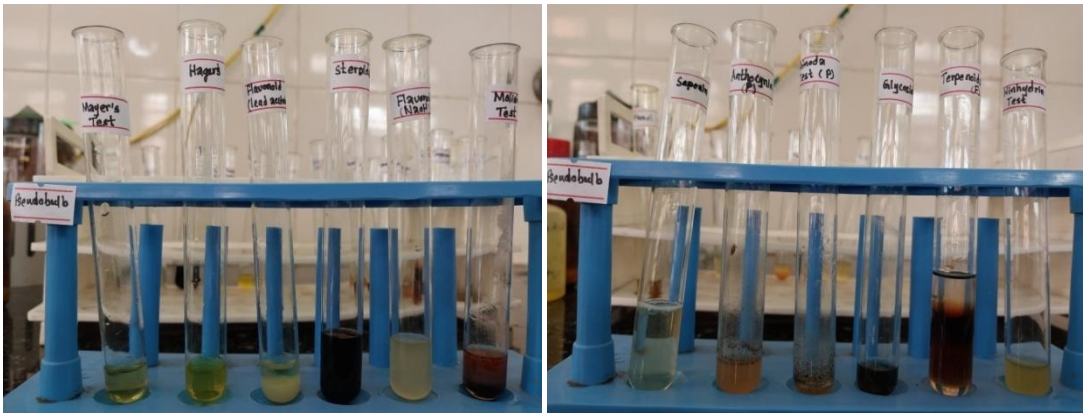
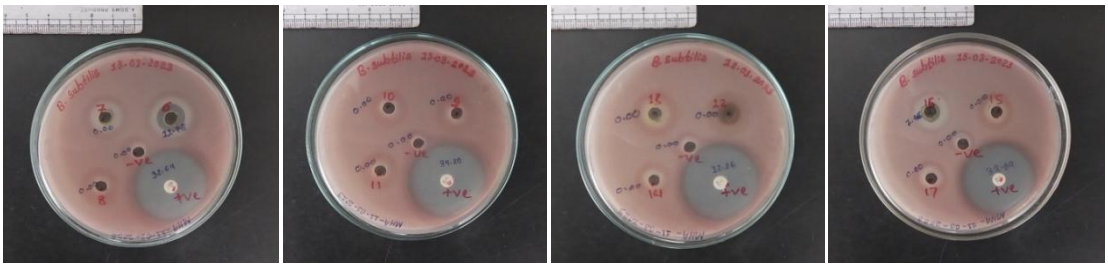


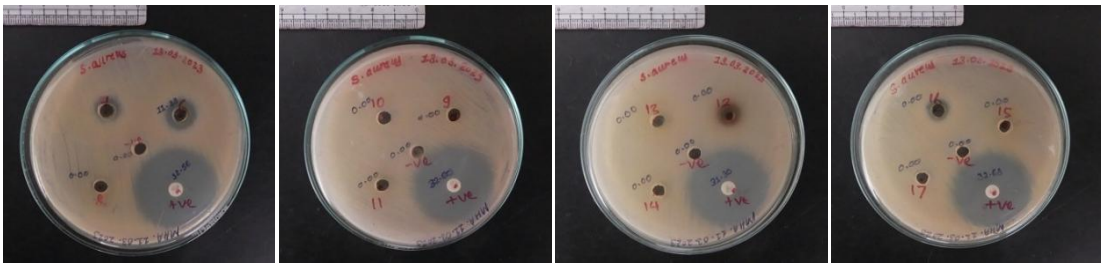
Figure: Phytochemical screening of Pseudobulb of *Coelogyne nitida*

Appendix-3

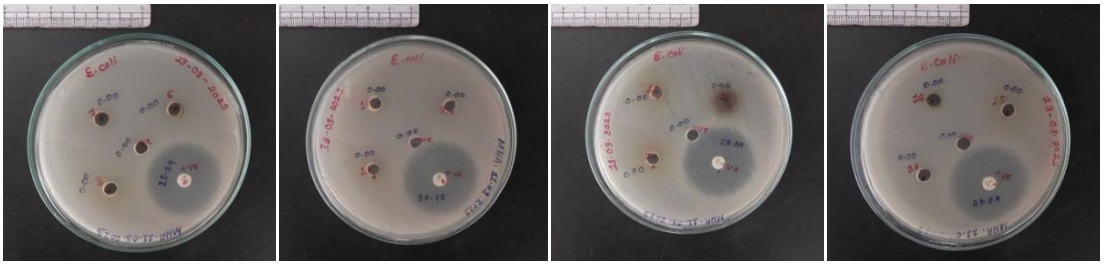
Screening of antimicrobial activity



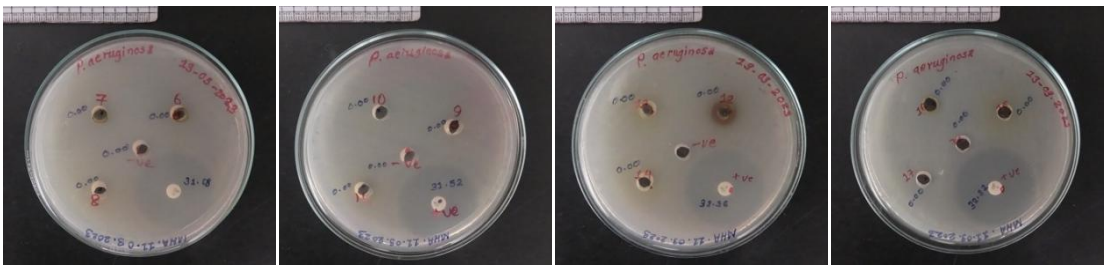
a) *Bacillus subtilis*



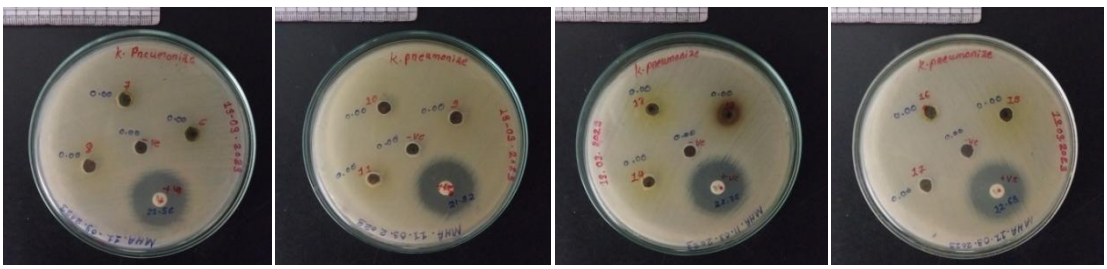
b)*Staphylococcus aureus*



c) *Escherichia coli*

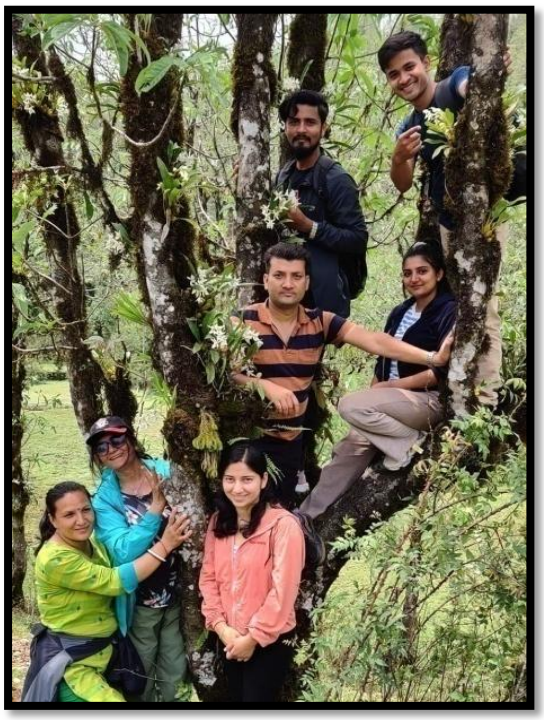


d) *Pseudomonas aeruginosa*



e) Klebsiella pneumoniae

Appendix-4



Field visit at Panchase (Photo with tree full of *Coelogyne nitida* flower)

Panchase Biodiversity Information Center

Phytochemical extraction

Phytochemical screening

Appendix-5

Taking absorbance of the sample with microplate reader

Plate preparation for screening of antimicrobial activities

Observation of the culture tube

Appendix-6

Conferences


CERTIFICATE
OF ACHIEVEMENT

THIS CERTIFICATE IS PRESENTED TO

Ms. Shanti Ranabhat

Tribhuvan University, Nepal

as a Best Poster Presenter

with Title

Antioxidant Activity and Antimicrobial Activity of Coelogyne nitida (Wall. ex D.Don) Lindl.

in the 4th International Conference on

Biotechnology: Academia to Industry (ICB - 2023)

held on 17-19 March, 2023 at Kathmandu

organized by

Nepal Biotechnology Association.

www.nepalbiotech.org

Prof. Dr. Bijaya Pant
Convener, ICB 2023

Prof. Dr. Tribikram Bhattarai
President, NBA

Certificate of Best Poster Presenter Award in 4th International Conference on Biotechnology: Academia to Industry (ICB-2023).


CERTIFICATE

OF APPRECIATION

The certificate is presented to

Shanti Ranabhat

in recognition of their POSTER PRESENTATION

contributions as a Member of the Organizing Committee of the First National Biotechnology Conference 2023, held at Kathmandu University on May 11-12, 2023. Your dedicated efforts are highly appreciated. We value what you have done and what you will do in the years to come.

Dr. Dhurva P. Gouchan
Convener
NBC-2023

Prof. Dr. Subodh K. Upadhyaya
Co-chairperson
NBC-2023

Prof. Dr. Janardan Lamichhane
Chairperson
NBC-2023

Certificate of Poster Presentation in First National Biotechnology Conference, 2023

Appendix- 7

ICB 2023 — The Biotechnology for Economic Prosperity! — Mar 17-19 — Kathmandu, Nepal — NEPAL BIOTECHNOLOGY ASSOCIATION

Antioxidant Activity and Antimicrobial Activity of *Coelogyne nitida* (Wall. ex D. Don) Lindl.

Shanti Ranabhat and Mukti Ram Paudel

Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

Abstract

Orchids are gaining much popularity in pharmacological studies these days. *Coelogyne* is one of the highly studied genus for antioxidant and antimicrobial activity. This study aims to determine the antioxidant activity and antimicrobial activity of *Coelogyne nitida*. The DPPH scavenging assay was employed to determine the antioxidant activity of flower, leaf, and pseudobulb extracts. DPPH of 0.2mM was used in this assay. DCM fractions of all three parts were found more active than hexane, methanol and aqueous fractions. DCM fraction of pseudobulb exhibits the highest antioxidant activity with IC_{50} value 51.87. Similarly, the DCM fraction of the flower and methanol fraction of the pseudobulb shows an IC_{50} value of 61.135 and 63.44 respectively. Leaf extracts of *Coelogyne nitida* exhibit high IC_{50} values resulting in low antioxidant activity. The active fractions of flower, leaves and pseudobulb will be subjected to the study of antimicrobial activity. This study will unearth a new avenue in the field of pharmacy.

Keywords: Antioxidant, antimicrobial, *Coelogyne nitida*, DPPH, pseudobulb

Introduction

Coelogyne nitida → Antioxidant activity, Antimicrobial activity → Pharmaceutical

C. nervosa	Shibu *et al.*, 2013	IC_{50} value 312 µg/mL
C. brachyptera	Buyun *et al.*, 2021	*S. aureus*
C. fimbriata	Wati *et al.*, 2021	*B. cereus*, *S. aureus*
C. lucantha	Yang *et al.*, 2012	*S. aureus*

This study aimed to determine the TPC, TFC, antioxidant activity and antimicrobial activity of *Coelogyne nitida* .

Materials and Methods

A. Plant Collection

Location : Panchase, Kaski
Latitude : 28°14' 31.4"N
Longitude: 83°48' 43.9"E

B. Extract Preparation

Drying and grinding

Habit photo of *Coelogyne nitida* at Panchase

a. Pseudobulb b. Leaves c. Flower d. Dried pseudobulb e. Powder

Extraction in 70 % methanol

i. DCM iii. Methanol
ii. Hexane iv. Aqueous

(a) Soaking of powder (b) Separating funnel (c) Extract

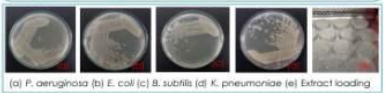
C. Determination Of Antioxidant Activity (DPPH Method)

DPPH method : Plant extract + 0.2 mM DPPH → Incubate in dark for 30 min → Absorbance at 517 nm

D. Determination Of Antimicrobial Activity

Agar well diffusion method:

Media preparation → Preparation of test sample → Bacterial suspension preparation → Carpet culture → Media boring and loading sample → Incubation (24 hrs)



(a) *P. aeruginosa* (b) *E. coli* (c) *B. subtilis* (d) *K. pneumoniae* (e) Extract loading

Results

Total Phenolic Content (TPC)

96 Well plate (TPC)

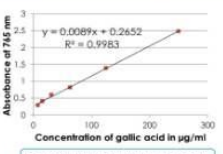


Fig 1: Standard curve of gallic acid

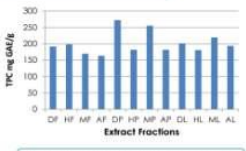


Fig 2: Total phenolic content of *C. nitida*

Total Flavonoid Content (TFC)

96 well plate (TFC)

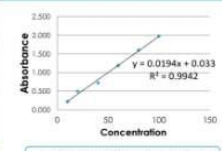


Fig 3: Standard curve of quercetin

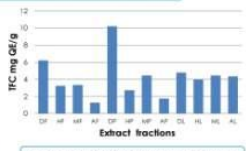


Fig 4: Total flavonoid content of *C. nitida*

Antioxidant Activity: DPPH Assay

96 well plate (DPPH)

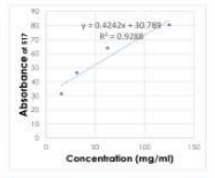


Fig 5: DPPH assay of pseudobulb

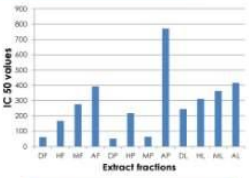
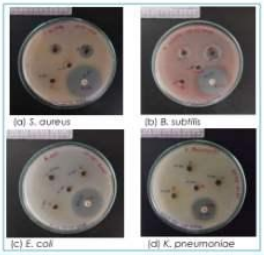


Fig 6: IC_{50} values of extracts of *C. nitida*

Antimicrobial Activity: Agar Well Diffusion Method



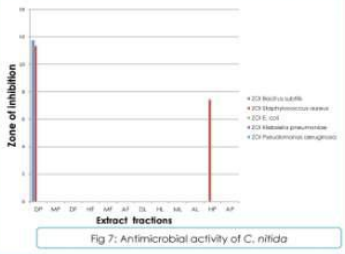


Fig 7: Antimicrobial activity of *C. nitida*

Discussion

The bioactivity of extract depends upon the total phenol and total flavonoid content. TPC and TFC values depends upon the polarity of the solvent used . Low amount of phenolic and flavonoid contents was estimated in aqueous fraction. Similar result was reported in *Dendrobium speciosum* (Moretti *et al.*, 2013) and *D. macraei* (Prajapati and Patal 2013).

Conclusion

Higher total phenol and total flavonoid contents results better bioassay.

Future direction

- Determination of compound of phenol and flavonoid group.
- Determination of antibacterial activity in broad range of bacterial strain.

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Appendix-8

Poster presented in first Nepal Biotechnology Conference-2023

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Appendix-9

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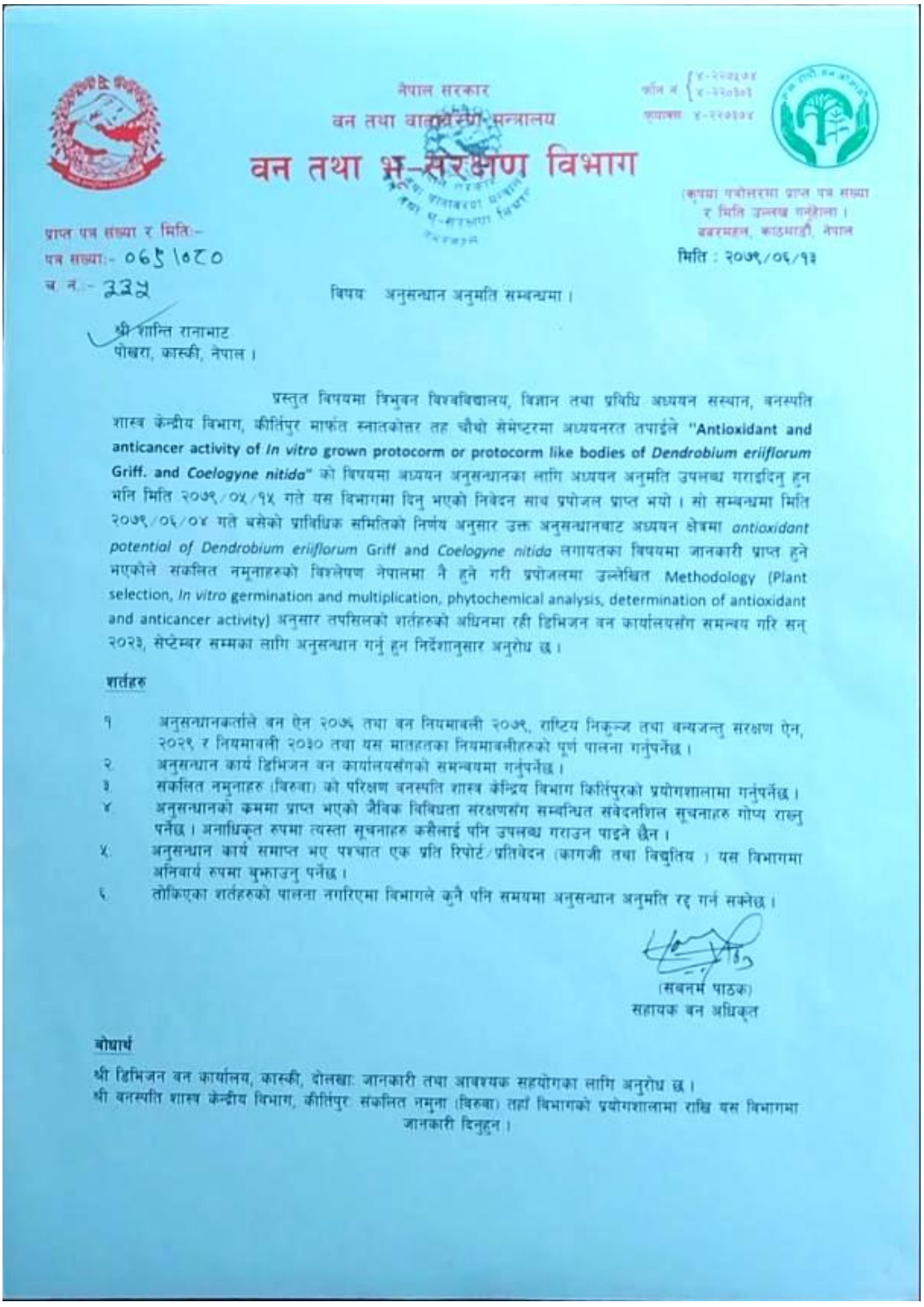
Best Poster Presenter Award in 4th International Conference on Biotechnology: Academia to Industry (ICB-2023).

Conference Grant Awardees

Appendix-10



नेपाल सरकार
वन तथा वातावरण मन्त्रालय
वन तथा भू-संरक्षण विभाग

फोन नं {४-२००५७४ ४-२२०३०१
फ्याक्स ४-२२०३७४

(कृपया माथीसरमा प्राप्त पत्र संख्या र मिति उल्लेख गर्नुहोला।)
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प्राप्त पत्र संख्या र मितिः-
पत्र संख्याः- ०६५\०८०
च.नं.- ३३५

मिति : २०७९/०६/१३

विषय अनुसन्धान अनुमति सम्बन्धमा।

श्री शान्ति रानाभाट
पोखरा, कास्की, नेपाल।

प्रस्तुत विषयमा त्रिभुवन विश्वविद्यालय, विज्ञान तथा प्रविधि अध्ययन संस्थान, वनस्पति शास्त्र केन्द्रीय विभाग, कीर्तिपुर मार्फत स्नातकोत्तर तह चौथो सेमेष्टरमा अध्ययनरत तपाईले "**Antioxidant and anticancer activity of *In vitro* grown protocorm or protocorm like bodies of *Dendrobium eriiflorum* Griff. and *Coelogyne nitida***" को विषयमा अध्ययन अनुसन्धानका लागि अध्ययन अनुमति उपलब्ध गराईदिनु हुन भनि मिति २०७९/०५/१५ गते यस विभागमा दिनु भएको निवेदन साथ प्रयोजन प्राप्त भयो। सो सम्बन्धमा मिति २०७९/०६/०४ गते बसेको प्राविधिक समितिको निर्णय अनुसार उक्त अनुसन्धानबाट अध्ययन क्षेत्रमा *antioxidant potential of Dendrobium eriiflorum* Griff and *Coelogyne nitida* लगायतका विषयमा जानकारी प्राप्त हुने भएकोले संकलित नमूनाहरुको विश्लेषण नेपालमा नै हुने गरी प्रयोजनमा उल्लेखित Methodology (Plant selection, *In vitro* germination and multiplication, phytochemical analysis, determination of antioxidant and anticancer activity) अनुसार तपसिलको शर्तहरुको अधिनमा रही डिभिजन वन कार्यालयसँग समन्वय गरि सन् २०२३, सेप्टेम्बर सम्मका लागि अनुसन्धान गर्नु हुन निर्देशानुसार अनुरोध छ।

शर्तहरु

१. अनुसन्धानकर्ताले वन ऐन २०७६ तथा वन नियमावली २०७९, राष्ट्रिय निकुन्ज तथा वन्यजन्तु संरक्षण ऐन, २०२९ र नियमावली २०३० तथा यस मातहतका नियमावलीहरुको पूर्ण पालना गर्नुपर्नेछ।
२. अनुसन्धान कार्य डिभिजन वन कार्यालयसँगको समन्वयमा गर्नुपर्नेछ।
३. संकलित नमुनाहरु (विरुवा) को परिक्षण वनस्पति शास्त्र केन्द्रिय विभाग किर्तिपुरको प्रयोगशालामा गर्नुपर्नेछ।
४. अनुसन्धानको क्रममा प्राप्त भएको जैविक विविधता संरक्षणसँग सम्बन्धित संवेदनशिल सूचनाहरु गोप्य राख्नु पर्नेछ। अनाधिकृत रुपमा त्यस्ता सूचनाहरु कसैलाई पनि उपलब्ध गराउन पाइने छैन।
५. अनुसन्धान कार्य समाप्त भए पश्चात एक प्रति रिपोर्ट/प्रतिवेदन (कागजी तथा विद्युतिय) यस विभागमा अनिवार्य रुपमा बुझाउनु पर्नेछ।
६. तोकिएका शर्तहरुको पालना नगरिएमा विभागले कुनै पनि समयमा अनुसन्धान अनुमति रद्द गर्न सक्नेछ।

(सबनम पाठक)
सहायक वन अधिकृत

बोधार्थ

श्री डिभिजन वन कार्यालय, कास्की, दोलखाः जानकारी तथा आवश्यक सहयोगका लागि अनुरोध छ।
श्री वनस्पति शास्त्र केन्द्रीय विभाग, कीर्तिपुरः संकलित नमुना (विरुवा) तहाँ विभागको प्रयोगशालामा राखि यस विभागमा जानकारी दिनुहुन।

Plant collection permission letter

Appendix-11

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