Biological Activities of *In Vitro* Generated Callus of *Neopicrorhiza scrophulariiflora* (Pennell) D. Y. Hong, a Himalayan Medicinal Plant

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

By

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Declaration

The dissertation entitled "**Biological Activities of *In Vitro* Generated Callus of *Neopicrorhiza scrophulariiflora* (Pennell) D. Y. Hong, a Himalayan Medicinal Plant**" 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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Recommendation

This is to recommend that **Kiran Tiwari** carry out the dissertation entitled "**Biological Activities of *In Vitro* Generated Callus of *Neopicrorhiza scrophulariiflora* (Pennell) D.Y. Hong, a Himalayan Medicinal Plant**" 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, Kritipur for the submission of the dissertation for the Master's Degree in Botany.

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Letter of Approval

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Abstract

Neopicrorhiza scrophulariiflora Pennell (D.Y.) Hong commonly known as "Kutki" has been prioritized for traditional medicinal use. Several studies were carried out previously on biological activities of wild plant parts of *N. scrophulariiflora* but there is no any study on biological activities of its *in vitro* induced callus. The goal of this research is to induce callus, to determine the total phenolic content, total flavonoid content, antioxidant activity, antidiabetic activity and toxicity of different extracts of callus of *N. scrophulariiflora*. The FC reagent and aluminium chloride method were used to determine the total phenol and total flavonoid content respectively. The DPPH scavenging and alpha glucosidase inhibition method was used to find out the antioxidant activity and antidiabetic activity respectively. In this study, maximum seed germination (41.11%) was observed in medium containing 10% coconut water. Interestingly, the vigorous rise of greenish callus was found in medium containing 1 mg/l NAA, which is followed by 1 mg/l BAP and 1 mg/l NAA. Maximum two shoots with small green leaves were observed in callus from 1 mg/l BAP and 0.5 mg/l NAA. Beside these, methanol extract of callus was determined to have i.e., 38.11 mg GAE/g total phenolic content and 23.94 mg QE/g flavonoid content. Moreover, methanol extract showed the maximum antioxidant potential with an IC_{50} value 887.08±0.87 μg/ml in comparison to the aqueous extract while there was no inhibition activity on alpha-glucosidase enzyme. Methanol extract of callus of *N. scrophulariiflora* was found to be less toxic with LC_{50} value 793 μg/ml while aqueous extract was non-toxic.The findings of the research suggest that callus of *N. scrophulariiflora* is also the sourceof phenolic and flavonoid compounds which can be used for the production of medicines but the evaluation of biological activities shows that callus has low therapeutic potential in comparison to wild plant.

सारांश

सामान्यतया "कुट्की" भनेर चिनिने *N. scrophulariiflora* (Pennell) D.Y. Hong लाई परम्परागत औषधीय प्रयोगका लागि प्राथमिकता दिइएको छ। *N. scrophulariiflora* का जङ्गली बोटबिरुवाका भागहरूको जैविक गतिविधिहरूमा पहिले धेरै अध्ययनहरू गरिएको थियो तर यसको *in vitro* callusको जैविक गतिविधिहरूमा कुनै अध्ययन गरिएको छैन। अध्ययनको उद्देश्य callusको मात्रा बढाउने र कुल फेनोलिक कन्टेन, कुल फ्लेभोनोइड कन्टेन, एन्टिअक्सिडन्ट गतिविधि, मधुमेह विरोधी गतिविधि र विषाक्तता निर्धारण गर्नु थियो। कुल फिनोल र कुल फ्ल्याभोनोइड कन्टेन निर्धारण गर्न क्रमशः FC reagent र एल्युमिनियम क्लोराइड विधि प्रयोग गरिएको थियो। callusको एन्टिअक्सिडन्ट गतिविधि र मधुमेह विरोधी गतिविधि निर्धारण गर्न क्रमशः DPPH स्क्याभेन्जिङ विधि र alpha glucosidase inhibition विधि प्रयोग गरिएको थियो। हाम्रो अध्ययनमा नरिवल पानी राख्दा बीउको उच्चतम अङ्कुरण प्राप्त भयो। 1mg/l NAA हर्मोन मा हरियो रंगको callusको पर्याप्त वृद्धि पाइयो। 1mg/l BAP र 0.5 mg/l NAA हर्मोनको संयोजनबाट callus मा साना हरियो पातहरू भएका अधिकतम दुई अङ्कुरहरू अवलोकन गरियो। यी बाहेक, callusको methanol एक्स्ट्र्याक्टमा उच्चतम कुल फिनोल र फ्ल्याभोनोइड कन्टेन पाइएको थियो। यसबाहेक, callusको methanol एक्स्ट्र्याक्टले aqueous एक्स्ट्र्याक्टको तुलनामा उच्चतम एन्टिआक्सिडन्ट गतिविधि प्रदर्शन गर्यो जबकि alpha glucosidase एन्जाइममा कुनै अवरोध गतिविधि थिएन। *N. scrophulariiflora* को callusको methanol एक्स्ट्र्याक्ट कम विषाक्त भएको पाइयो जबकि aqueous एक्स्ट्र्याक्टले गैर-विषाक्तता देखायो। अनुसन्धानको निष्कर्षले *N. scrophulariiflora* को callus पनि फिनोल र फ्ल्याभोनोइड कम्पाउन्डको स्रोत हो जुन औषधिहरूको उत्पादनमा प्रयोग गर्न सकिन्छ तर जैविक गतिविधिहरूको मूल्याङ्कनले जंगली बिरुवाको तुलनामा callus मा कम उपचारात्मक क्षमता भएको देखाउँछ।

List of Abbreviations and Acronyms

NAA	Naphthalene acetic acid
BAP	Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
Kn	Kinetin
IAA	Indole-3-Acetic Acid
IBA	Indole-3- Butyric acid
Mg/l	Miligram per litre
g/l	Gram per litre
MS	Murashige and skoog's medium (1962)
ROS	Reactive Oxygen Species
DPPH	2, 2-diphenyl-1-picrylhydrazyl
IC_{50}	Inhibitory concentration at 50%
LC_{50}	Lethal concentration at 50%
$AlCl_3$	Aluminium chloride
Na_2CO_3	Sodium carbonate
FC	Folin Ciocalteau reagent
PNPG	para-Nitrophenyl-β- D-glucopyranoside
TUCH	Tribhuvan University Central Herbarium
DNA	Deoxyribose nucleic acid
TPC	Total phenolic content
TFC	Total flavonoid content
GAE	Gallic acid equivalent
QE	Quercetin equivalent

V	Volume of extract
µL	microlitre
µg	microgram
mg	miligram
ml	mililitre
SD	Standard deviation
RSA	Radical Scavenging Activities
UGC	University Grants Commission
°C	Degree Celsius
ANOVA	Analysis of variance

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1. Introduction

1.1Background

Medicinal plants reflect the deep-rooted tradition of herbal medicine (Shrestha et al., 2022). Economically important medicinal plants such as *Neopicrorhiza scrophulariflora*, *Ophiocordyceps sinensis*, *Nardostachys jatamansi*, and *Fritillaria cirrhosa* have garnered attention due to their pharmaceutical value (Pyakurel et al., 2019). The historical and cultural significance of medicinal herbs transcends geographical boundaries, with their utilization dating back to ancient time. Each civilization developed distinct perspectives on health and illness, contributing to the diverse practices observed in traditional medicine (Sohani, 2021). In Nepal, rural communities predominantly rely on medicinal, aromatic, and non-timber plants for traditional healthcare and supplementary income (Angelsen et al., 2014; Lamichhane et al., 2021). Ethnomedicinal research serves as a crucial repositoryof knowledge, identifying valuable medicinal plants for cultivation and conservation (Njoroge et al., 2004). The pharmacological potential of plants lies in their bioactive compounds, including alkaloids, phenols, tannins, cardiac glycosides, volatile oils, and terpenoids (Sharma et al., 2020). However, the escalating demand for phytoconstituents has led to overharvesting and illicit trade, posing a threat to both plant biodiversity and the livelihoods of Himalayan communities (Bhattarai and Tamang, 2017). Consequently, concerted efforts, both nationally and internationally, are underway to safeguard these invaluable resources (Shukla, 2023). Tissue culture emerges as a promising strategy to enhance the production of endangered medicinal plants sustainably.

1.1.1 Plant tissue culture and callus formation

Plant tissue culture, involving the laboratory establishment of plant cells or tissues under regulated conditions, offers a controlled environment for mass propagation of medicinal plants, ornamentals, and food crops (Kumlay et al., 2015). This technique holds particular relevance for the pharmaceutical industry, facilitating the production of plant-derived compounds essential for drug development (Rahayu et al., 2016). Callus culture is one of the types of plant tissue culture which involves the production of mass of undifferentiated cells derived from somatic tissues through high rate of cell division and proliferation while subjected in nutrient media (Sokmen et al., 2001). The manipulation of auxin and cytokinin concentrations influences callus induction and growth, with auxin playing a pivotal role in cell differentiation and expansion (Sharma and Agrawal, 2018; Ikeuchi et al., 2013).

1.1.2 Callus for the secondary metabolites production

Callus, a mass of undifferentiated cells, derived from somatic tissues in *in-vitro*, serves as a key intermediate in production of valuable plant metabolites having medicinal properties (Khurshid et al., 2020). Numerous secondary compounds were determined in callus culture of medicinal plants such as psoralen, daidzein and genistein from *Cullen corylifolium* (Singh et al., 2020), meroterpene bakuchiol from *Psoralea drupacea* (Lystvan et al., 2010), flavonoids, phenylpropanoids, alkalods, fatty acids and aromatic glycosidase from *Carthamus tinctorius* (Liu et al., 2021), epigallocatechin and chlorogenic acid from *Oryza sativa* (EL-Beltagi et al., 2022), Picroside I and Picroside II from *Picrorhiza kurroa* (Yamjala et al., 2017; Partap et al., 2022).

1.1.3 Biological activities of the callus

Callus tissue, enriched with phenolic and flavonoid compounds, presents an alternative source for valuable phytochemicals, exhibiting potential biological activities such as antifungal, anticancer, antidiabetic, and antioxidant properties (Lian et al., 2019). Antioxidants mitigateROS-induced damage caused by imbalance between the formation of reactive oxygen species (ROS) and antioxidantdefense systems (Kasote et al., 2015). Furthermore, Diabetes mellitus is a seriousmetabolic disorder which leads to hyperglycemic syndrome that causes numerous health problems. This disorder can be regulated by lowering the glucose level using alpha-amylase and alpha-glucosidase (Kajaria et al., 2013). Moreover, it was also reported that the diabetes is directly related to oxidative stress due to rise in the production of ROS (Sarian et al., 2017). It is assumed that the therapy for diabetes liesin the herbal approach because medicinal plants contain phytoconstituents to treat diabetes such as glycosides, terpenoids, saponins, alkaloids, flavonoids and carotenoids (Afrisham et al., 2015). Due to the rising demand for herbal medicines, sustainable production methods i.e. callus culture is imperative to prevent the depletion of wild plant populations.

In this scenario, the current study aims to assess the efficiency of *N. scrophulariiflora* callus in managing oxidative stress and inhibiting glucose level. By harnessing the potential of plant tissue culture, researchers endeavor to explore novel avenues for herbal medicine production, ensuring sustainability and conservation of medicinal plant.

1.2 Rationale

The global landscape is currently grappling with the emergence of novel illnesses, imposing constraints on the duration of good health for living organisms. Consequently, researchers are increasingly drawn to exploring diverse chemical compounds sourced from medicinal plants, seeking effective remedies for a spectrum of ailments. Natural compounds derived from wild plants are favored due to their comparatively lower adverse effects. Many plants were traditionally utilized as remedies to treat a variety of ailments due to the presence of bioactive compounds that endow them with curative properties. The discovery and isolation of these chemicals hold promise for broader applications in the pharmaceutical sector, while also validating their traditional uses through scientific inquiry. Among the methodologies employed for the production of medicinally valuable compounds, callus culture from medicinal plants emerges as a pivotal technique (Hendrawati et al., 2012).

Neopicrorhiza scrophulariiflora stands out as an economically significant medicinal plant, with its rhizomes predominantly exported to international markets (Pyakurel et al., 2019). It is being emphasized for preservation and revenue generation (GoN/MoFSC/DPR, 2006) and has a rich history of traditional use in treating various ailments. Furthermore, scientific research has underscored its multifaceted therapeutic properties, demonstrating its efficacy as an anti-atherosclerotic, anti-diabetic, antioxidant, hepatoprotective, immune-modulatory, and anti-inflammatory agent (Rokaya et al., 2020).

Due to the medicinal significance of *Neopicrorhiza scrophulariiflora*, it is imperative to investigate inhibitory effects of its callus extract on the activity of alpha-glucosidase and ascertain whether it contributes to the reduction of oxidative stress. Additionally, the lack of studies on the biological activity of this plant's callus extract underscores the need for further research in this area.

1.3 Research questions

Seed derived shoot culture produced mass of callus on nutrient medium and its extract has high total phenolic and flavonoid contents may show antioxidant and antidiabetic activity.

1.4 Objectives

1.4.1General Objective

The key goal of the current research is to produce callus from *Neopicrorhiza scrophulariiflora*’s shoot of seedlings and evaluate its antioxidant and antidiabetic properties.

1.4.2 Specific Objectives

The specific objectives of this study are:

- To induce the mass of callus from shoot culture using various growth hormones.
- To quantify the total phenolic and flavonoid content in the callus extract.
- To investigate of the toxicity, antioxidant and antidiabetic potential of the callus extract.

2. Materials and Methods

2.1 Materials

2.1.1 Plant Material (*Neopicrorhiza scrophulariiflora*)

Neopicrorhiza, a genus endemic to the Eastern Himalayas and belonging to the Plantaginaceae family, holds significant importance as a medicinal plant genus, commonly referred to as Kutki. *Neopicrorhiza scrophulariiflora*, a prominent species within this genus, thrives in altitudes ranging from 3500 to 5000 meters above sea level across Nepal, China, Bhutan, India, and Myanmar (Ghimire et al., 2005; Press et al., 2000). It prefers damp, north-facing hillsides, predominantly inhabiting meadows, shrublands, moraine gravel areas, and woodlands throughout the Western, Central, and Eastern regions of Nepal (Figure 1) (Shrestha et al., 2022).

Figure 1: *Neopicrorhiza scrophulariiflora.* A. Habit. B. Flower. C. Inflorescence.

Neopicrorhiza scrophulariiflora holds a profound significance in the lives and cultural practices of local communities, being deeply ingrained in traditional medicine practices (Ghimire et al., 2023). Its therapeutic applications extend to a wide spectrum of ailments, including blood-related disorders, respiratory issues, skin conditions, urinary problems, gastrointestinal troubles, liver ailments, and heart conditions (Smit, 2000). Utilized primarily for its rhizome, this plant is employed in the management of fever and gastralgia, while its root powder serves as a remedy for constipation and alleviation of abdominal discomfort. Extracts from *Neopicrorhiza scrophulariiflora* yield approximately 124 significant phytochemicals such as caffeoyl glycoside, cyclopentanoid monoterpenes, hydroquinone glycoside, non-glycosidic iridoid, phenyl ethyl, secoiridoid glycoside, and flavonoids, among others (Smit, 2000), iridoid glycoside, phenyl glycoside, cucurbitacin glycoside, phenylpropanoid (Sah and Varshney, 2013), apocynin, androsin, picroside (Chen et al., 2011), scroside, phenylpropanoids, gallic acid, isoferulic acid, and vanillic acid (Huanget al., 2009), rendering it valuable as an anthelmintic, antioxidant, anti-inflammatory, hepatoprotective agent, among other therapeutic applications (Rokaya et al., 2020). Furthermore, pharmacological investigations have elucidated the plant's efficacy *in vitro*, demonstrating antifungal (Jiang et al., 2020), antidiabetic (Xu et al., 2020), and immunomodulatory activities (Smit, 2000). Studies on *N. scrophulariiflora*'s iridoid and glycoside constituents have highlighted their anti-diabetic effects when administered to rats (Sasidharana and Elyas, 2019).

In Ayurvedic medicine, this plant features prominently, with forty-five formulations incorporating elements of *Neopicrorhiza scrophulariiflora* to address diverse health concerns (Kafle et al., 2018). Among Nepalese communities, it is utilized for treating various ailments such as headaches, coughs, colds, heart conditions, diarrhea, bile disorders, hypertension, snake bites, intestinal discomfort, and gastric distress (Hasan et al., 2013). Moreover, it is enlisted for managing chronic health conditions like diabetes and high blood pressure (Ghimire et al., 2023).

2.1.2 Chemicals

A variety of chemicals were employed throughout the experiment, including methanol, ethanol, gallic acid, sodium chloride, sodium carbonate, Folin-Ciocalteu reagent, aluminium chloride, quercetin, sodium acetate, ascorbic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH), alpha glucosidase enzyme, p-nitrophenyl α-D-glucopyranoside (pNPG), potassium dihydrogen phosphate and dipotassium hydrogen phosphate.

In addition, for tissue culture purposes, stocks A, B, C and D, sucrose, myoinositol, coconut water, hormones such as BAP (6-benzyl amino purine) and NAA (α-Naphthalene acetic acid), 2,4-dichlorophenoxyacetic acid (2,4-D), Kinetin (Kn), and distilled water were utilized for media preparation. Furthermore, ethanol, sodium hypochlorite, and bavistin were employed for sterilization procedures.

2.1.3 Apparatus

The apparatus used in this experiment was a micropipette, glass jars, test tubes, measuring cylinder, conical flask, separating funnel, stand, pipette, glass rods, glass vials, a round bottom flask, and 96-well microplates.

2.1.4 Instruments

A microplate reader, autoclave, hot air oven, rotary evaporator, electric grinder, laminar air flow, shaker, distillatory, weighing machine, water bath, and refrigerator were used.

2.1.5 Test Organisms

To determine the toxicity of callus extracts of *N. scrophulariiflora*, eggs of brine shrimp (*Artemia salina*) were used.

2.2 Methods

A. Tissue Culture

2.2.1 Collection of Seeds

The seeds were gathered from the Khumbu valley, situated within Sagarmatha National Park and its adjacent buffer zone in the Solukhumbu district of the Nepalese Himalayas. The geographic coordinates of the collection site are approximately 27° 49’ 1.9” N and 86° 42’ 1.9” E, spanning an altitude range of 3,300 meters to 8,848 meters above sea level. Taxonomic identification of the plant was carried out by esteemed botanists, namely Prof. Dr. Suresh Kumar Ghimire from the Central Department of Botany, along with Prof. Dr. Hari Datta Bhattrai, Dr. Mukti Ram Paudel, and Dr. Narayan Prasad Ghimire, during a field expedition conducted from October 21 to November 2, 2021. The herbarium of the collected plant was submitted to Tribhuvan University Central Herbarium (TUCH) (Voucher specimen no: UGC 2019-05). Subsequent experiments were conducted in the laboratory of the Central Department of Botany, Tribhuvan University, located in Kathmandu.

2.2.2 Preparation of Stock Solution

In accordance with the formulation by Murashige and Skoog (1962), stock solutions were comprised of macronutrients, micronutrients, an iron source, and vitamins. During the preparation of these stock solutions, all constituents were precisely weighed and dissolved in distilled water. The mixture was then vigorously stirred using a magnetic stirrer to ensure complete dissolution. Subsequently, the prepared stock solutions were transferred into clean, labeled bottles and stored in a refrigerated environment at 4°C to maintain their integrity and stability.

2.2.3 Sterilization of Glass Wares and Metal Instruments

The experiments were carried out utilizing sanitized metal and glassware exclusively. The glassware underwent thorough cleaning by soaking in detergent water for 24 hours, followed by scrubbing with a bottle brush and rinsing with tap water. Subsequently, a final rinse with distilled water was performed before sterilization. The glassware was then sterilized in a hot air oven at 120°C for 3-4 hours. To assure sterilization, metal equipment was also cleaned with tap water and autoclaved for 45 minutes at 121°C.

2.2.4 Preparation of Medium

Stocks A, B, C, and D were dispensed into a 1 L measuring cylinder in increments of 100 ml, 1 ml, 10 ml, and 1 ml, respectively. Subsequently, 30 g of sugar and 0.1 g of Myo-inositol were added and dissolved. Distilled water was then added to the medium to reach a total volume of 1000 ml. Cytokinin and auxin were carefully supplied using a micropipette in appropriate quantities. The pH of the medium was adjusted to 5.8. To solidify the medium, 0.8 g/l of agar was incorporated. The mediumwas heated for approximately 6-7 minutes in a shaker to ensure proper dissolution of the agar. Approximately 16.5-17 ml of the medium was poured to each test tube, while1 L of medium was distributed into two jars or six test tubes, depending on the vessel type. Test tubes and jars were securely sealed using aluminum foil and elastic bands. Subsequently, the medium was sterilized in autoclave at 121°C and 15 lb./sq.inch pressure for approximately 30 minutes. Test tubes containing the medium were positioned in a slanted manner for optimal storage.

2.2.5 Preparation for Inoculation

Sterilization of the laminar airflow chamber was done before inoculation. The tubes and jars containing the medium, along with all essential inoculation supplies including media, tissue paper, ethanol, spray bottle, lamp matches, sterile water, rubbers and aluminium foils, underwent a 45-minute exposure to ultraviolet (UV) light.

Additionally, forceps, petri dishes, beakers, and blades required for the culture were sterilized by autoclaving.

2.2.6 Seed Inoculation

The viable seeds underwent a cleaning process using washing powder for 30 to 40 minutes under running tap, followed by rinsed using distilled water. After that, the seeds were sterilized for ten minutes with 1% sodium hypochlorite and then immersed for one minute in 70% ethyl alcohol. Afterward, they were rinsed about four times with sterile water within a laminar airflow cabinet. The sterilized seeds were then placed into sterile Murashige and Skoog (MS) basal media, with five seeds inserted into each tube. The cultures were grown under monitored condition at temperature of 25 ± 2°C.

2.2.7 Culture of Explant and Subculture of Callus

Due to the minute size of the leaves on the seedlings, only the shoots were utilized for callus induction in the experiment. Shoots measuring approximately 4-5 mm were excised from 6-7-week-old seedlings derived from healthy seeds. These excised shoots were then inoculated into MS media with several concentrations of NAA, BAP, 2, 4-D, Kn, and their combinations. The weight of the resulting callus was also assessed. Additionally, for callus subculture (Figure 2), the *in-vitro*-grown callus was aseptically transferredinto media containing hormones. The culture was grown at 25 ± 2 °C and a 16-hour light cycle. Subculturing was performed regularly every four weeks.

Figure 2: Subculture of callus.

B. Callus Extraction

Callus extraction was done by the maceration method reported by Begashaw et al. (2017) with slight alteration.

2.2.8 Collection, Drying and Storage of Callus

After approximately six to seven weeks, callus generated *in-vitro* was carefully taken out from the jars and let to air dry at ambient temperature in the shade for approximately 3-5 days (Figure 3, A). Subsequently, the dried callus was finely ground into a powder using a mortarand pestle, and then stored in a glass jar for further utilization.

2.2.9 Preparation of Crude Extract

Extraction was conducted using the maceration process. The dried callus powder, weighing approximately 3 grams, was separately weighed three times for three solvents using a high-precision balance. The measured callus was then added to clean conical flasks, each containing approximately 30 mL of 90% methanol, 90% ethanol, and distilled water, respectively (Figure 3, B). It was left in dark at room temperature for duration of 48 hours. Following the 48-hour period, filtration was performed using Whatman filter paper, with the filtrates collected into separate conical flasks. The process of maceration was repeated three times for each solvent. Subsequently, the filtrates were passed through Whatman filter paper and subjected to vaporization using a vacuum rotary evaporator at 37°C. This yielded crude extracts, which were carefully collected for the determination of the extract yield percentage and subsequently stored in a refrigerator.

A B

Figure 3: A-Dried callus B-Callus soaked in different solvents.

2.2.10 Percentage Yield of Crude Extracts

The percentage yield (%) = weight of extract/weight of powdered callus sample taken × 100%

2.3 Total Phenolic Content (TPC)

With a little modification, the Folin-Ciocalteu technique of Ainsworth and Gillespi's (2007) standard methodology was used to quantify the total phenolic content.

2.3.1 Preparation of Reagents

To prepare 700 mM sodium carbonate (Na_2CO_3), 0.74 grams of Na_2CO_3 were dissolved in 10 mL of distilled water. Additionally, 10% Folin-Ciocalteu (FC) reagent was prepared by diluting 1 mL of Folin-Ciocalteu (FC) in 10 mL of methanol.

2.3.2 Preparation of Gallic Acid Solution

By dissolving 1 mg of gallic acid in 1 ml of distilled water, 1mg/ml of stock solution was prepared.

2.3.3 Preparation of Sample Extract Solution

The sample extract of 1mg/ml is prepared by diluting 1 mg extract in 1 ml of distilled water.

2.3.4 Procedure

Initially, the microplate was loaded with 10 µl of the extract, 20 µl of FC reagent, and 80 µl of Na_2CO_3 to create the reaction mixture. Subsequently, the microplate was placed in a warm for approximately two hours. Following incubation, the absorbance was taken at 765 nm using a microplate reader.

Next, a standard curve was generated using various concentrations of gallic acid 50 µg/ml to 3.125 µg/ml. Following this, the microplate was loaded with 20 µl of FC reagent, and 80 µl of sodium carbonate. Once again, it was left in warm for approximately 2 hours before measuring the absorbance at 765 nm using a microplate reader. The total phenolic content in the callus extract was then quantified as milligrams of gallic acid equivalent per gram of dry weight of callus extract, utilizing the gallic acid standard curve.

2.3.5 Calculation of TPC

The total phenolic content was measured by using the following formula:$C = c\ V/m$

Where, C = total content of the phenolic compounds (mg/gm) in gallic acid equivalent c = concentration of gallic acid obtained from the calibration curve (µg/ml)

V = volume of extract in ml and m = mass of extract in gm

2.4 Total Flavonoid Content (TFC)

After a minor modification to Majid et al. (2018)'s standard methodology, the total flavonoid content was ascertained using the aluminum chloride method.

2.4.1 Preparation of Reagents

A 10% solution of aluminum chloride was created by adding 1 gram of $AlCl_3$ in 10 ml of distilled water. Additionally, a 1 M solution of sodium acetate was created by adding 0.82 grams of sodium acetate in 10 ml of distilled water.

2.4.2 Preparation of Standard Quercetin Solution

By adding 1 mg quercetin in 1 ml methanol, 1 mg/ml of stock solution was prepared. Again, it was serially diluted to obtain a concentration of 62.5 μg/ml-7.81 μg/ml.

2.4.3 Preparation of Sample Extract Solution

It was made by adding 1 mg of the methanol, ethanol, and aqueous callus extract separately into 1 ml of distilled water in individual Eppendorf tubes, resulting in a final concentration of 1 mg/ml.

2.4.4 Procedure

The aluminum chloride technique was used to calculate the total flavonoid content. First, 20 μl of the extract, 160 μl of distilled water, 10 μl of aluminum chloride, and 10 μl of sodium acetate were added to the microplate. The microplate was then kept in a warm for about half an hour. After that, a microplate reader was used to measure the absorbance at 415 nm.

A standard curve was generated using different concentrations of standard quercetin 62.5 μg/ml to 7.81 μg/ml. Following this, the microplate was loaded with 160 μl of distilled water, 10 μl of aluminum chloride, and 10 μl of sodium acetate. Once again, it was incubated in warm for approximately 30 minutes before measuring the absorbance at 415 nm using a microplate reader.

The TFC in the callus extract was then quantified as milligrams of quercetin equivalent per gram of dry weight of callus extract, utilizing the quercetin standard curve.

2.4.5 Calculation of TFC

The total flavonoid content was measured by using the following formula:$C = c\ V/m$

Where, C = total content of the flavonoid compounds (mg/gm) in quercetin equivalent

c = concentration of quercetin obtained from the calibration curve (μg/ml)

V = volume of extract in ml, m = mass of extract in gm

2.5 Antioxidant Activity

With a little change, the standard methodology of Blois (1958) and Desmarchelier et al. (1997) was used to determine the antioxidant ability of callus extract using the DPPH free radical scavenging assay.

2.5.1 Preparation of 0.1 mM DPPH

By dissolving 3.943 mg (mol wt. 394.32 g) DPPH in 100 ml methanol, 0.1 mM DPPH was prepared and stored in the dark.

2.5.2 Preparation of Sample Extract Solution

A 1 mg/ml extract solution was prepared by adding sample extract i.e., 1 mg in 1 ml methanol.

2.5.3 Procedure

Initially, the stock solution of the callus extract was diluted in 100μl of methanol to generate concentrations of 1000 μg/ml to 31.25 μg/ml through serial dilution. Furthermore, ascorbic acid was utilized as the reference standard compound. A 1 mg/ml stock solution was prepared by dissolving 1 mg of ascorbic acid in 1 ml of methanol. This stock solution was then serially diluted to obtain concentrations of 5 μg/ml to 0.5 μg/ml. For the negative control, a reaction mixture consisting of methanol and DPPH was utilized.

Next, a reaction mixture was prepared by combining 100 μl of the stock solutions of methanol and aqueous extract at different concentrations with 100 μl of freshly prepared 0.1 mM DPPH methanol solution in a 96-well plate. The plate was then incubated in darkness for half an hour. Subsequently, the absorbance was measured at 517 nm using a microplate reader. Each test was conducted in triplicate.

The % inhibition was plotted against the extract concentration to create a calibration curve. Similarly, the curve for the sample was plotted, and the IC_{50} values were determined.

2.5.4 Measurement of DPPH Radical Scavenging Activity and IC_{50} Value

The percentage of inhibition was calculated by

% inhibition = (A0-At)/A0 ×100

Where, A0= Absorbance of DPPH, At = Absorbance of callus extract.

A standard curve was created with concentrations on the x-axis and inhibition percentage on the y-axis from the resulting value. Using the linear equation of the obtained curve, the IC_{50} value was also determined by the following equation:

$Y = mx + c$, $IC_{50} = (50-c)/m$

Where, x = concentration, y = %inhibition, m = coefficient of linear equation and c =constant of the linear equation

2.6 Brine Shrimp Lethality Assay (Toxicity Test)

The toxicity assessment of the plant extract was conducted based on the procedure outlined by Maurer-Jones et al. (2013) and Kakar et al. (2020), with minor modification.

2.6.1 Preparation of Stock Solution

This solution was formulated by adding 1 mg of each extract, namely methanol, ethanol, and aqueous extract, in 1 ml of distilled water separately. Subsequently, 1000 μg/ml to 62.5 μg/ml extracts were prepared through serial dilution with distilled water.

2.6.2 Preparation of Brine Solution

Brine solution was made by adding 3.8 g of NaCl in 100 ml water which is also known as artificial sea water.

2.6.3 Procedure

Artemia salina (brine shrimp) eggs, approximately one-fourth of a tablespoon, were introduced into artificial sea water and placed under light conditions to maintain a temperature of approximately 25-28°C for 48-72 hours (Figure 4). Subsequently, a solution containing counted larvae (10) and 1000 to 62.5 μg/ml extracts was preparedin a vial. The vials were then exposed to continuous light for 24 hours. All experiments were conducted in triplicate. After 24 hours, surviving nauplii were enumerated and the mortality percentage was evaluated by

Mortality % = (number of dead nauplii / initial number of live nauplii) × 100%

The procedure described above was used to get the extracts LC_{50} value (lethality concentration). The mortality percentage was then plotted on a probit scale against the logarithm of the inhibitor concentration to create a linear regression line. The experiment was replicated three times for accuracy.

Figure 4: Brine shrimp larvae cultured at 25-28°C for 48-72 hours.

2.7 Antidiabetic activity

The antidiabetic activity of callus extract was determined by using alpha glucosidase inhibitory assay following the standard protocol of Bhatia et al. (2019) with slight alteration.

2.7.1 Preparation of Sample Stock Solution

First 1 mg callus extracts were weighed in separate Eppendorf tubes and dissolved in 1 ml water to make a 1 mg/ml stock solution.

2.7.2 Preparation of Phosphate Buffer (100 mM)

A phosphate buffer solution of 100 mM and pH 6.8 was prepared by adding 0.739 gm of potassium dihydrogen phosphate (K_2HPO_4) and 0.795 gm dipotassium hydrogen phosphate (KH_2PO_4) in 100 ml distilled water.

2.7.3 Preparation of 5 mM PNPG Solution

For 5 mM PNPG solution, 7.532 mg of para-Nitrophenyl-β- D-glucopyranoside substrate (mol wt. 301.3 g) was dissolved in a 5 ml phosphate buffer.

2.7.4 Preparation of 1-unit alpha-Glucosidase Solution

By adding 50 µl 200 units stock enzyme solution in 9950 µl buffer, 1-unit alpha glucosidase solution was prepared.

2.7.5 Procedure

Initially, the sample stock solution was serially diluted to obtain a concentration of 1000 μg/ml-1.95 μg/ml but it did not show any activity against the enzyme. So, again thevarying concentrations were made from 5 mg/ml stock solution. A microplate was loaded with 20μl of extract fraction at different concentrations, 40 μl buffer and 20 μl alpha glucosidase enzyme of 1 unit. The solution was then incubated at room temperature for 15 minutes. After the incubation period, 20 μl of PNPG were taken in a96 well plate. It was then incubated at room temperature for 15 minutes. Later, to terminate enzyme-substrate reaction, 100 μl Na_2CO_3 was added in reaction mixture to inhibit enzyme activity. Furthermore, absorbance of sample was measured at 405 nm. The mixture of buffer, enzyme and PNPG was used as a negative control. Unfortunately, again the sample stock solution did not show any activity against enzyme.

2.7.6 Measurement of Inhibition of alpha-Glucosidase

The inhibition of alpha glucosidase can be calculated by

% inhibition = Ab control- Ab extract / Ab control ×100

Where, Ab control= Absorbance of the negative control, Ab extract= Absorbance of the callus extract

A calibration curve can be obtained by plotting the graph between % inhibition on the Y axis and concentration of extract on the X-axis. The curve of the sample should be plotted and IC_{50} values can be determined.

2.8 Data Analysis

Each tissue culture experiment was done thrice, and data analysis was done using Microsoft Excel and SPSS. Data were presented as mean ± standard deviation (SD). Regression analysis was performed using Microsoft Excel, while ANOVA test was conducted using SPSS software. Similarly, all experiments investigating the biological activities of callus extracts were repeated thrice, and data were analyzed using Microsoft Excel and SPSS. IC_{50} values for antioxidant activity and LC_{50} values for the lethality assay were determined using a linear regression. Paired T-tests were conducted using SPSS software. The significance level was set at $p<0.05$.

2.8.1 Flow Chart for Callus Culture, Extraction, and Analysis

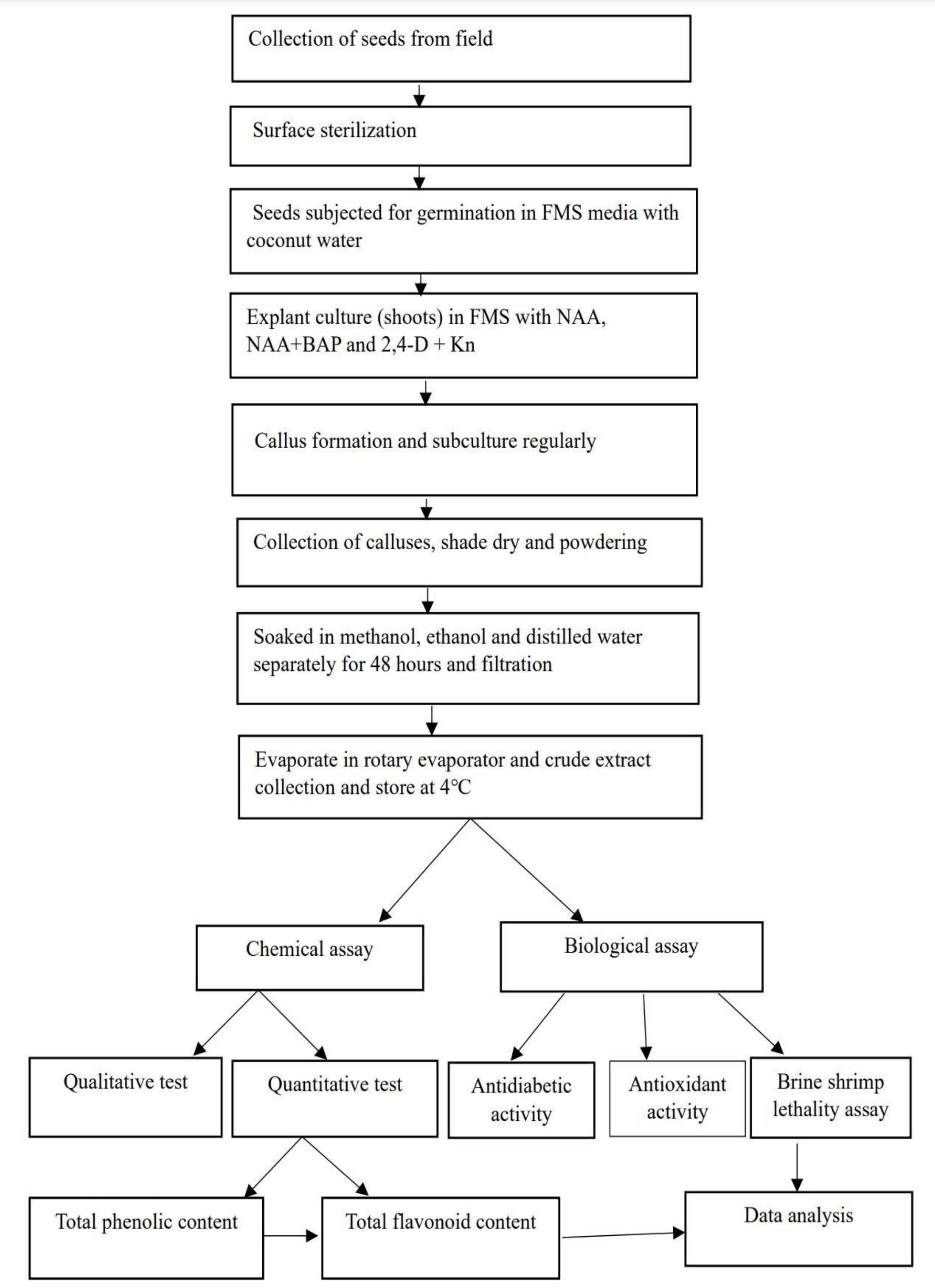

Figure 5: Flow Chart of Research

3. Results

3.1 Seeds Culture

The sterilized seeds were cultivated on MS media with varying concentrations of coconut water (CW), including 5%, 10%, and 15%, as well as without coconut water. Each test tube contained five seeds, with six replicates per concentration. This experiment was conducted three times. The cultures were maintained under controlled conditions at a temperature of 25 ± 2°C. Seed germination commenced within three weeks (Figure 6, A).

Table 1: Seeds germination percentage.

S.N.	Media + C.W. (%)	Number of seeds germinated (Mean ± S.D.)	Germination%
1.	FMS	1.06±0.63	21.11
2.	FMS + 5% C.W.	1.33±0.68	26.66
3.	FMS + 10% C.W.	2.06 ±0.93	41.11
4.	FMS + 15% C.W.	1.61 ±0.85	32.22

Culture condition: MS. 25 ± 2℃, 6 Replicates, 3-4 weeks of culture. All the experiments were performed triplicate, p- value from two-way ANOVA test of number of seeds germinated versus different concentrations of coconut water in media (p-value <0.05, i.e.0.02) and germination% (p-value<0.05 i.e., = 0.02) showed significant difference.

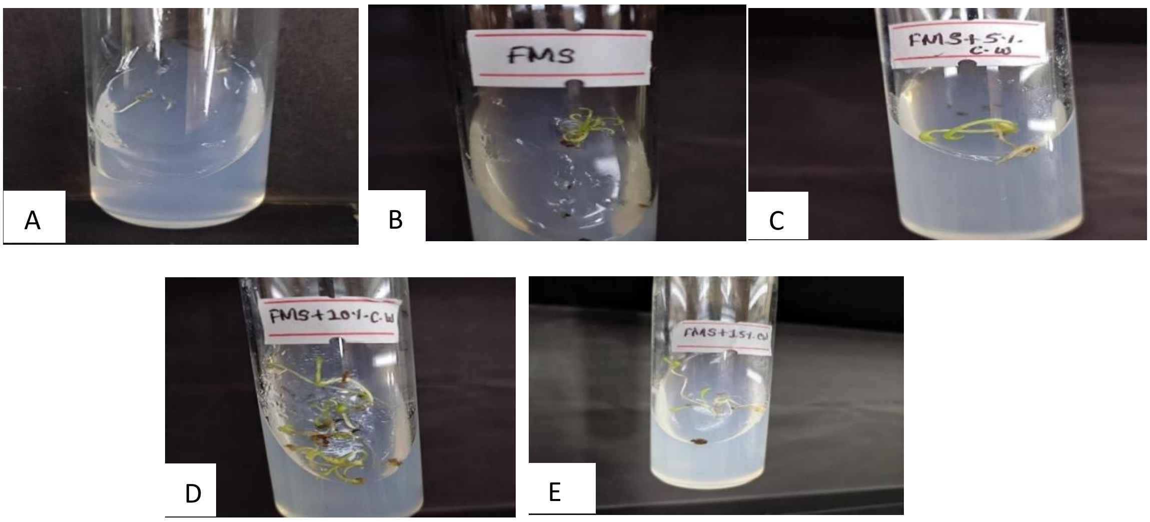

Figure 6: Seed germination of *N. scrophulariiflora* in media without and with different concentration of coconut water.

After one month, seed germination only in FMS was 21.11 % (Figure 6, B) and 26.66% in 5% coconut water (Figure 6, C). The highest total germination rate was observed in media with 10% coconut water (Figure 6, D), reaching 41.1%, followed by FMS with 15% CW (Figure 6, E) at 26.85%. All resulting plantlets exhibited normal root and shoot growth. The mean number of germinated seeds and the percentage of germination at different coconut water concentrations (Table 1).

3.2 Callus Formation from Shoot

To induce callus, young shoots were cut into 4-5 mm segments and subjected to MS media with BAP and NAA, as well as 2,4-D and Kn. After 6-7 weeks of culture, callus was successfully generated under specific conditions: 0.5 mg/l NAA, 1 mg/l NAA, 1.5 mg/l NAA, 0.5 mg/l BAP + 0.5 mg/l NAA, 1 mg/l BAP + 1 mg/l NAA, 0.5 mg/l 2,4-D + 0.5 mg/l Kn, and 1 mg/l 2,4-D + 0.5 mg/l Kn. The resulting callus exhibited diverse characteristics in different plant growth regulators, including hairy callus in 0.5 mg/l NAA (Figure 7, A), greenish callus in 1 mg/l NAA (Figure 7, B) and 1.5 mg/l NAA (Figure 7, C), as well as greenish callus in 0.5 mg/l BAP + 0.5 mg/l NAA (Figure 7, D) and 1 mg/l BAP + 1 mg/l NAA (Figure 7, E). Yellowish and soft callus was observed in both 0.5 mg/l 2,4-D + 0.5 mg/l Kn (Figure 7, F) and 1 mg/l 2,4-D + 0.5mg/l Kn (Figure 7, G). Particularly vigorous growth of green and friable callus was noted in 1 mg/l NAA, followed by of 1 mg/l BAP + 1 mg/l NAA. Subsequently, the callus produced under the conditions of 1 mg/l NAA was sub cultured in media with 1mg/l NAA to enhance the biomass for further study of its biological activity.

The details regarding callus induction, their characteristics, and biomass in MS media from shoot explants of seedlings are shown in Table 2.

Table 2: Callus induction, callus nature and biomass production in MS media with different concentrations of BAP, NAA, 2,4-D and Kn from shoot explant of seedlings.

S.N.	Media FMS + Hormone (mg/l)		Callus induction	Callus nature	Fresh weight (g) (Mean ± S.D.)
	BAP	**NAA**			
1.	0	0	No callus	- -	
2.	0	0.5	Callus	Hairy callus	0.43±0.06
3.	0	1	Callus	Greenish friable callus	0.56±0.13
4.	0	1.5	Callus	Greenish friable callus	0.42±0.05
5.	0.5	0	No callus	- -	
6.	1	0	No callus	- -	
7.	1.5	0	No callus	- -	
8.	0.5	0.5	Callus	Greenish callus	0.49±0.12
9.	1	1	Callus	Greenish callus	0.51±0.18
	2,4-D	**KN**			
10.	0.5	0.5	Callus	Yellowish and soft callus	0.43±0.019
11.	1	0.5	Callus	Yellowish and soft callus	0.44±0.08

Culture condition: MS, 25 ± 2℃, 6 Replicates, 6-7 weeks of culture. (- -) = no callus formation. All the experiments were performed in triplicate, p-value from the one-way ANOVA test of the weight of callus (g) versus different plant growth hormones (p-value <0.05, i.e., 0.011) showed a significant difference.

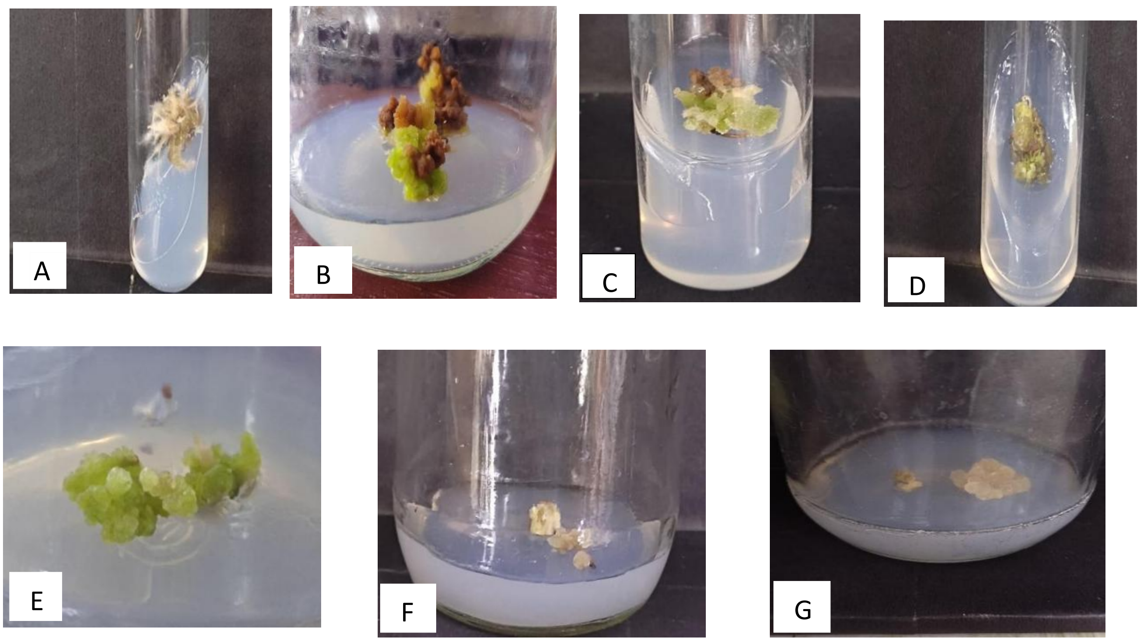

Figure 7: Callus induction in different hormones. A-0.5mg/l NAA B-1 mg/l NAA C-1.5 mg/l NAA D-0.5mg/l NAA + 0.5 mg/l BAP E- 1mg/l NAA + 1mg/l BAP F- 0.5 mg/l 2,4-D + 0.5mg/l Kn G- 1mg/l 2,4-D + 0.5mg/l Kn.

3.3 Response of NAA and BAP on *In vitro* Shoot Proliferation from Callus of *N. scrophulariiflora*

To induce shoot from *in vitro*-generated calluses, various combinations of BAP and NAA were employed. Interestingly, single hormone treatments with NAA and BAP did not elicit any significant response in terms of shoot induction from the callus. However, there was a slight increase in biomass in the case of 0.5 mg/l NAA, but later subsequent browning of the callus was observed while there was significant increase in biomass of callus with 1 mg/l NAA. After 9 weeks of callus culture, shoot initiation was observed. Shoots were successfully formed with the combinations of 0.5 mg/l BAP + 0.5 mg/l NAA (Figure 8, A), 1 mg/l BAP+ 0.5 mg/l NAA (Figure 8, B), and1 mg/l BAP + 1 mg/l NAA (Figure 8, C). Notably, only one shoot with small green leaves was generated from 0.5 mg/l BAP + 0.5 mg/l NAA. Similarly, predominantly one shoot, occasionally two shoots with small green leaves, were generated from hormone 1 mg/l BAP + 1 mg/l NAA. Additionally, two shoots with small green leaves were found in 1 mg/l BAP + 0.5 mg/l NAA.

Table 3: Response of callus for shooting (%), average number of shoots and general description of shoots formed in NAA and BAP.

S.N.	Media FMS + Hormone (mg/l)		Response for shooting %	Average numberof shoots	General description
	BAP	NAA			
1.	0	0	00.0	-	No response
2.	0	0.5	00.0	-	Less increase in callus biomass and start browning
3.	0	1	00.0	-	Only increase in callusbiomass
4.	0.5	0	00.0	-	No response
5.	1	0	00.0	-	No response
6.	0.5	0.5	33.33	1±0.81	Only 1 shoot, small greenleaves
7.	1	0.5	66.6	1.66±0.47	2 shoots, small green leaves
8.	1	1	50	0.66±0.47	Mostly 1 shoot, 2 shoots,small green leaves

Culture condition: MS, 25 ± 2°C, 3 Replicates, 9 weeks of culture. (-) = no shoot formation occurs. All the experiments were performed in triplicate, p-value from regression analysis of a number of shoots versus different combinations of BAP and NAA (p-value <0.05, i.e., 0.07) showed no significant difference.

Figure 8: Shoot proliferation from callus. A- 0.5 mg/lBAP + 0.5 mg/l NAA B- 1mg/l BAP + 0.5 mg/l NAA C- 1mg/l BAP + 1 mg/l NAA.

3.4 Extraction and Percentage Yield of Crude Extracts

A total of 0.48 g of methanol crude extract was obtained from 3 g of dry callus powder, resulting in a yield percentage of 16%. Likewise, 0.39 g of ethanol crude extract and 0.33 g of aqueous extract were obtained from the dry callus powder, yielding percentages of 13% and 11%, respectively (Figure 9).

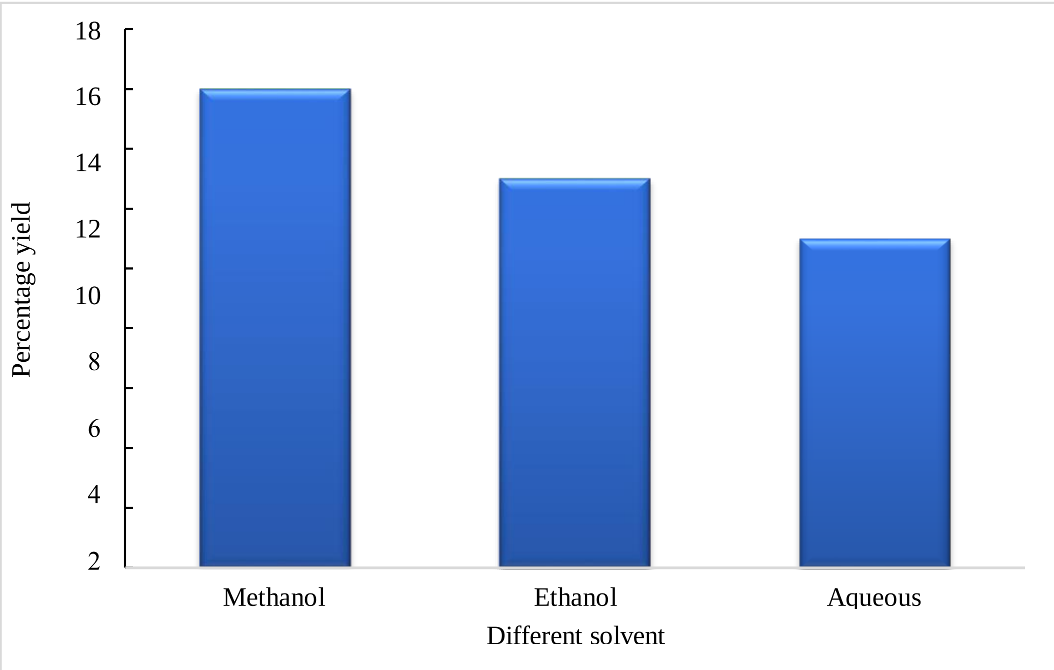

Figure 9: Percentage yield of crude callus extracts from different solvents.

3.5 Total Phenolic Content

The calculation of the flavonoid content in the extracts was conducted using the linear equation of standard curve of gallic acid ($y = 0.007x + 0.0542$, $R^2 = 0.9988$). The calibration curve for gallic acid (Figure 10) was used to estimate the total phenolic content of *N.scrophulariiflora* callus extract (Figure 11). Results indicated that the methanol extract exhibited the maximum phenolic content (38.11 mg GAE/g), whereas the ethanol extract showed the minimum flavonoid content (15.97 mg GAE/g). The phenol content of the aqueous extract was 27.97 mg GAE/g.

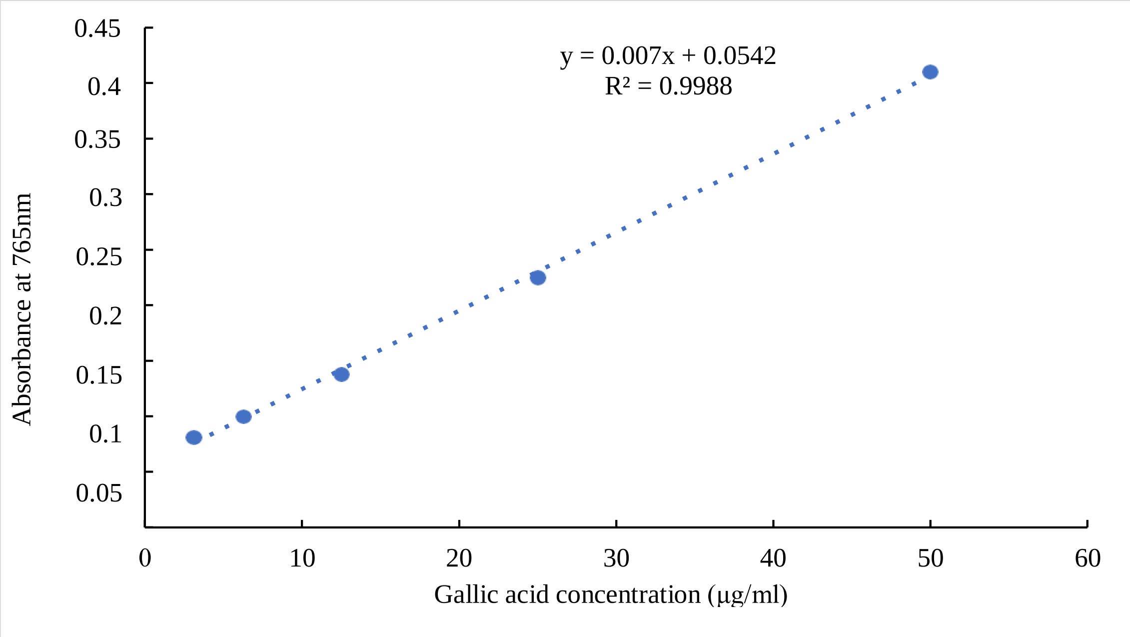

Figure 10: Calibration curve of gallic acid.

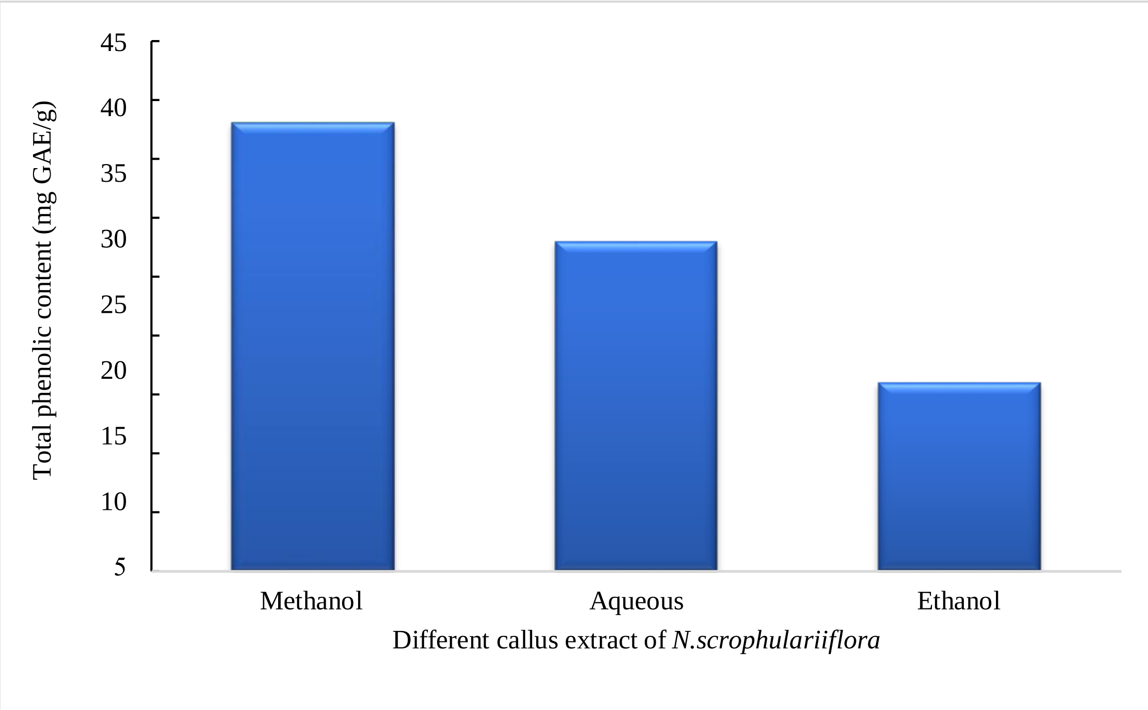

Figure 11: Total phenolic content of different callus extracts of N. scrophulariiflora.

3.6 Total Flavonoid Content

The calculation of the flavonoid content in the extracts was conducted using the linear equation of standard curve of quercetin (y = 0.0057x + 0.0635, R2 = 0.9963. The calibration curve for quercetin (Figure 12) was used to estimate the total flavonoid content of *N.scrophulariiflora* callus extract (Figure 13). Results indicated that the methanol extract exhibited the maximum flavonoid content (23.94 mg QE/g), whereas the ethanol extract showed the minimum flavonoid content (7.4 mg QE/g). The flavonoid content of the aqueous extract was 19.38 mg QE/g.

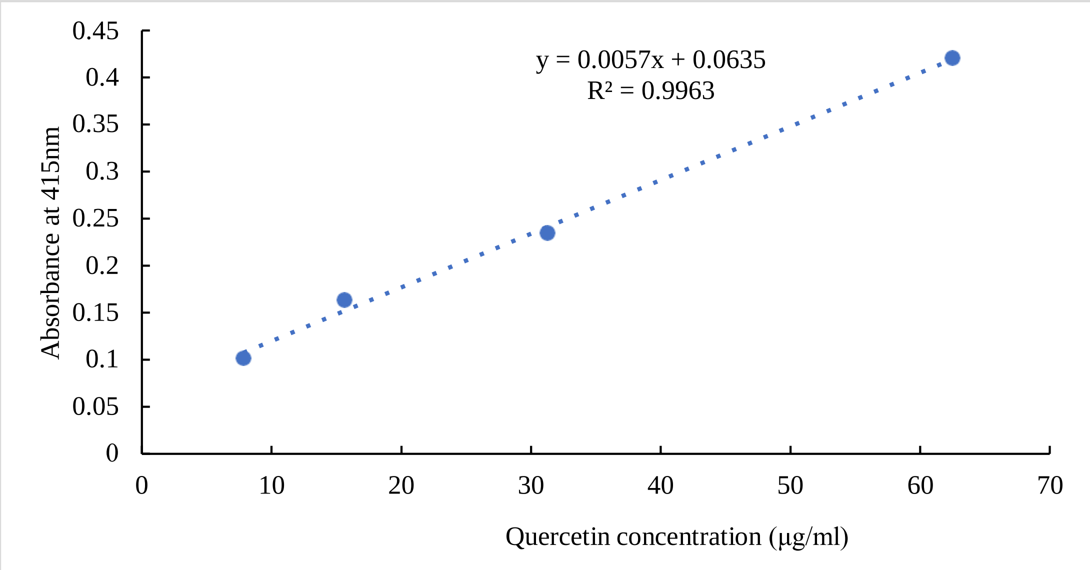

Figure 12: Calibration curve of quercetin.

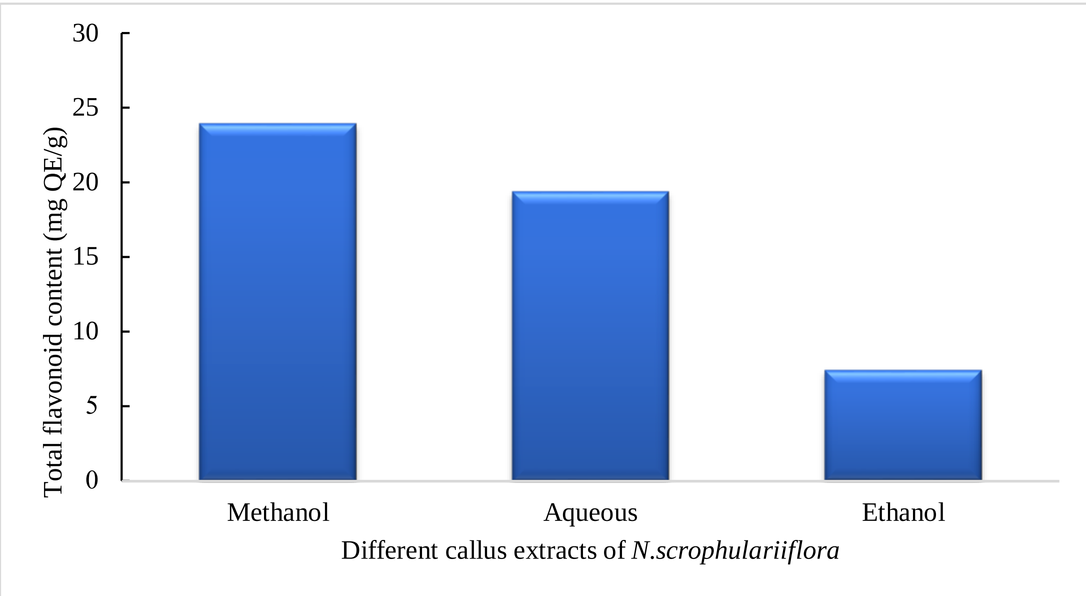

Figure 13: Total flavonoid content of different callus extract of N. scrophulariiflora.

3.7 Biological Assay

3.7.1 Antioxidant Activity

The antioxidant potential of extracts was assessed through the DPPH free radical scavenging assay, with the percentage inhibition of various extracts (Figure 14) calculated accordingly. Furthermore, the percentage inhibition of ascorbic acid (Figure 15) was used to calculate its antioxidant potential.

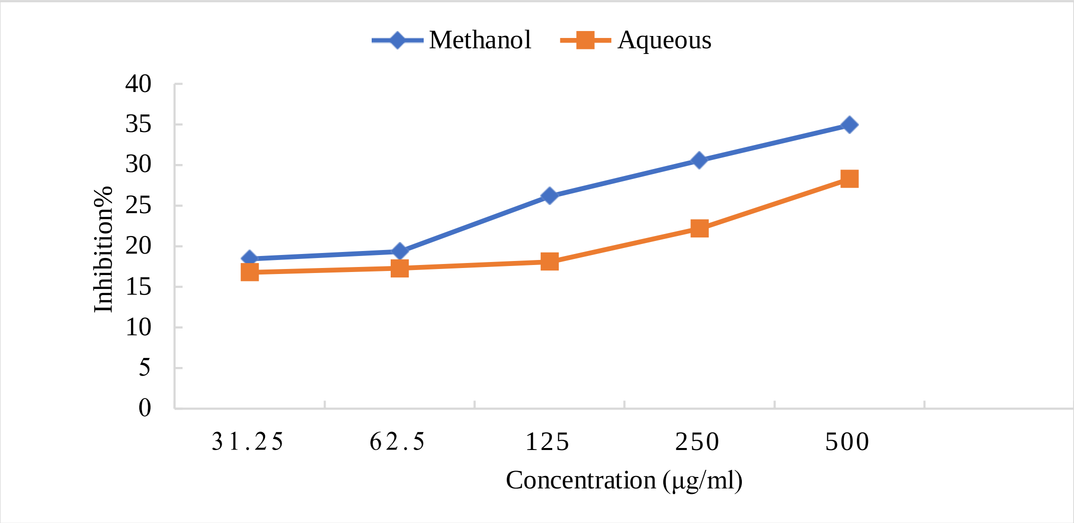

Figure 14: Percentage inhibition of DPPH by methanol and aqueous callus extract.

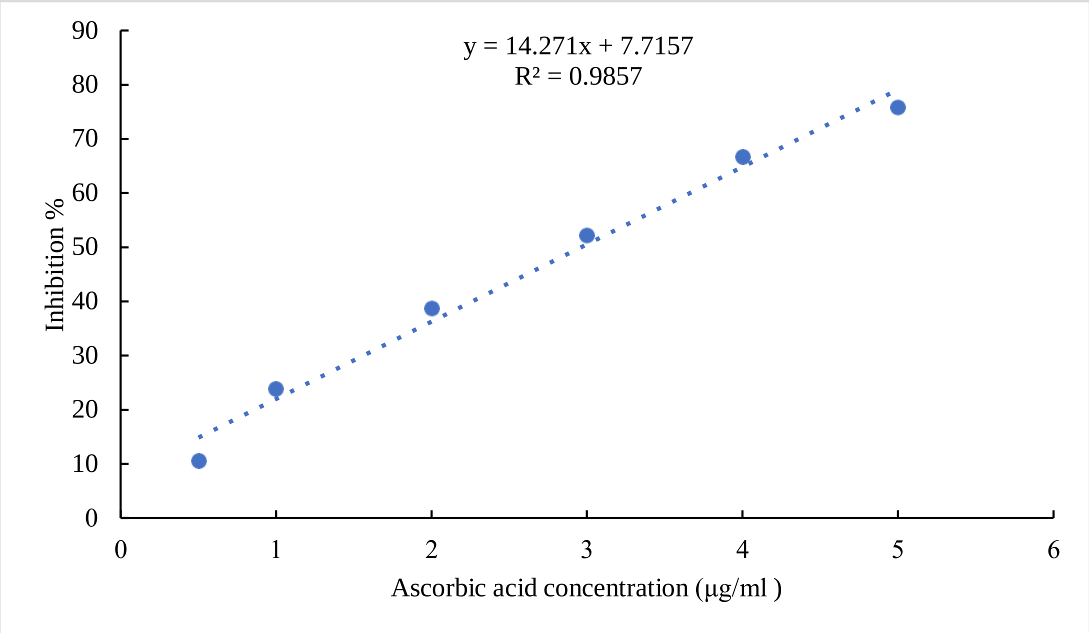

Figure 15: Percentage of Radical Scavenging activity of Ascorbic acid.

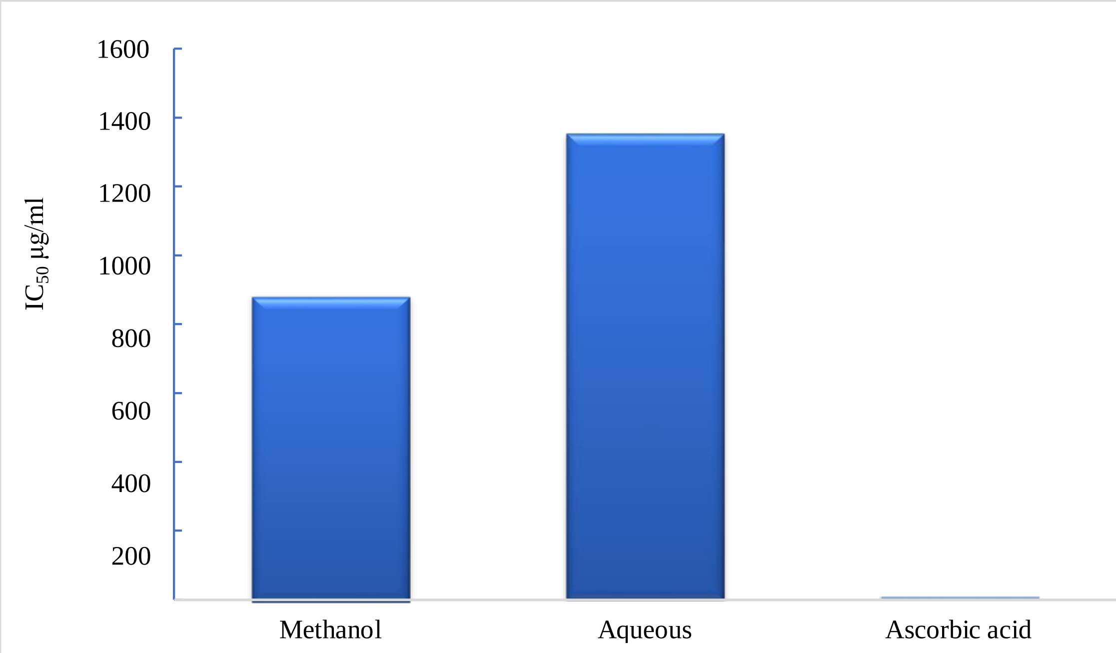

Figure 16: IC_{50} value of methanol callus extract, aqueous callus extract and standard ascorbic acid.

The above graph represents the linear increment of the DPPH inhibition percentage with the increase in the concentration of extracts. The half inhibitory concentration (IC_{50}) of various extracts was determined from the percentage inhibition using linear regression in Microsoft Excel. The IC_{50} value of the ascorbic acid was calculated 2.96 µg/ml (R^2 = 0.9857). The methanol extract exhibited the maximum antioxidant potential, with an IC_{50} value 887.08±0.87 µg/ml (R^2 = 0.873)but the aqueous extract exhibited the lowest activity with IC_{50} value 1356.617±8.40 µg/ml) (R^2 = 0.992) (Figure 16). There was a significant variance (p- value <0.05, i.e., 0.001) between the IC_{50} values of methanol and aqueous extract with ascorbic acid in paired T-test. Additionally, the ethanol extract exhibited no inhibition in qualitative analysis and was therefore excluded from antioxidant assays and subsequent analyses.

3.7.2 Brine Shrimp Lethality Test

The toxicity of the extract was evaluated using brine shrimp larvae. The lethal concentration of 50% (LC_{50}) represents the concentration of a chemical that causes mortality in half of a population of larvae within a specified duration of exposure. In this investigation, both the aqueous and methanol extracts exhibited mortality in brine shrimp larvae at concentrations ranging from 1000 µl to 250 µl. The aqueous extract of *N. scrophulariiflora* exhibited an LC_{50} of 1047 µg/ml, while the methanol extract displayed LC_{50} value 793µg/ml (Table 4).

Table 4: Results of Brine shrimp lethality assay showing mortality rate and LC_{50} of different concentrations of methanol, and aqueous extract of callus of *N. scrophulariiflora*.

Extract	Concentration of extract (µl)	Log C	Number of nauplii taken for each experiment	Number of nauplii dead			Average number of nauplii dead	Mortality rate (%)	Probit value	LC_{50} (µg/ml)
				T 1	T2	T3				
Methanol extract	1000	3	10	4	4	3	4	40	4.75	793
	500	2.6	10	2	3	1	2	20	4.16	
	250	2.39	10	1	2	1	1	10	3.72	
	125	2.09	10	0	0	0	0	0	0	
Aqueous extract	1000	3	10	2	2	3	2	20	4.16	1047
	500	2.6	10	1	1	2	1	10	3.72	
	250	2.39	10	0	1	1	1	10	3.72	
	125	2.09	10	0	0	0	0	0	0	

All the experiments were performed in triplicate, and p-value from paired t-test of different concentrations of extracts versus mortality rate (p-value <0.05, i.e., $p = 0.006$) showed a significant difference.

3.7.3 Antidiabetic activity

The effectiveness of *N. scrophulariiflora*'s callus as an antidiabetic was assessed using an alpha glucosidase inhibition test. The first two column wells of the 96-well plate (Figure 17) showing alpha- glucosidase inhibition assay has no activity due to no change in colour of enzyme.

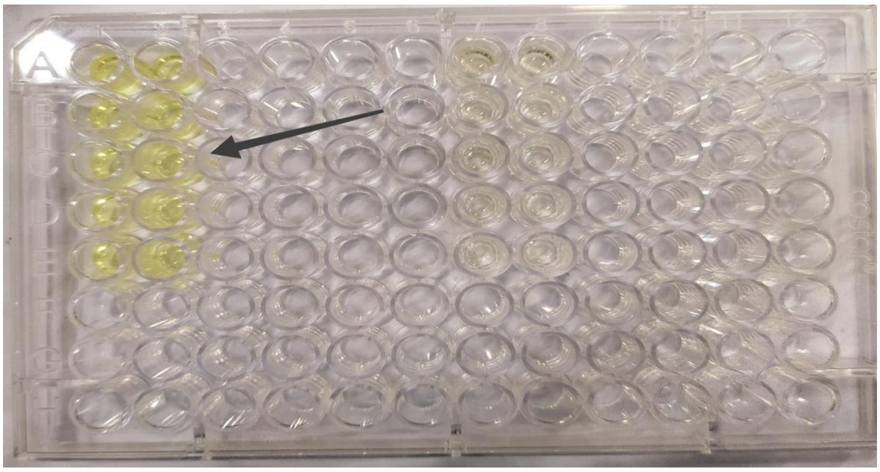

Figure 17: Alpha glucosidase inhibition test of methanol and aqueous extract of *N. scrophulariiflora*.

4. Discussion

4.1 Seed Culture

The germination response of *N. scrophulariiflora* seeds was influenced by various factors such as seed nature, age, and culture conditions, as supported by relevant literature. Our study revealed that MS full basal media devoid of hormones supported seed germination, corroborating findings in *Psoralea corylifolia* Linn (Pandey et al., 2014). Notably, the highest germination rate was noted in MS media containing coconut water of different concentrations. Among them, 10% coconut water exhibited the most favorable outcome for *N. scrophulariiflora* seed germination. Coconut water is known to contain various growth regulators, including cytokinins, gibberellins, and auxins, which promote seed germination and plant growth (Irwanto et al., 2019). Additionally, it is rich in the composition of vitamins, sugars, amino acids, and minerals further enhance seed germination and plant growth (Prades et al., 2012).

4.2 Callus Formation from Shoots

The response of shoots to callus induction was assessed after 6-7 weeks of culture. In this investigation, varying concentrations of NAA, 2,4-D + Kn and NAA + BAP (0.5 mg/l 2,4-D + 0.5 mg/l Kn, and 1 mg/l 2,4-D + 0.5 mg/l Kn, as well as 0.5 mg/l NAA + 0.5 mg/l BAP and 1 mg/l BAP + 1 mg/l NAA) exhibited callus formation. Notably, supplementation of NAA alone to the culture medium was sufficient to induce callus formation, aligning with findings in *Solanum tuberosum* (Kawochar et al., 2017) and *Clinacanthus nutans* (Ghazali et al., 2021).

Previously, in *Picrorhiza scrophulariiflora*, NAA alone did not induce callus formation significantly, whereas combining BAP with NAA resulted in higher callus production (Bantawa et al., 2011). However, in our study, NAA alone demonstrated substantial callus biomass production. Notably, the most efficient concentration was 1 mg/l NAA, leading to faster callus formation on MS media. Similar outcomes were reported in *Melastoma malabathricum* (Nauli et al., 2020), *Aconitum vilmoriniaum* (Zongmin et al., 2022), and *Hemidesmus indicus* (Saha et al., 2003). The callus induction procedure is known to be influenced by both explant type and growth regulators (Hansen et al., 1999). Additionally, NAA has been observed to stimulate both callus induction and root formation in various species (Kaviani et al., 2013).

Generally, equal concentrations of auxin and cytokinin are conducive to callus formation from different explants, as observed in our study. Equal concentrations of BAP and NAA (1 mg/l) resulted in high callus biomass following treatment with NAA alone (1 mg/l). Similar findings were reported for callus biomass from leaf explants of *Atropa acuminate* at 1 mg/l BAP and 1 mg/l NAA (Dar et al., 2021).

Interestingly, when various combinations of 2,4-D + Kn (0.5 mg/l and 1 mg/l) were utilized, a lesser amount of callus was generated. This contrasts with previous studies where 2,4-D was implicated in increased callus formation in other plants such as Indica rice (Binte Mostafiz et al., 2018). Additionally, a combination of 2,4-D and Kn (3.0 mg/l-1 mg/l Kn with 2.5 mg/l-2,4-D for stem explant) was found to be optimal for callus formation (Bisht et al., 2019). However, in our study, we observed a reduced formation of callus when employing 2,4-D and Kn, potentially attributed to the existing phytohormones within the system, impeding the achievement of the requisite ratio essential for increased callus yield (Binte Mostafiz et al., 2018).

4.3 *In vitro* Shoot Proliferation from Callus

The maximum number of shoots observed per callus in hormone NAA and BAP was two. When the callus was exposed solely to either NAA or BAP, no shooting response was elicited. However, the combination of NAA and BAP, specifically 0.5 mg/l BAP+ 0.5 mg/l NAA, 1 mg/l BAP + 1 mg/l NAA, and 1 mg/l BAP + 0.5 mg/l NAA, resulted in shoot formation from the callus. Alternatively, in a prior investigation on *N. scrophulariiflora*, it was documented that over seven shoot buds were induced per explant on woody plant medium (WPM) treated with 0.23 μM Kn and 0.53 μM NAA (Bantawa et al., 2011). This disparity may because of variations in the media composition and hormone concentrations employed between our experiment and prior research.

4.4 Extraction and Percentage Yield of Crude Extract

The characteristics of the extraction matrix, including the plant component, solvent, pressure, temperature, and time, play crucial roles in the extraction process (Azmir et al., 2013). Among the various extraction methods, maceration is commonly employed for extracting medicinal herbs (Abubakar & Haque, 2020). The efficacy of extracting bioactive constituents from plant materials relies on the polarity of the solvent and its concentration (Mustapa et al., 2015). Since a single solvent may not effectively extract all phytochemicals present in flora (Nawaz et al., 2020), we employed different polar solvents, namely water, methanol, and ethanol, for extracting the callus of *N. scrophulariiflora*.

Our results indicated that the choice of solvent significantly influenced the extraction yield. The differences in extraction yields can be ascribed to variances in solvent polarity, potentially resulting in fluctuations in the levels of bioactive compounds extracted. Our research demonstrates that methanol, ethanol, and distilled water yielded higher extraction rates, indicating that solvents with high polarity are more effective for extraction purposes. This finding aligns with similar observations in studies on *Limophila aromatica* (Do et al., 2014) and *Severinia buxifolia* (Truong et al., 2019). Methanol and ethanol solvents exhibited higher extract yields compared to water, likely because the addition of water to organic solvents enhances extraction efficiency (Do et al., 2014), and using organic solvents or mixes of organic solvents with water may be more successful for extraction than utilizing only water (Boeing et al., 2014).

4.5 Total Phenolic Content

Phenolics, also referred to as polyphenols, represent crucial plant secondary metabolites known for their antioxidant activity attributed to redox-active metal ion chelation and free radical inactivation (Sahreen et al., 2010). Several studies have investigated the total phenolic content of *Picrorhiza* species. For instance, Kumai et al. (2023) reported a total phenolic content of 141.271 mg GAE/gm in the rhizome methanol extract, while Nisar et al. (2022) found the total phenolic content of *Picrorhiza kurroa* rhizome methanol extract to be 26.18 mg GAE/gm. Furthermore, Sinha et al. (2012) reported the total phenolic content of *Picrorhiza kurroa* aqueous extract at 20, 10, and 0.5 mg/l concentrations as 42, 29.2, and 11 mg QE/gm, respectively.

The maximum phenolic content was observed in methanol extract (38.11 mg GAE/gm), followed by an aqueous extract (27.97 mg GAE/gm) and the ethanol extract (15.97 mg GAE/gm) in our experiment. While the phenolic content in our callus methanol extract was lower compared to the rhizome methanol extract of *Neopicrorhiza scrophulariiflora* (Kumai et al., 2023) but higher than the methanol extract of *Picrorhiza kurroa*'srhizome (Nisar et al., 2022) and rhizome aqueous extract of *P. kurroa* (Sinha et al. 2012). The estimation of phenolic compounds in plants is influenced by various factors including their chemical composition, extraction process, solvent type, particle size, and the presence of interfering chemicals. Solvents with high polarity typically yield higher polyphenol content (Hanachi et al., 2018). Additionally, factors such as the plant organs used, extraction methodology, environmental conditions, and geographic location can all impact the phenolic content of a plant (Dbeibia et al., 2022). It was also reported that callus, being undifferentiated cells, contains minimal concentrations of plant-specific chemicals (Hasan et al., 2022)

4.6 Total Flavonoid Content

As secondary metabolites, flavonoids have antioxidant action, although this activity depends on the availability and configuration of free OH groups (Panche et al., 2016). Several investigations have assessed the total flavonoid content of *Picrorhiza* species. Kumai et al. (2023) reported a total flavonoid content of 43.48 mg QE/gm in the methanol extract of *Neopicrorhiza scrophulariiflora*, while Nisar et al. (2022) found the total flavonoid content of *Picrorhiza kurroa* to be 19.26 mg QE/gm. Furthermore, Sinha et al. (2012) determined the total flavonoid content of *Picrorhiza kurroa* aqueous extract at concentrations of 20, 10, and 0.5 mg/ml as 13.3, 9.2, and 5.6 mg QE/gm, respectively.

The maximum flavonoid content was observed in the methanol extract (23.94 mg QE/gm), followed by an aqueous extract (19.38 mg QE/gm) and ethanol extract (7.4 mg QE/gm) in our experiment. Although the flavonoid content in our callus methanol extract was lower compared to the rhizome methanol extract of *Neopicrorhiza scrophulariiflora* (Kumai et al., 2023) but higher than the rhizome methanol extract of *Picrorhiza kurroa* (Nisar et al., 2022) and rhizome aqueous extract of *P. kurroa* (Sinha et al., 2012). The variations in flavonoid content among studies of *Picrorhiza* species may beattributed to differences in extraction methodologies, solvents used, and sample variations encompassing diverse chemical constituents (Kalt et al., 2001). Our result aligns with the concept that callus, being undifferentiated cells, contains minimal concentrations of plant-specific chemicals (Hasan et al., 2022)

4.7 Biological Assay

4.7.1 Antioxidant Activity

Cellular metabolism naturally generates reactive oxygen species (ROS), which, when produced rapidly, can damage cells and contribute to various illness. Antioxidants play a vital function in eliminating reactive oxygen species from the cells throughout the body, thereby mitigating oxidative damage by scavenging ROS or bolstering the antioxidant defense mechanism (Ummamaheswari et al., 2008). Moreover, antioxidants are beneficial in the food industry for preventing food spoilage and nutrient loss (Kant et al., 2013).

In the context of *Picrorhiza* species, limited research has explored their antioxidant properties. The IC_{50} value of the methanolic extract of *Neopicrorhiza scrophulariiflora* rhizome was reported 57.49 µg/ml (Kumai et al., 2023). Similarly, the methanolic extract of *Picrorhiza kurroa* rhizome exhibited more antioxidant potential with IC_{50} value 0.8942 mg/ml (Nisar et al., 2022) and the aqueous extract of *Picrorhiza kurroa* rhizome show more antioxidant potential with IC_{50} 0.8 ± 0.03, 0.6 ± 0.02, 0.3 ± 0.02 µg/ml (Sinha et al., 2012). However, there is no available data on the DPPH free radical scavenging activity of *Neopicrorhiza scrophulariiflora* callus. Notably, callus extract from *Pouteria bullata* demonstrated an EC_{50} of approximately 85.59 ± 6.09 µg/ml, suggesting the accumulation of antioxidants in the callus (Zaman et al., 2020). Additionally, the methanolic extract of *Crotolaria pallida* callus exhibited more scavenging potential with IC_{50} value of 23.57 ± 0.07 µg/ml (Kiruthiga et al., 2014).

In our study, crude callus extracts exhibited lower antioxidant activity compared to the control, ascorbic acid. Aqueous extracts were shown to have lesser antioxidant capacity than methanol extracts. This difference may have resulted from the methanol extract's greater concentration of phenolics and flavonoid components, which are known to have free radical scavenging action. Therefore, methanol appears to be a more suitable solvent for extracting antioxidants (Amid et al., 2011). The results of our study align with the notion that callus, being undifferentiated cells, contains minimal concentrations of plant-specific chemicals (Hasan et al., 2022).

Our investigation revealed a positive correlation between the total phenolic content TPC, TFC, and antioxidant activity of the examined extracts. Extracts with lower IC_{50} values (indicating better antioxidant activity) tended to have higher total phenolic and flavonoid contents. Thus, the overall phenolic and flavonoid concentration of the extracts may influence their antioxidant activity.

4.7.2 Brine Shrimp Lethality Test

Utilizing *Artemia salina* larvae, the toxicity of the callus extract of *N. scrophulariiflora* was evaluated, employing a simple and cost- effective method. Toxicity levels of extracts are determined based on their LC_{50} values.

Toxicity is inversely related to LC_{50} values. LC_{50} values are typically categorized as highly toxic (0-100 μg/ml), moderately toxic (100-500 μg/ml), low toxic (500-1000 μg/ml), and non-toxic (greater than 1000 μg/ml) (Clarkson et al., 2004; Meyer et al., 1982).

While previous research focused solely on the wild plant *N. scrophulariiflora*, there is a notable absence of studies on the callus extract of this plant species. In earlier investigations, rhizome methanol extract exhibited more toxicity, with LC_{50} value of 173.78 μg/ml. Furthermore, Rahman and Venkatraman (2011) conducted a toxicity test on albino rats, revealing no toxic reactions induced by *N. scrophulariiflora* toxicity.

In this study, the methanol extracts demonstrated low toxicity, with LC_{50} value 793 μg/ml while the callus aqueous extract was found to be non-toxic with LC_{50} value exceeding 1000 μg/ml indicating safety and non-hazardous effects on cells. This suggests that it can be the potential source of non-toxic drugs. Similar findings were observed in *Trigonella foenum graceum,* where the callus aqueous extract exhibited minimal toxicity (1801.52 μg/ml). The cytotoxic effects of plants are due to presence of secondary metabolites (Rahman et al., 2015). The results of our study align with the notion that callus, being undifferentiated cells, contains minimal concentrations of plant-specific chemicals (Hasan et al., 2022).

4.7.3 Antidiabetic Activity

In the previous study conducted on wild plants parts of *N. scrophulariiflora*, reported that *Neopicrorhiza scrophulariiflora* had the strongest alpha-amylase activity with IC_{50} value 192.88 μg/ml (Kumai et al., 2023). Beside this, there were numerous researches on *in vivo* antidiabetic activities of *N. scrophulariiflora* which showed remarkable hypoglycemia response in type 2 model rats when extracts were ejected orally to the rats (Manandhar, 2015; Skyler et al., 2017). In our experiment, there was no inhibition shown by methanol and aqueous while using alpha-glucosidase enzyme, this might be due to the presence of low content of secondary metabolites (Hasan et al., 2022) in callus. There was lacking further research on antidiabetic activity of *in vitro* generated callus of *Neopicrorhiza scrophulariiflora.*

5. Conclusion and Recommendations

5.1 Conclusion

In conclusion, *N. scrophulariiflora* seeds were germinated best on media containing 10% coconut water after 3-5 weeks. The callus was robustly proliferating from shoots using FMS with several hormones, including BAP, Kn,2, 4-D, and NAA. Notably, the combination of 1 mg/l BAP and 1 mg/l NAA yielded high biomass after 1 mg/l NAA treatment, particularly from stem explants. Moreover, callus exposed to 1 mg/l BAP and 0.5 mg/l NAA exhibited significant *in vitro* shoot induction, underscoring callus as a viable source for new plantlet regeneration.

The methanol extract demonstrated the highest phenolic content (38.11 mg GAE/gm) and flavonoid content (23.94 mg QE/gm). Notably, methanol extract exhibited low toxicity while the aqueous extracts exhibited non toxicity in the brine shrimp lethality test. Regarding antioxidant activity, the methanol extract showed superior activity with an IC_{50} value of 887.083±7.33 µg/ml compared aqueous extract. None of the callus extracts showed inhibition activity against alpha-glucosidase enzyme while assessing antidiabetic activity.

Overall, our findings shows that callus is also the source of phenolic and flavonoid component used in medicine but the evaluation of biological activities underscore the lower therapeutic potential of *N. scrophulariiflora* callus extracts, characterized by low toxicity, and low antioxidant activity. So, callus extract of *N. scrophulariiflora* is less effective for therapeutic purposes.

5.2 Recommendations

Callus culture stands out as a prominent method for augmenting the production of therapeutic compounds. This study delved into seed germination, callus generation, plant regeneration from callus, biological activity assessment, and quantitative analysis of phenols and flavonoids. Drawing from our findings, the following recommendations are proposed:

1. Explore a broader spectrum of hormones to ascertain their effects on explants, thereby optimizing callus production and organogenesis.
2. Given the moderate levels of phenolic and flavonoid content observed, consider

employing various elicitors to stimulate the production of diverse phytochemicals within the callus.

3. Extend the scope of extraction beyond polar solvents like water, methanol, and ethanol. Conduct assays across different biological activities and identify various bioactive compounds by fractionating extracts using solvents such as hexane, dichloromethane, chloroform, ethyl acetate, petroleum ether, and others.

4. Intensify efforts towards identifying and isolating chemical compounds from callussamples to uncover their therapeutic potential.

5. Broaden the investigation to encompass diverse biological activities such as anticancer, anti-inflammatory, antifungal, among others, to comprehensively evaluate the therapeutic capabilities of the callus extracts.

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Appendix

नेपाल सरकार
वन तथा वातावरण मन्त्रालय
राष्ट्रिय निकुञ्ज तथा वन्यजन्तु संरक्षण विभाग
(......................इकोलोजी........................ शाखा)

फोन नं. : ४२२०८५० / ४२२०९१२ / ४२२७९२६
फ्याक्स नं. ४२२७६७५

पो. ब. नं. - ८६०
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०७८/७९
पत्र संख्या :- इको ४९६
चलानी नं :-

मितिः २०७८/६/१८

विषयः अध्ययन अनुमति सम्बन्धमा ।

श्री सगरमाथा राष्ट्रिय निकुञ्ज कार्यालय, नाम्चेबजार, सोलुखुम्बु ।

प्रस्तुत प्रस्तुत विषयमा तहाँ राष्ट्रिय निकुञ्ज क्षेत्रमा निम्नानुसारको अध्ययन अनुसन्धान अनुमति प्रदान गरिएको व्यहोरा मिति २०७८/०६/१८ को विभागीय निर्देशनानुसार अनुरोध छ ।

अनुसन्धानकर्ताको नाम	कुशल के.सी., आरति गुरुङ, प्रा. डा. सुरेश कुमार घिमिरे, प्रा.डा. हरिदत्त भट्टराई, डा. नारायण प्रसाद घिमिरे, डा. मुक्तिराम पौडेल		
ठेगाना	काभ्रेपलान्चोक	इमेलः kecy17koosall@gmail.com	फोन नं. ९८६०४३१४३४
समद्व संस्था	वनस्पतिशास्त्र केन्द्रीय विभाग, त्रिभुवन विश्वविद्यालय, किर्तिपुर, काठमाण्डौं ।		
अनुसन्धानको प्रकृती	व्यक्तिगत		
पद	विद्यार्थी		
अनुसन्धानको तह	स्नातकोत्तर		
अनुसन्धानको शिर्षक	Population Status and Threats Assessment of Medicinal Herb *Neopicrorhiza Scrophulariiflora* (Pennell) D.Y. Hong in Khumbu Region		
अनुसन्धान विधि	Ecological Survey	नमुना संकलन गर्ने	नमुना परिक्षण कहाँ गर्ने: विश्वविद्यालयको प्रयोगशालामा
अनुसन्धानको अविध	०४/०७/२०७८ देखि ०४/०८/२०७८ सम्म		

शर्त :

१.अनुसन्धानकर्ताले राष्ट्रिय निकुञ्ज तथा वन्यजन्तु संरक्षण ऐन, २०२९ र नियमावली, २०३० तथा मातहतका सवै नियमावलीहरुको पूर्ण पालना गर्नु पर्नेछ ।

२. अध्ययन गर्दा सम्बन्धित संरक्षित क्षेत्र कार्यालयसंग समन्वय गरी कार्यालयमा कार्यरत कर्मचारीको रोहबरमा गर्नु पर्ने पर्नेछ ।

३. अनुसन्धानकर्ताले आफ्नो अनुसन्धानको प्रस्ताव सम्बन्धित संरक्षित क्षेत्र कार्यालयमा समेत पेश गर्नु पर्नेछ ।

४.अनुसन्धानकर्ताले अनुसन्धान समाप्त भएपछि एक प्रति कागजी प्रतिवेदन र एक प्रति इलोक्ट्रोनिक प्रतिवेदन यस विभाग र सम्बन्धित संरक्षित क्षेत्र कार्यालयमा बुझाउनु पर्नेछ ।

५. अनुसन्धानकर्ताले नतिजाहरु प्रकाशित गर्दा अनुसन्धानमा संलग्न यस विभाग र अन्तरगतका कर्मचारीको योगदानको आधारमा सहलेखकको रुपमा समावेश गराउनु पर्नेछ ।

६. सङ्कलित नमुना विदेश लैजान पाईने छैन ।

७. तोकिएका शर्तहरुको पालना नगरेमा विभागले कुनैपनि समयमा अनुमतिपत्र रद्द गर्न सक्नेछ ।

हेम राज आचार्य
सहायक इकोलोजिष्ट

बोधार्थः

कुशल के.सी., आरति गुरुङ, प्रा. डा. सुरेश कुमार घिमिरे, प्रा.डा. हरिदत्त भट्टराई, डा. नारायण प्रसाद घिमिरे, डा. मुक्तिराम पौडेलः सम्बन्धित संरक्षित क्षेत्र कार्यालयसंग समन्वय गरी अध्ययन अनुसन्धान गर्नु हुन र अनुसन्धान समाप्त भएपछि एक प्रति कागजी तथा विघुतीय प्रतिवेदन सम्बन्धित कार्यालय र विभागमा बुझाउनु हुन अनुरोध छ ।

वनस्पतिशास्त्र केन्द्रीय विभाग, त्रिभुवन विश्वविद्यालय, किर्तिपुर, काठमाण्डौं : जानकारीको लागि अनुरोध छ ।

Plant collection permission letter received by project team.