In vitro Morphogenesis of nodes and Bioactivities of Different Part extracts of *Rubia manjith* Roxb. Ex Fleming

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RECOMMENDATION

This is to certify that the Project work entitled "***In vitro* Morphogenesis of nodes and Bioactivities of Different Part extracts of *Rubia manjith* Roxb. Ex Fleming**'' has been carried out by **Salina Nagarkoti**. This is her original work and has been carried out under my supervision. To the best of my knowledge, this work has not been submitted for any other degree in any institutions. I hereby recommended for the approval of this dissertation for the partial fulfillment of the requirement of Master's degree in Botany, Central Department of Botany at Tribhuvan University, Nepal.

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LETTER OF APPROVAL

The M.Sc. dissertation entitled "***In vitro* Morphogenesis of nodes and Bioactivities of Different Part extracts of *Rubia manjith* Roxb. Ex Fleming**'' submitted at the Central Department of Botany, Tribhuvan University, by **Ms. Salina Nagarkoti** for the partial fulfillment of the requirement of Master's degree in Botany has been approved.

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DECLARATION

The dissertation entitled "***In vitro*** **Morphogenesis of nodes and Bioactivities of Different Part extracts of *Rubia manjith* Roxb. Ex Fleming**" is my original work that I completed under the guidance of Dr. Krishna Kumar Pant, Associate Professor of the Central Department of Botany at Tribhuvan University and is being submitted to the Institute of Science and Technology (IoST) as a dissertation for the completion of a Master's Degree in Botany. To the best of my knowledge, my thesis does not infringe upon anyone's copyright, and I adhered to it with all academic honesty and integrity. I further declare that the work reported in this research has not previously been deposited, in whole or in part, for the merit of any degree at this or any other institution or University.

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Salina Nagarkoti

25th September, 2023

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ABSTRACT

Rubia manjith, a perennial herbaceous climber is one of the important medicinal and dye plants that have been prioritized for research and development as well as categorized as a vulnerable plant (CAMP 2001) in Nepal. This is a significant plant used for medicine, cosmetic and dye purpose. Locally this plant is being used by various communities to cure different diseases. It is heavily exploited unsustainably and exported in a large amount. All these factors are leading to it's the depletion of its population in nature and hence it becomes necessary to study its potentials and conserve *in situ* and *ex situ*. Single nodes as an explant of *R. manjith* were cultured in Murashige and Skoog (MS) medium supplemented with plant growth regulators, BAP and NAA. The highest mean shoot number 5.33±0.33 was observed on MS media without any growth regulators followed 4.33±0.33 mean shoots on 0.1 mg/L BAP and 1.0 mg/L BAP. The highest mean root number 8.00±1.00 was also observed in MS media without any growth regulators. Highest shoot length 14.33±1.54cm was observed in 0.1 mg/L BAP followed by MS media 14.13±1.12cm whereas the highest root length 6.80±1.27 in 2.0 mg/L BAP. High amount of callus (+++) were observed in 2.0 mg/L NAA, 0.1 mg/L BAP + 1.0 mg/L NAA, 0.1 mg/L BAP + 2.0 mg/L NAA and 1.0 mg/L BAP + 1.0 mg/L NAA. For the analysis of phytoconstituents and their bio-activities, methanol, dichloromethane and hexane extract of the root, stem and leaves were prepared by fractionation. Total phenolic content was estimated using FC-reagent and the highest TPC was found in dichloromethane extract of root with 77.5±0.01 µg GAE/mg phenols. Brine shrimp lethality assay was performed to examine the toxic activity of plant and highest toxicity was found in the hexane extract of the stem, with an LC_{50} value of 194.99±34.58 g/mL. DPPH assay was done to analyze the antioxidant activities and strong antioxidant activity was exhibited by hexane extract of root, dichloromethane extract of root and methanol extract of stem with an IC_{50} value 16.92±2.97 µg/mL, 35.17±1.42 µg/mL and 46.49±0.85 µg/mL respectively. *Rubia manjith* showed high TPC, antioxidant and toxic activities in the hexane fraction, especially in the stem and root, suggesting that the nonpolar compounds and essential oils present in the species may play a role in their active bioactivities, which can be further used in several pharmaceutical and other applications. It can be cultivated easily *in vitro* from the nodes and utilized on a large scale if needed to maintain its population sustainability.

सारांश

Rubia manjith लहरे जडिबुटी हो, जसलाई अनुसन्धान र विकासको लागि प्राथमिकता दिनुका साथसाथै नेपालमा जोखिमपूर्ण बिरुवा (CAMP, 2001) को रूपमा वर्गीकृत गरिएको छ। यसलाई औषधि, कस्मेटिक र रंगाईका लागि प्रयोग गरिन्छ। बजारको माग परिपुर्ती गर्न यसको जथाभावी रूपमा संकलन गर्ने, कलिलो बिरुवाहरु नाश गर्ने जस्ता कार्यहरुले प्रकृतिमा यसको जनसंख्याको ह्रास गरिरहेका छन् , त्यसैले यसको *in situ* तथा *ex situ* अवस्थामा संरक्षण साथै यसका सम्भाव्यता अध्ययन गर्न जरुरी छ। बिरुवाको एकल नोडहरू Murashige and Skoog (MS) medium र पूरकको रुपमा बिरुवा वृद्धि हर्मोनहरू, BAP तथा NAAका साथ कल्चर गरिएको थियो। कुनै पनि वृद्धि हर्मोनहरू बिनाको MS mediumमा उच्चतम औसत शूट 5.33±0.33 र उच्चतम औसत जरा 8.00±1.00 अवलोकन गरियो। उच्चतम शुट लम्बाइ 14.33±1.54cm र 14.13±1.12 cm क्रमश: 0.1 mg/L BAP र MS mediaमा देखियो भने उच्चतम जरा लम्बाइ 6.80±1.27 2.0 mg/L BAP मा देखियो। उच्च मात्रामा callus 2.0 mg/L NAA, 0.1 mg/L BAP + 1.0 mg/L NAA, 0.1 mg/L BAP + 2.0 mg/L NAA and 1.0 mg/L BAP + 1.0 mg/L NAAहरुमा देखियो। बिरुवामा उच्चतम Total phenolic content 77.5±0.01 µg GAE/mg phenols जराको dichloromethane extractमा पाइएको थियो। बिरुवाको विषाक्त गतिविधि परीक्षण गर्न Brine shrimp lethality assayको प्रयोग गरिएको थियो र उच्चतम विषाक्तता LC_{50} 194.99±34.58 g/mL काण्डको hexane extractमा प्राप्त भयो। एन्टिअक्सिडेन्ट गतिविधिको परीक्षण गर्न DPPH assayको प्रयोग गरिएको थियो, जसमा उच्चतम एन्टिअक्सिडेन्ट गतिविधिहरू IC_{50} 16.92±2.97 µg/mL, 35.17±1.42 µg/mL तथा 46.49±0.85 µg/mL क्रमश: जराको hexane extract, जराको dichloromethane extract र काण्डको methanol extractहरुमा पाइएको थियो। विशेष गरी *Rubia manjith*को काण्ड र जराको hexane fractionमा जैविक गतिविधिहरू देखिएकोले nonpolar essentials oil तथा यौगिकहरुले यसको सक्रिय जैविक गतिविधिहरूमा भूमिका खेल्न सक्ने अनुमान गर्न सकिन्छ र ति सक्रिय यौगिकहरुलाई विभिन्न औषधि र अन्य कार्यहरूमा प्रयोग गर्न सकिन्छ। साथै यसलाई नोडहरूबाट सजिलैसँग *in vitro* खेती गर्न सकिने भएकाले यसको दिगोपन कायम राख्न आवश्यक भएमा ठूलो मात्रामा यसलाई प्रयोग गर्न सकिन्छ।

ABBREVIATIONS AND ACRONYMS

°C = Degree Celsius

µg = Microgram

µl = Microliter

2,4-D = 2,4- Dichlorophenoxyacetic acid

AQs = Anthraquinones

BAP = Benzylamino purine

CAMP = Conservation Assessment and Management Plan

cm = Centimeter

DMSO = Dimethyl sulfoxide

DPR = Department of plant resources

Fe^{2+} = Ferrous oxide

Fe^{3+} = Ferric oxide

FRAP = Fluorescence recovery after Photobleaching

GAE = Gallic Acid euqivalance

gm = Gram

HCL = Hydrochloric acid

HEPA = High efficiency particulate air

$HgCl_2$ = Mercuric chloride

IAA = Indole-3-acetic acid

IBA = Indole-3-butyric acid

IC_{50} = Half-Maximal Inhibitory Concentration

IUCN = International Union for Conservation of Nature

Kn = Kinetin

LC50 = Half-Maximal Lethal Concentration

m = Meter

MAPDON = The Medicinal and Aromatic Plants Database of Nepal

mg = Miligram

MS = Murashige and Skoog

NAA = 1-Naphthalene acetic acid

NaOH = Sodium hydrochloride

NPRL = Natural Product Research Laboratory

PBS = Phosphate-buffered Saline

POWO = Plants of the World Online

ppm = Parts per million

PVP = Polyvinylpyrrolidone

SAWTEE = South Asia Watch on Trade, Economics and Environment

TDZ = Thidiazuron

TEAC = Trolox Equivalent Antioxidant Capacity

TUCH = Tribhuvan University Central Herbarium

UV = Use Value

μM = Micro molar

LIST OF FIGURE

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Background

Tissue culture has been a remarkable method for generating millions of healthy, virus-free plants from a single leaf, meristem, root, node or even a cell. The concept of tissue culture originated from the groundbreaking research of Gottlieb Haberlandt (1902), who cultivated isolated plant cells and discovered the totipotency of plants. Through this tissue culture approach, we can carry out commercial plant production as well as the conservation of rare and endangered plant species. Micropropagation is the tissue culture procedure used for clonal propagation, which consists of rapidly multiplying the plant parts, known as explants, resulting in a large number of progeny plants. Today, an extensive range of plant varieties are propagated through the micropropagation technique for a number of reasons, including large-scale virus-free plant production, pest and disease-resistant plants, genetic modification, plant breeding, germplasm preservation, embryo rescue, hybrid and cybrid production, and many more (Gaspar *et al.,* 2002; Thorpe, 2007; Doran, 2009). Morphogenesis is kind of the micropropagation which simply means process of development, for instance, callus, root, shoot formation from the preexisting material called 'explant' in different responses such as growth hormone, temperature, light, etc. For that, we can use various plant parts such as the root or shoot tip, node, meristem, leaf, tuber, anther, pollen, protoplast, or even particular cell types as an explant. The best tissue for tissue culture generally consists of tissue that is younger, more rapidly growing, or at an early stage of development. Micropropagation can also be used to supply and produce pharmaceutical crops of various components, including small therapeutic molecules, standard therapeutic extracts, large therapeutic molecules, and functional foods used for medicinal purposes (Moraes *et al*., 2021).

Phytochemistry is a branch of research whose main objective is the study of plant chemical constituents. Fossil evidence suggests that humans have been using plants as remedies for at least 60,000 years (Rabizadeh *et al*., 2022). Along with nutrients for their growth, plants also produce a variety of phytochemicals and secondary metabolites. Higher plants tend to have significantly higher levels of secondary metabolites, especially those that have medicinal properties. It can be exclusive to certain plant developmental phases and stressful times. Nitrogen and sulphur compounds, phenolic compounds, and

terpenoids are some major groups of secondary metabolites (Mendoza and Silva, 2018). Other chemical compounds include alkaloids, glycosides, flavonoids, saponins, tannins, anthraquinones, essential oils and steroids (Yalavarthi and Thiruvengadarajan, 2013). Plants use those phytochemicals for biological functions, including defense against microorganisms, insects, fungi and herbivorous mammals. Such compounds are investigated for their chemical composition, physiology ecological distribution, biological function, extraction, and qualitative and quantitative analysis. Due to the chemical compounds they produce, plants have been recognized as a natural source of several antibacterial, anticancer, antioxidant, anti-diarrheal, and different medicinal agents (Zhou, 2021). Those phytoconstituents are extracted in different solvents through different methods according to their polarity, pH, thermostability, etc. After that, evaluation and pharmacological activities can be studied through a series of processes, i.e. fractionation, isolation, characterization of the constituents, and investigation of the biosynthetic pathways of a particular compound (Yalavarthi and Thiruvengadarajan, 2013).

The production of free radicals during cellular processes may have negative impacts on cellular integrity, resulting in cell damage and weakened antioxidant defense mechanisms in cells. Those free radicals can be eliminated through the consumption of antioxidative agents that help to lower oxidative stress and hence chronic diseases (Kamath *et al.*, 2015; Rahman *et al.*, 2015). The main reason behind this is that antioxidants may bind these free radicals and prevent cellular damage. Phenolic molecules are one of the crucial antioxidant components that deactivate free radicals by donating hydrogen atoms to them according to their capacity (Aryal *et al.*, 2019). The phenolic hydroxyl groups in phenolic compounds have a redox potential that enables them to function as hydrogen donors, reducing agents, and singlet oxygen quenchers. (Kamath *et al.*, 2015).

All living organisms have defense mechanisms that remove damaged molecules from their systems, but those mechanisms might be inefficient and can be fulfilled by consuming antioxidants in their diet (Rahman *et al.*, 2015). The majority of vegetables, fruits, nuts, seeds, roots and barks naturally contain phenolics and antioxidants (Lobo *et al*., 2010; Pratt and Hudson, 1990). Many antioxidant compounds are found in our

plasma, including ceruloplasmin, haptopexin, uric acid, ascorbic acid, and others, each with distinct mechanisms of action. For example, uric acid acts as a radical scavenger, ascorbic acid scavenges both radical and stable oxygen, and ceruloplasmin binds copper ions and prevents reinitiation (oxidizes Fe^{2+} to $Fe^{3+)}$ reactions (Krinsky, 1992). Vitamins C, E, b-carotene, anthocyanins, and others are other typical examples of antioxidants. Various techniques, such as TEAC test, FRAP assay, and DPPH assay, can be used to perform antioxidant activities (Magalhaes, 2008).

Toxicity simply means poisoning. There are generally five types of toxicity: chemical, biological, physical, radiation and behavioral toxicity and it depends upon the amount and concentration used, regularity of use, and interaction with the substances (Tisserand and Young, 2013). The qualitative and quantitative study of toxic effects is important for the evaluation of the potential hazards that a particular chemical compound can cause (Gregus, 2008).

1.2. Botanical description of Genus *Rubia manjith* Roxb. Ex Fleming

Synonyms: *Rubia cordifolia* var. *khasiana* G.Watt, *Rubia cordifolia* var. *munjista* (Roxb.) Miq., *Rubia cordifolia* f. *tetramera* Makino, *Rubia munjista* Roxb. (POWO, 2023)

Common name: Indian Madder, Common Madder

Vernacular name: Majitho (मजिठो), Tiro Lahara (तिरो लहरा) (Nepali).

Distribution: The plant is native to the Himalayan regions of Assam, South-Central China, East Himalaya, India, Nepal, Qinghai, Tibet and West Himalaya in Asia (POWO, 2023). It is distributed in the temperate and sub-tropical regions of Nepal between 1200 and 3000 meters (Ghimire *et al.*, 2008).

Rubia manjith Roxb. Ex Fleming is a terrestrial, climbing herb with braches up to 3 meter height, quadrangular, glabrous, weakly strigose-hooked to almost smooth, stem slightly woody at the base, red pith. Leaves are present in a four whorls, 2-4 cm long, shape-ovate-lanceolate to ovate, base rounded or slightly cordate lower leaves, apex-acuminate, petiole 0.5-3.5 cm with many sharp recurved prickles, greenish adaxially and purplish red abaxially. Inflorescences thysoid, paniculate with several minute flowers. Calyx tube

globose, glabrous. Corolla red, purplish red or orange, rotate, glabrous, lobes 5, lanceolate to triangle. Fruits black, 0.3-0.5 cm, dark red in colour. Flowering and fruiting from July-August to October (eFloras, 2023; Malla and Vibhāga, 1986).

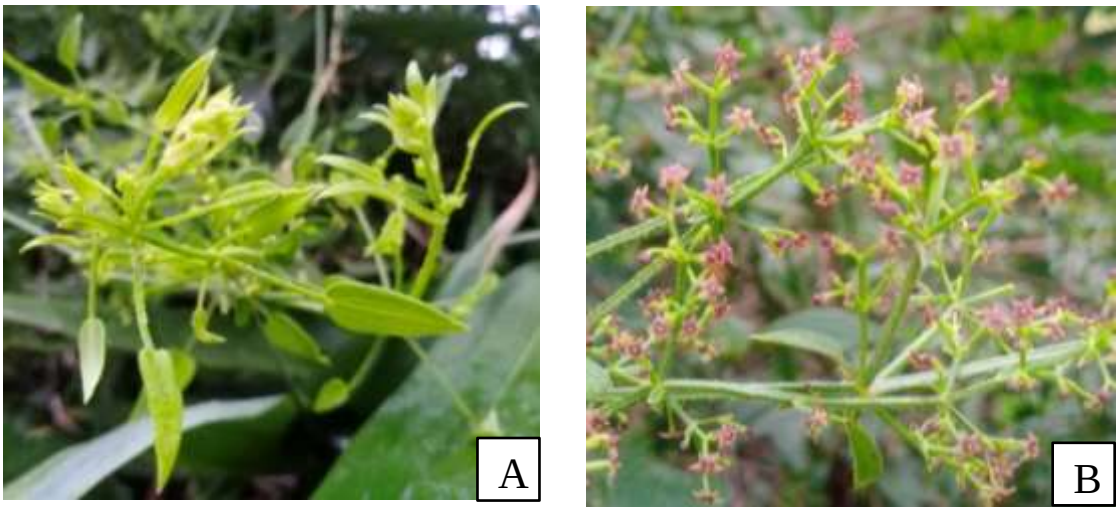


Figure 1: Photographs of *Rubia manjith* from Shikharpa, Lalitpur. **A:** Whole plant in natural habitat; **B:** Inflorescence.

There are a total of 78 species of the genus *Rubia* whose names are accepted (The Plant List, 2010). A total of six species of the genus *Rubia* are recorded in Nepal according to Shrestha *et al.*, (2022). They are:

1. *R. alata* Wall
2. *R. charaefoila* Wall.ex G.Don
3. *R. hispidicaulis* D.G.Long
4. *R. manjith* Roxb. Ex Fleming
5. *R. sikkimensis* Kurz
6. *R. wallichiana* Decne.

1.3. Importance of *Rubia manjith*

The government of Nepal has prioritized 33 medicinal plants for research and economic development (DPR 2006, 2016). One of the significant plants among them, *Rubia manjith*, is utilized for medicinal, cosmetics and dyeing purposes. Most of the *Rubia* species contain anthraquinones (AQs), particularly alizarin and purpurin, which is also

known as a natural dye. For dyeing and cosmetics purpose, roots of 2-3 years plant are used. Dye extracted from *R. cordifolia* L. is used to dye cotton, silk wool, jute, nylon, and other textiles. It is also used to make hair dyes and cosmetics. It is also used in Korea and Japan as a colorant in ice cream, chewing gum, and noodles (Caro *et al.*, 2012; Murthy *et al.*, 2022). In Nepal, the root and stems of *Rubia manjith* are widely used to create a demanded red dye for clothing as well as wool used for preparing carpet (Manandhar, 2002; DPR, 2006; Gurung and Pyakurel, 2017). Different parts like root, stem and leaves are also used by different ethnic group to cure several diseases.

1.4. Conservation status of *Rubia manjith*

Rubia manjith Roxb. is categorized as vulnerable in Nepal by the Conservation Assessment and Management Plan (CAMP) 2001 (Pradhan *et al*., 2021). The species is one of the 30 non-timber forest products that Nepal's government has identified as being particularly important for the nation's economic development and therefore prioritized for conservation and research purposes (DPR 2006). According to Pandit and Thapa (2003), the majority of collectors followed destructive collection practices, which involve cutting stems to the ground with no scope for regeneration. In most parts of Nepal, supplies of middle and low-altitude herbs like *Rubia manjith* have already decreased to the point of almost extinction, along with other herbs including *Asparagus racemosus, Dioscorea deltoidea, and Valeriana Jatamansi* (Sharma, 2015).

1.5. Research question

1. What can be the best strategy to rapidly multiply this species under *in vitro* conditions for *ex situ* conservation?
2. What is the amount of total phenolic content in the species?
3. At what strength do different extracts of *Rubia manjith* exhibit antioxidant properties and show toxic effects?

1.6. Objectives

1.6.1. General objectives

In-vitro morphogenesis and phytochemical evaluation of *Rubia manjith* for conservation and therapeutic potentials, respectively.

1.6.2. Specific objectives

1. To establish a standard protocol for rapid multiplication and callogenesis of the plant *Rubia manjith* by using nodes as explants.
2. To estimate the total phenolic content present in the wild *Rubia manjith*.
3. To test the antioxidant and toxic properties of the wild *Rubia manjith*.

1.7. Rationale of the study

Rubia manjith is one of the medicinal plants that the Government of Nepal has identified and prioritized for conservation, agro-technology and research purposes (DPR 2006, 2016). There is no similar work available so far related to tissue culture and biological activities and hence the present attempt is the first of its kind on this plant. Various ethnic communities and Ayurvedic formulas both use this plant to treat several ailments. It is a significant plant used for medicine, dye and cosmetic purposes thus, this plant is in high demand, particularly on the international market. The current commercial demands on medicinal plants drive premature harvesting and rush collection leads to habitat destruction and also threatens their population. Therefore, a conservation strategy is urgently needed to avoid its extinction. By using the standard protocol for *in-vitro* propagation of this species, we can effectively grow the plants and cultivate them commercially to meet market demand. This would also aid in maintaining their population in nature by planting the *in-vitro* grown and hardened plants whenever and wherever required.

Since *Rubia manjith* is a plant with a high potential as a medicine, there is a strong possibility that this species contains significant bioactive substances and exhibits biological activity with high efficiency. In the future, potential drug formulations against various diseases could be made possible by understanding the bioactive components of this plant species and their mechanisms of action. The market value of the plant species

will rise, promoting local industry and entrepreneurship as well as the economy at both the local and national levels.

1.8. Limitation

1. The analysis of the phytochemicals was limited to wild plants.
2. The only method used for quantitative analysis was total phenolic content.
3. For tissue culture, only nodes were used as explants.

2. LITERATURE REVIEW

2.1. Traditional uses of *Rubia manjith*

About 60% of the world's population and 80% of the population of Nepal are reported to rely on traditional medicinal herbs to cure health ailments (Shrestha & Dillion 2003). The Medicinal and Aromatic Plants Database of Nepal (MAPDON) estimates that there are 1,624 different kinds of medicinal plants in Nepal, and about 100 of those species are traded each year (SAWTEE, 2015). One of them is *R. manjith*, which is a shrub that grows in both sunny and shaded areas. It is distributed throughout the Himalayan region, from Simla to Bhutan, and in Khasia. In Nepal, the species is found in the central, eastern and western regions (Ghimire *et al.,* 2008). The nearest species of *R manjith*, *R. cordifolia* has been used for years in medical practices as a part of traditional Chinese medicine (Chen, 2022). The Latin word ruber, which means "red" is the origin of the genus name *Rubia* (St Clair, 2016). *R. manjith* has astringent, antidysenteric, antiseptics, and deobstruent properties that are also used in rheumatism, ulcers, inflammations and skin troubles (IUCN Nepal, 2000). According to Balami (2004), the Pharphing Newar community uses paste or liquid of *R. manjith* root for antiseptic and rheumatism. The root and entire plant are used to treat fever in the Chepang population, while the stem and root are used to treat diarrhea, burns, and scorpion stings. Additionally, they use this plant to make dye (Tamang *et al.,* 2017).

In the review paper by Pradhan (2021), he has mentioned the ethnomedicinal uses of *R. manjith*. This plant has been used as an astringent, blood purifier, urinary tract infection, antidote for cobra and scorpion, to cure eye and ear problems, stomachache, headache, skin diseases, blood, liver and menstrual disorders, jaundice, etc. Root and stem are taken for the treatment of heart attacks, ulcers, and skin problems in various parts of Nepal. Similarly, the paste of stem and petiole is used in cobra or scorpion bites (Gurung and Pyakurel, 2017). This plant is used to treat skin and urinary problems as well as purify the blood (Kunwar *et al.,* 2009). In Sikkim, a decoction of *R. manjith*'s roots, about 10–20 gram taken 2-3 times a day, is used as a tonic and treatment for jaundice, urinary tract infections, and liver complaints. It is considered a remarkable blood purifier that is also used in treating conditions like irregular menstruation, joint discomfort, leucoderma, and skin disorders, as well as eye and ear diseases (Maity *et al.,* 2004).

Bhatia *et al.*, (2015) conducted an investigation on the use of *R. manjith* along with other medicinal plants for menstrual disorders in Udhampur, India, by interviewing users who used the dried powdered root of *R. manjith* about one teaspoon three times a day with lukewarm milk. It was found that *R. manjith* has a significant effect on menorrhagia with a use value (UV) of 0.56 and a fidelity level of 100%.

2.2. Chemical constituents

By combining the several annual reports, the compilation report of DPR (NPRL/DPR, 2014) included phytochemical and biological screening tests of some important and most traded MAPs in Nepal. Phytochemical groups like glycoside, steroid, terpenoid, tannin, saponin, flavonoid, volatile oil, reducing molecule, polyurenoid, polyoses, emodin, fatty acid, and carotenoid were present in the stem of *R. manjith*, but alkaloid was not found, which was also absent in the root of *R. cordifolia* of the eastern ghats, India (Sisubalan, 2016). *R. cordifolia* and *R. tinctorum*, which both contain the common colours alizarin, purpurin, emodin, and rhein as anthraquinone sources (De Santis and Moresi, 2007). Alizarin, munjistin, rubiadin, purpurin, techoquinone, and xanthopurpurin are some of the examples of anthraquinones found in *R. cordifolia* (King, 1992). More than 100 chemicals, mainly anthraquinones, naphthoquinones, anthraquinone glycosides, naphthoquinone glycosides, bicyclic hexapeptides, triterpenoids, and polysaccharides have been found to be rich in *R. cordifolia* through several studies (Wen *et al.*, 2022).

2.3. Trade of *Rubia manjith*

This plant has considerable demand, particularly on the international market because of its significant medicinal value and dyeing nature. The majority of naturally occuring quinones (biological pigments) are anthraquinones. Natural and synthetic anthraquinones have been widely used as colouring agents in food, drugs, cosmetics, dyes and textiles (Mori *et al.*, 1990). In several regions of Asia, Europe, and Africa, *Rubia* was a significant source of the red pigment (St Clair, 2016). About 80 metric tons of total annual trade and about 56 Metric tons of average annual quantity of *R. manjith* for 28–30 Rs/kg had been exported from Nepal to India (Poudel, 2011). Altogether, 176,423 kg of high-value MAps were produced during the period of 2011–2015, in which *Swertia chirayita* and *R. manjith* covered 60% and 35% of the total production respectively,

produced from only national and community-managed forests that were exported to India from the Basantapur NTFP market hub. In 2015, a total of 8,995 kg of *R. manjith* were produced, worth a total of 1,349,000 US dollars. (Bhujel and Pokharel, 2018). According to data from the plant quarantine and pesticide management center (2019), during the fiscal year 2075/076, a total of 219.4 metric ton of *R. manjith* were exported from various quarantine posts in Nepal for a total value of Rs. 52,442,000 to India and Bangladesh. Nepal is the third most exporters in world for Manjith after India and Afghanistan (Volza Grow Global, 2023). This plant is often exported at a low cost and collected haphazardly. The dried sample of *R. cordifolia*'s root has been sold under the name Manjith (Pathania, 2006).

2.4. Tissue culture

During the time period of 1972–1976, the earliest tissue culture of the family Rubiaceae, Edwards and LaMotte (1976) attempted to obtain bud, shoot and root from the stem, bud and callus as explants of *Psychotria punctata* through different experiments. They used zeatin with basal media of the "stock callus medium" of LaMotte and Lersten (1972) in different concentrations and concluded that exogenous cytokinin is essential to develop the shoot in tissue culture of the species.

Radha *et al.,* (2011) studied the micropropagation of *R. cordifolia* L. using shoot tip, nodal and split nodal half explants on MS media with various hormones. The maximum number of shoots was obtained on 1 mg/l BAP and 0.02 mg L-1 IAA which produced 5.9 and 5.2 shoots per explant where 0.5 mg/l BAP found to be best for rapid shoot multiplication. 1 mg L-1 IAA was best for rooting, and the survival rate was high at that concentration. The study also suggested that the nodal explant was a better option than the shoot tip.

According to Gaurav *et al.,* (2017), nodal explant of *R. cordifolia* L. with MS medium in combination with 0.5 mg/l TDZ and 0-1% PVP liquid media was suitable for shoot development (20–25), whereas MS with 2 mg/l IBA was appropriate for rooting (2-3 roots).

Hapsari and Ermayanti (2020) studied the *in-vitro* micropropagation of *R. akane*, a dye and medicinal plant, using shoot tips and node explants in an MS media containing Kn and BAP as cytokinin hormones. The highest number of shoots (8.36 cm) and the highest number of roots (9.14 per shoot) were produced after 8 weeks of culture in MS media without cytokinins. The highest leaves (45.86) were obtained in the MS media containing 0.5 ppm Kn and the highest internode (4.72 segments) was produced in the MS media containing 0.5 ppm BAP.

M and Siril (2022) had used several growth hormones such as BAP, Kin, 2-iP, TDZ, IAA, IBA and NAA singly or in combinations and discovered that 2.5 μM of BAP and TDZ (9.37 shoots) was the most effective for the multiple shoots through axillary bud proliferation in a short time period. For the *ex-vitro* rooting of *in-vitro* grown plants, 2.5 μM IBA for 5 min produced maximum roots i.e. 79.17%. They also concluded that to produce clonally uniform plants, direct regeneration through nodal explants is more effective than from callus.

Regarding the *ex vitro* propagation, the seed of *R. manjith* had been sown in different places, for instance, Godawori and Khokana where 95% of the seeds germinated after one month in Khokana (Shrestha *et al.*, 2017).

2.5. Biological activities

Several studies have shown that the closest species, *R. cordifolia* has antioxidant properties. In *R. cordifolia*, the hydroxyl group on the benzene ring plays an important role in scavenging free radicals, and the hydroxyl structure in hydroxyanthraquinone can significantly enhance this ability (Cai *et al.*, 2004).

Phytochemical evaluation and the antihyperglycemic effect of *R. cordifolia* leaves have been investigated by Khan *et al.*, (2021). The ethanolic extract had the strongest antioxidant activity in the DPPH assay, followed by n-butane and methanol, which had IC_{50} values of 34.0 g/mL, 36.86 g/mL, and 38.19 g/mL, respectively. With 207.306 g GAE/mg, 119.12 g GAE/mg, and 99.732 g GAE/mg correspondingly, the total phenolic content indicated a positive correlation with the antioxidant activities.

Humbare *et al.,* (2022) investigated the antioxidant properties of *R. cordifolia* in different solvents. They used the DPPH, hydrogen peroxide, nitric oxide, and total antioxidant procedures to evaluate the antioxidant properties and found that root extracts had higher antioxidant levels than leaf and stem extracts in all assays. Particularly in the DPPH assay, the IC_{50} value of methanol root extract was 79.1 ± 1.92 µg/mL in the presence of PVPP and 89.47 ± 0.79 µg/mL in the absence of PVPP. Ethanol extract was potent as compared to methanol, aqueous, and PBS extracts in DPPH, hydrogen peroxide, and reducing power assays.

Aqueous root extract of *R. cordifolia* was tested for toxicity using brine shrimp lethality test and half lethal concentration (LC_{50}) value was found to be 370 µg/ml (Krishnaraju *et al.,* 2006). The stem of *R. cordifolia* was examined with other medicinal plants from Gulmi, Nepal, and it was reported that the stem did not exhibit toxicity. The LC_{50} value was found to be $5 \times 10^{\wedge 9}$ µg/mL and had the lowest potential for antioxidants (Subba and Paudel, 2014).

In the annual reports of DPR 063/064 and 065/066, the acute toxicity of stem was tested on mice, and the half lethal dose (LD-50) values were 600 mg/kg and 100 mg/kg, respectively. In an anti-helmentic test, the stem of *R. manjith* was 50% effective at 300 mg/kg and 75% effective at 500 mg/kg (NPRL/DPR, 2014).

Ullah *et al.,* (2011) studied the antibacterial properties of *R. manjith* and found that the methanol extract of the aerial part is ineffective against bacteria like *Staphylococcus aureus, Staphylococcus methacilline, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexenari,* and *Pseudomonas sesame.*

There is a lot of confusion between the plants *R. manjith* Roxb. Ex Fleming and *R. cordifolia* Linn. because *R. manjith* has the synonym "*R. cordifolia*" which has different authors. Nearly all databases consider *R. manjith* as a synonym of variable *R. cordifolia* but they are very distinct (Dar *et al.,* 2020), and many researches and reviews were done by considering them as the same plant. Dar *et al., (*2020) observed that two species can be

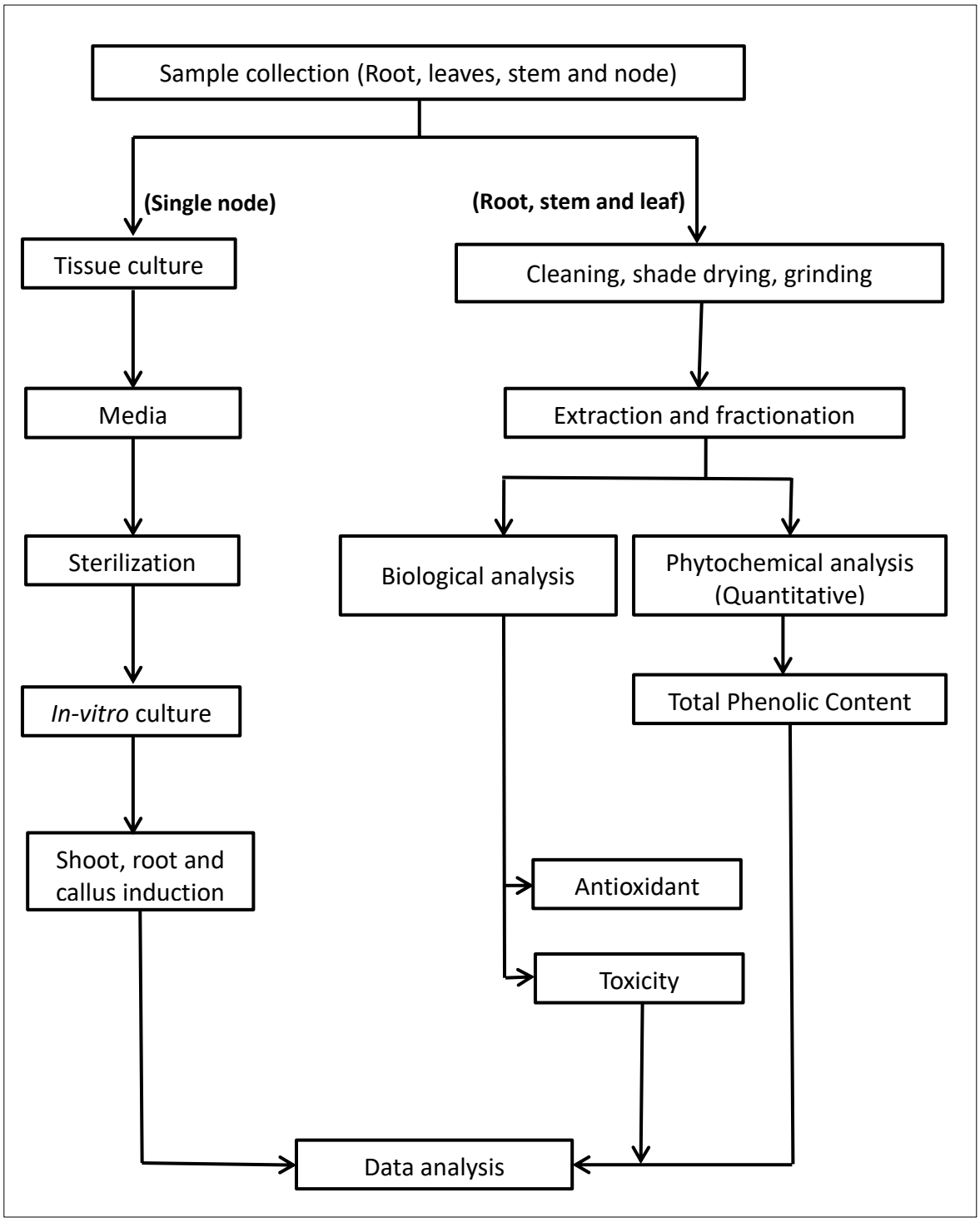
separated from one another very easily. *R. manjith* has a dark reddish flower and dark red mature fruit with a smooth surface, while *R. cordifolia* has a greenish yellow flower and orange mature fruit with a rough surface. In addition, due to similar vegetative appearances, the same common name and confusion in the systematics, the results from the phytochemical research may have been mixed up with *R. cordifolia.*

The above-mentioned literatures indicate that scientific investigation has been carried out on the nearby species, *R. cordifolia* in various aspects, including tissue culture, the production of secondary metabolites, clinical trials and more (Wen *et al.,* 2022). However, limited information on phytochemical analysis is available, there are no published scientific papers regarding the tissue culture of *R. manjith*.

3. MATERIALS AND METHODS

3.1. Research Design

Figure 2: Flow chart of research design



3.2. Collection of plant materials

The plant material *Rubia manjith* roxb. (Fig. no. 1) was collected without destructing the natural habitat from Godawari municipality-6, Lalitpur, Nepal at a 1650 meter elevation above sea level. The upper portion of the plant i.e. the stem was carefully collected, kept in the plastic bag and stored in the refrigerator at 4°C for tissue culture purposes. For the phytochemical analysis, different parts of plants like roots, stems and leaves were collected separately, cleaned, and then kept in shade for drying. Herbarium was prepared then identified by using different available literature and the herbarium of TUCH which was checked with the expert of Tribhuwan University. Herbarium was kept in TUCH with the Voucher specimen number: 101-2023.

3.3. *In vitro* propagation

3.3.1. Sterilization techniques

Sterilization is a vital step in the tissue culture technique that should proceed before, during, and after the inoculation process. In order to maintain the aseptic condition, the essential glassware, metallic instruments, chemicals, plant explants, and culture rooms should be adequately sterilized. For the sterilization in tissue culture, techniques from Torres, 1988 were followed. These sterilization methods were used throughout the process:

3.3.1.1. Wet sterilization

All the required glassware such as culture tubes, culture jars, beakers, test tubes, funnels, petri plates and metallic instruments such as forceps and scalpels were sterilized by moist heat. The filter papers were placed inside the dry petri plate and wrapped with aluminium foil. Likewise, aluminium foil was used for wrapping all metallic equipment. By using this method, distilled water is additionally sterilized. This procedure involves autoclaving at 121 °C with 15 psi pressure for 35 minutes.

3.3.1.2. Dry sterilization

All of the metal and glass instruments that had previously undergone wet sterilization were once again given the dry treatment in a hot air oven at 150 °C for 30 minutes.

3.3.1.3. UV sterilization

All of the glassware, metallic instruments, surgical blades, culture media, empty jars, chemicals like mercuric chloride, 70% ethanol, distilled water and other necessities for culture except the explant should be placed inside the laminar air flow chamber. The procedure involves exposing all the materials to ultraviolet (UV) radiation for at least 45 minutes followed by turning on the HEPA filter for 15 minutes. Then the process of culture was carried out closer to the flame. The HEPA filter is continuously activated throughout the culture and every single item of equipment was further sterilized by flame inside the chamber.

3.3.1.4. Surface sterilization of plant explant

The plant stem is gently cleaned with tap water to remove the dirt. The stem is then cut into single nodes. All of these nodes were soaked in tween-20 for 5 minutes and rinsed off using running water for 20 minutes. The single nodes were then transferred to a laminar chamber and sterilized with a 0.1% mercuric chloride solution for 5–6 minutes, followed by a rinse with distilled water three times. Then they were once again dipped in 70% ethanol for 1 minute, followed by a series of rinses with distilled water.

3.3.2. Preparation of MS media

The MS (Murashige and Skoog) medium (Murashige and Skoog, 1962) used for the experiment was a standard stock solution of macronutrients (stock A), micronutrients (stock B), an iron source (stock C), and a vitamin source (stock D). Sucrose (30 g/L) and Myo-inositol (0.1 g/L) were added to the media, and the pH was maintained at 5.6 ± 0.1 by adding either 0.1 N NaOH or 0.1 N HCL. Agar (0.8%) was then added to the media as a gelling agent. Plant growth regulators like NAA and BAP were used separately or in combination as needed. The prepared media was then transferred to the appropriate culture jars or culture tubes, depending on the needs, and tightly sealed with aluminium foil using rubber bands. The media was autoclaved to sterilize at 121 °C and 15 psi pressure for 35 minutes.

3.3.3. Inoculum of nodes

Explants (single nodes) were transferred after a series of surface sterilization processes to a sterile petri plate with filter paper on it. The excessive parts of the single nodes were cut off carefully with a surgical blade and the single node (1.5 cm ± 2) was inoculated in front of a flame in the prepared MS media accordingly. Each culture tube and jar were tightly sealed by aluminium foil with the help of rubber bands after inoculation.

Throughout the inoculation process, the chamber, surface, and instruments were frequently sterilized. After the inoculation, all of the inoculated tubes and jars were moved to a culture room, which had been maintained at a temperature of 25 °C, a relative humidity of 60-70%, and luminescent light exposure with a dark/light cycle of 8-16-hours.

3.4. Phytochemical and biological assay

3.4.1. Preparation of plant materials

The various mature plant parts, including the stem, root, and leaf, had been collected from Godawori municipality-6, Lalitpur District, Nepal at 1650 m altitude. All those plant materials were cleaned with water to remove the unwanted substances. The plant parts were then air-dried separately in the shade and finely grinded with an electric grinder. All of the plant parts were weighed and labeled. The weights of the powdered root, stem, and leaf were 90, 57.9, and 57 gram respectively. It was then stored in a sealed bag for further purposes.

3.4.2. Extraction

The extraction of phytochemicals was done with simple maceration techniques described by Harborne (1998). The powdered forms of various parts were soaked separately in 100% methanol for 48 hours and filtered through filter paper. The process was repeated up to three times for each part. The filtrate was then evaporated in a rotatory evaporator at reduced pressure. After decreasing in filtrate volume, the residue was suspended in 50 ml of water and partitioned successively with hexane and dichloromethane. The fractionation of methanol, hexane and dichloromethane was done gradually using a separating funnel and the dried crude extract of each fraction was obtained using a rotatory evaporator.

Those crude extracts were named with a distinctive letter, such as methanol root (8.925 gm) as MR, methanol stem (2.289 gm) as MS, methanol leaf (3.349 gm) as ML, hexane root (0.459 gm) as HR, hexane stem (0.4583 gm) as HS, hexane leaf (0.6381 gm) as HL, dichloromethane root (0.658 gm) as DR, dichloromethane stem (0.4059 gm) as DS and dichloromethane leaf (0.243 gm) as DL. The crude extracts were weighed, kept in sterile vials, and refrigerated to 4°C for further phytochemical analysis.

3.5. Phytochemical analysis

3.5.1. Quantitative phytochemical analysis

3.5.1.1. Total phenolic content (TPC)

Folin-Ciocalteu's reagent (F-C reagent) method was used to determine the total phenolic content in several extracts with a slight modification to the Poudel *et al.,* (2015) procedure. For the analysis, 1 mg/mL methanolic solution of each extract was prepared. In each well of the sterilized 96-well plate, 20 µL of the methanolic extract solution, 100 µL of the 10% F-C reagent, and 100 µL of the 7.5% sodium carbonate solution were added together and thoroughly mixed by pipetting. For the control sample, 20 µL of absolute methanol was used instead of the plant sample. Then the samples were well covered and left in the dark at room temperature for 45 minutes. The absorbance of samples at 765 nm was measured using micro-plate reader. For each analysis, the samples were taken in triplicate form. Gallic acid was used as a standard to construct a calibration curve.

3.5.2. Biological analysis

3.5.2.1. Antioxidant activity

1,1-Diphenyl -2 picrylhydrazyl (DPPH) assay had been used to evaluate the antioxidant activity through free radical scavenging potential with a slight modification of the Blois protocol (Blois, 1958). For the analysis, 1 mg/mL methanolic solution of each extract was prepared. Then, different concentrations of sample solution at 500, 250, 125, 62.5, 31.25, 15.62 and 7.812 µg/mL were prepared by serial dilution from the stock solution. 0.1 mM of DPPH solution was freshly prepared by dissolving 3.93 mg of DPPH powder (394.32 g/mol molecular weight) into 100 mL of absolute methanol and then protecting it from direct light by wrapping it with aluminium foil. In a sterilized 96-well plate, 100 mL of

each sample solution were then tested against 100 µl of DPPH in triplicate. For the control sample, 100 µL of absolute methanol and 100 µL of DPPH were used. The samples were then incubated for 30 minutes at room temperature under dark conditions. The absorbance of samples at 517 nm was measured using micro-plate reader.

Radical scavenging activity of the plant extracts was estimated by using the formula:

$$\text{DPPH\% scavenging activity} = \frac{control\ absorbance\ of\ methanol - sample\ absorbance}{control\ absorbance} \times 100\%$$

On the standard curve, a standard graph was plotted against concentration and percentage radical scavenging activity on the X- and Y-axis. The IC_{50} value of plant extract was calculated from the linear equation of that graph:

$Y = mX + C$

$IC_{50} = \frac{50 - C}{m}$

Where, X is the concentration i.e. IC_{50}

Y is Radical scavenging activity percentage of plant extract

m and C are the coefficient and constant of the linear equation, respectively.

3.5.2.2. Toxic activity

For the toxic activity, Brine Shrimp lethality assay (BSLA) was done with a slight modification to the Meyer protocol (Meyer *et al.*, 1982). 8 mg of crude extract from different plant parts such as the root, stem and leaf of *R. manjith* was dissolved in 100 µl of dimethyl sulfoxide (DMSO) solution followed by 900 µl distill water to make 1 ml of total volume of stock solution (10% DMSO). Then different concentrations of solution at 8mg/l, 4mg/l, 2 mg/l, 1 mg/l, 0.5 mg/l, 0.25 mg/l and 0.125 mg/l were prepared by serial dilution from the stock solution. In a sterilized 96-well plate, 100 µl volume of stock solution at different concentrations was taken in triplicate. Then, 100 µl of salt water with 10±1 newly hatched Brine shrimp (*Artemia salina*) called nauplii were transferred to a 96-well plate after counting them in a micropipette. For control, distill water with 10% DMSO was used instead of stock solution. The samples were left to incubate for 24 hours

at room temperature. The percentage of lethality of the nauplii for each concentration and control was listed and estimated:

$$\text{Mortality \%} = \frac{Number\ of\ dead\ nauplii}{Initial\ number\ of\ live\ nauplii} \times 100$$

The lethal concentration (LC_{50}) was determined using probit analysis. On the standard curve, a standard graph was plotted against concentration and mortality at the probit scale on the X-axis and Y-axis. The LC_{50} (median lethal concentration) values of plant extract were calculated from the linear regression equation of that graph data and expressed as the mean ± standard error.

$Y = mX + C$

Where, X is the concentration i.e. LC_{50}

Y is mortality

m and C are the coefficient and constant of the linear equation, respectively.

3.6. Data analysis

Microsoft Excel 2007 was used to construct each graph and table, and the t-test (Independent sample) was used to determine the significance of the tissue culture data. For the analysis of all the observed data, Microsoft Excel 2007 was used, and each analysis was performed in triplicate for the standard data. The data were presented as mean ± Standard error.

4. RESULT

4.1. Plant tissue culture

For the micropropagation of *Rubia manjith*, single nodes from mature plants were used as explants and cultured in different compositions from 0.1 mg/L to 2.0 mg/L of growth regulators (BAP and NAA) along with MS media. Approximately 80% of the nodes were lost due to microbial contamination, even after treatment with 0.1% $HgCl_2$ for 4 minutes. For that reason, the immersion time was increased to 5-6 minutes for the nodes. Young nodes were discarded because they were more likely to be damaged and die during the sterilization process. The grown plants from cultures were again subcultured in MS basal media for four weeks to reduce the residual effect of the hormone, and then their nodes were again used as explants. Cultured nodes were observed for up to 8 weeks, and the data were recorded and analyzed as shown in Appendix-I. Three distinct parameters were measured and analyzed in this study: shoot induction, root induction, and callus induction, cultured singly and in combination of cytokinin and auxin. Overall, the induction of shoot and root was comparatively more favored by MS media and the single media compositions of BAP and NAA.

4.1.1. Shoot induction of *Rubia manjith*

Two shoot tips were initially observed in the first week but in most of them, only one shoot elongated and the other did not elongate further. The majority of elongated shoots have 5 nodes, with a maximum of 7 nodes. Although less number of individual shoots (2.33) was reported in our study, long lateral shoots measuring approximately 5±2 cm were obtained in the second and third nodes in every plant in single BAP treatments and MS media. The total number of shoots will also significantly rise if they are counted separately because every single one of them has the potential to regenerate roots. If these lateral branches are *in-vitro* rooted, they can function as independent plants, advancing our goals to grow many plantlets with the least amount of resources and labor. So in this study, we also counted the number of those lateral shoots present in second and third nodes. Overall, the MS medium with cytokinin i.e. BAP was found to be suitable to induce the long shoot of *R. manjith*.

The highest mean shoot number was observed in MS medium with 5.33±0.33 shoots. In the single hormone treatment, MS media containing 0.1 mg/L BAP and 1.0 mg/L BAP

had the highest mean shoot number, which was 4.33±0.33 while the lowest mean shoot number was observed in MS media containing 0.1 mg/L NAA with 0.67±0.33 and 2.0 mg/L NAA 0.67±0.67 shoots (P value < 0.05) (Fig. 3).

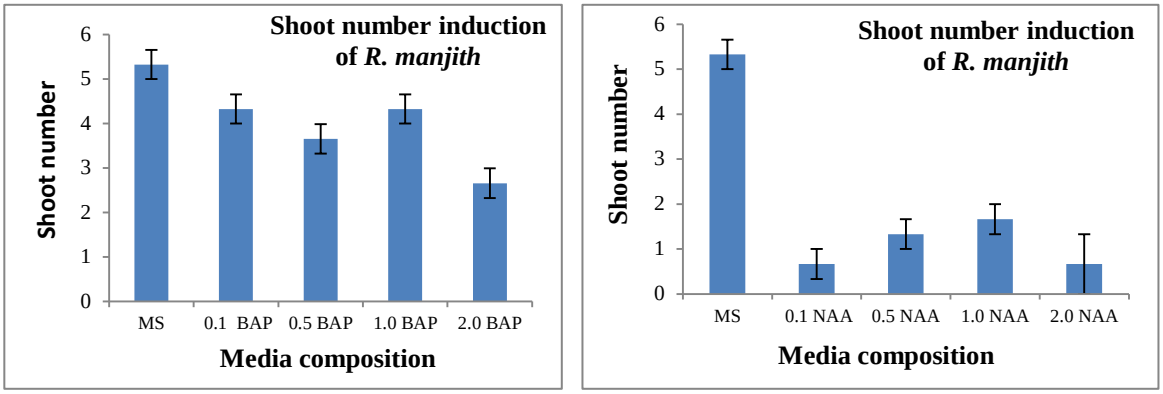


Figure 3: Mean shoot number in BAP and NAA treatment of *R. manjith*

In the hormone combination treatment, the highest mean shoot number, i.e. 2.00±0.0 was observed in MS media containing 0.5 mg/L BAP + 2.0mg/L NAA and 2.0 mg/L BAP + 1.0 mg/L NAA (P value < 0.05). There was an absence of shoot in the MS media containing 0.1 BAP mg/L + 0.1 mg/L NAA and 0.5 mg/L BAP + 0.1 mg/L NAA (P value < 0.05) (Fig. 4, Appendix-I).

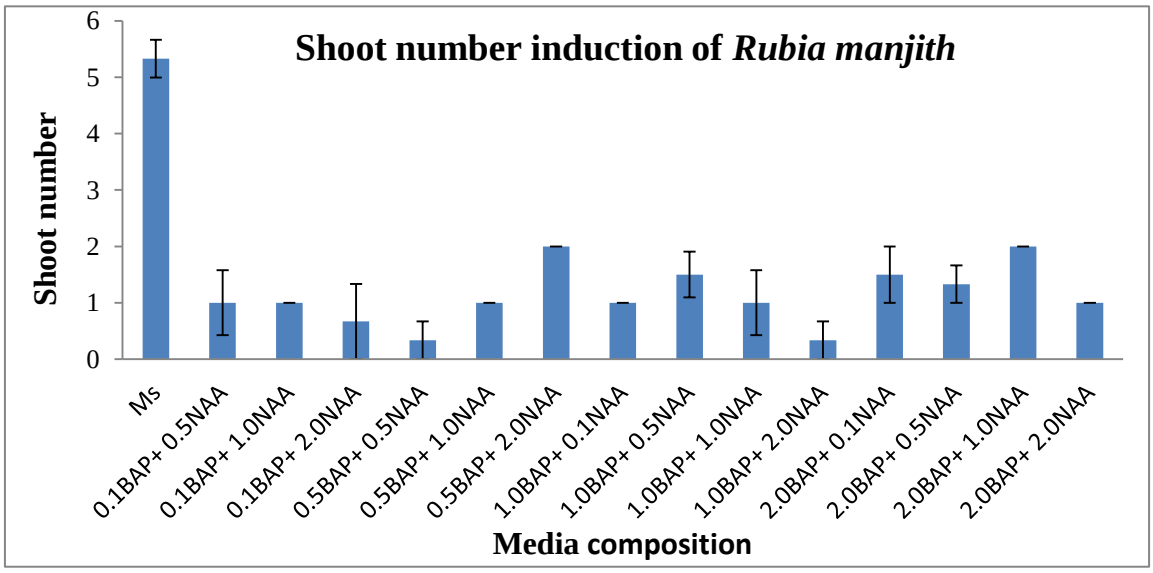


Figure 4: Mean shoot number in BAP and NAA combination treatment of *R. manjith*

The results of single hormone treatment showed that 0.1 mg/L BAP was most effective to induce long shoots, with a mean shoot length 14.33±1.54 cm (P value > 0.05), followed

by MS media with a mean shoot length 14.13±1.13 cm. Minute shoot tips were observed in the MS media containing 0.5 mg/L NAA with a mean shoot length of 0.10±0.1 cm and 2.0 mg/L NAA with 0.3±0.3 (P value < 0.05) (Fig. 5).

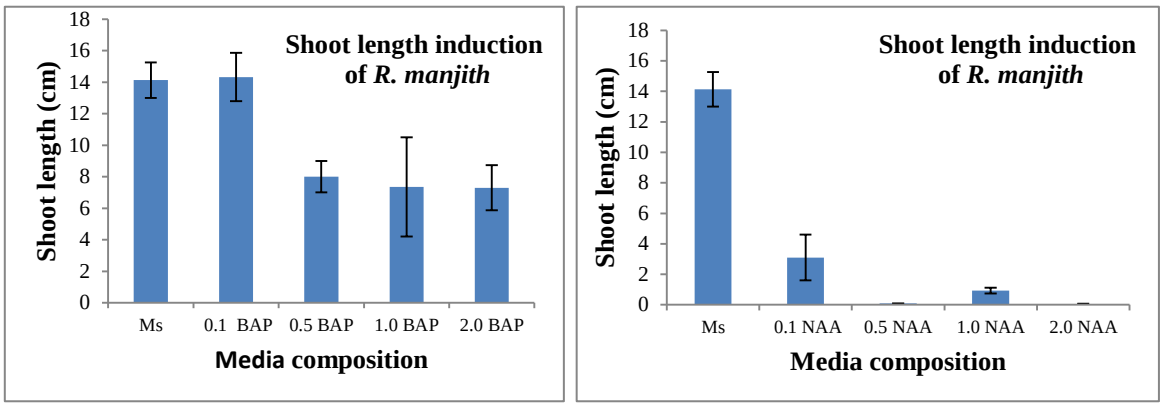


Figure 5: Mean shoot length in BAP and NAA treatment of *R. manjith*

In the hormone combination treatment, 2.0 mg/L BAP + 1.0 mg/L NAA showed the highest mean shoot length, which is 11.0±0.50 cm. Small shoot tips (0.10±0.0 cm) were observed in the MS media containing 0.1 mg/L BAP + 1.0 mg/L NAA, 0.1 mg/L BAP + 2.0 mg/L NAA and others. No shoots were observed in the MS media containing 0.1 BAP mg/L + 0.1 mg/L NAA and 0.5 mg/L BAP + 0.1 mg/L NAA (P value < 0.05) (Fig. 6; Appendix-I).

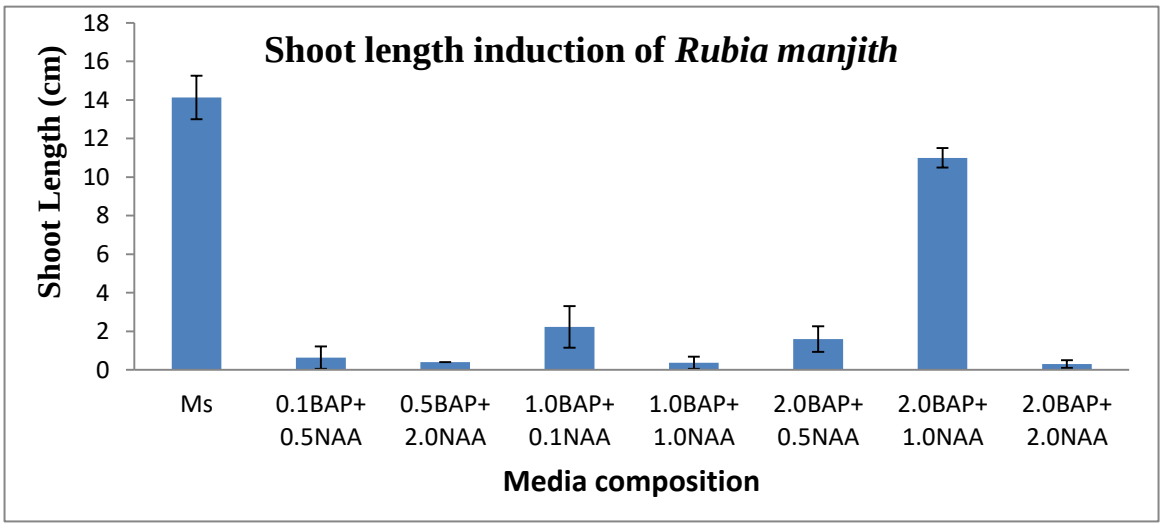


Figure 6: Mean shoot length in BAP and NAA combination treatment of *R. manjith*

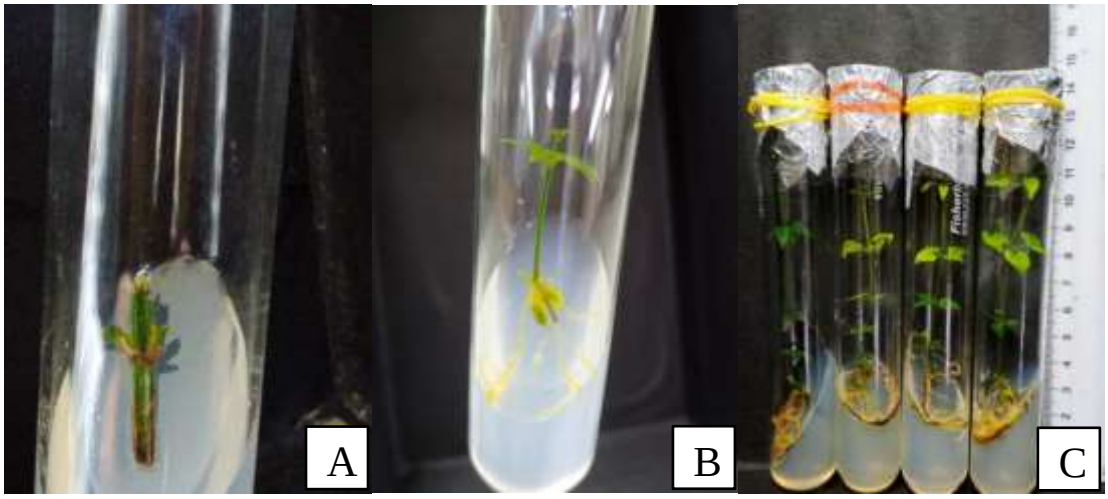


Figure 7: Different stages of shoot induction of *R. manjith.* **A:** Initial stage of node after one week of inoculation; **B:** Initiation of shoot, along with few roots after 3 weeks; **C:** Elongated shoots, along with long roots and callus on them.

4.1.2. Root induction of *Rubia manjith*

The MS media and the single hormone treatment were found to be more effective in inducing roots in *R. manjith*. Roots varied in number and size. It was observed that only long roots had secondary roots and root hairs. Likewise, in MS media, every single BAP treatment and the 2 mg/L BAP + 1 mg/L NAA combination treatment showed long roots (4-5cm) in the second and third nodes as well. The small roots in the MS media containing a 2 mg/L NAA combination were developed from the callus at the base. The root emerged to grow from the upper portion of the cultured node in the MS media containing 1mg/L BAP+2 mg/L NAA combination, where there was no shoot and in addition, a structure resembling fuzzy tufts (Fig. 12: **B**) appeared, which was also seen in the MS media containing 0.5 mg/LBAP + 1.0 NAA combination.

For the induction of root numbers, MS media and NAA were found to be most effective. The highest mean root number 8.0±1.0 was observed in the MS media. In the single hormone treatment, MS media containing 2 mg/L NAA had the highest mean root number with 7.67±0.33 roots followed by MS media containing 0.1 mg/L BAP with 6.0±1.15 roots (P value > 0.05). There was an absence of root in MS media containing 0.5 mg/L NAA (Fig. 8).

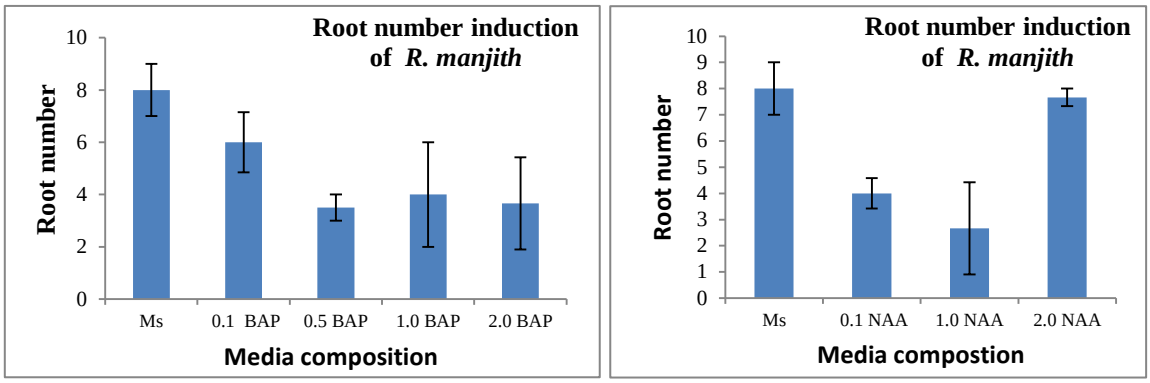


Figure 8: Mean root number in BAP and NAA hormone treatment of *R. manjith*

In the hormone combination treatment, the highest mean root number 4.6±0.88 was observed in 2.0 mg/L BAP+ 0.5 mg/L NAA, followed by 0.5 mg/L BAP+ 1.0 mg/L NAA with 4.50±1.50 roots. Some of the treatments did not respond to root induction. There was an absence of root in MS media containing 0.1 mg/L BAP+ 0.1 mg/L NAA, 0.1 mg/L BAP+1.0 mg/L NAA and others (P value < 0.05) (Fig. 9; Appendix-I).

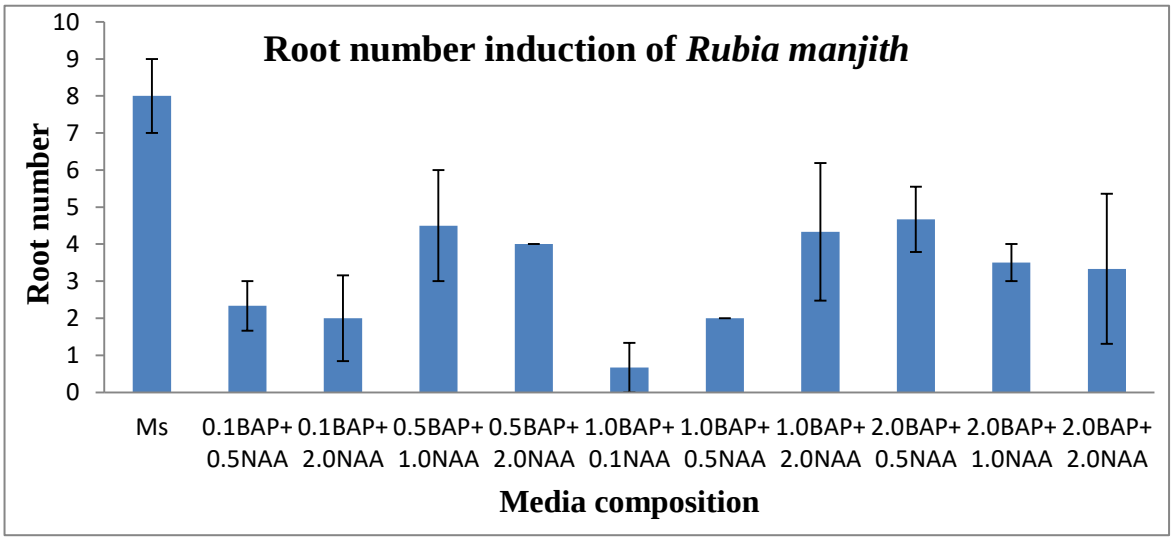


Figure 9: Mean root number in BAP and NAA combination treatment of *R. manjith*

In the single hormone treatment, MS media containing 2.0 mg/L BAP had the highest mean root length, which was 6.08±1.27cm followed by MS media containing 0.1 mg/L BAP with 4.27±1.07cm. There was an absence of root in the MS media containing 0.5 mg/L NAA (P value < 0.05) (Fig. 10).

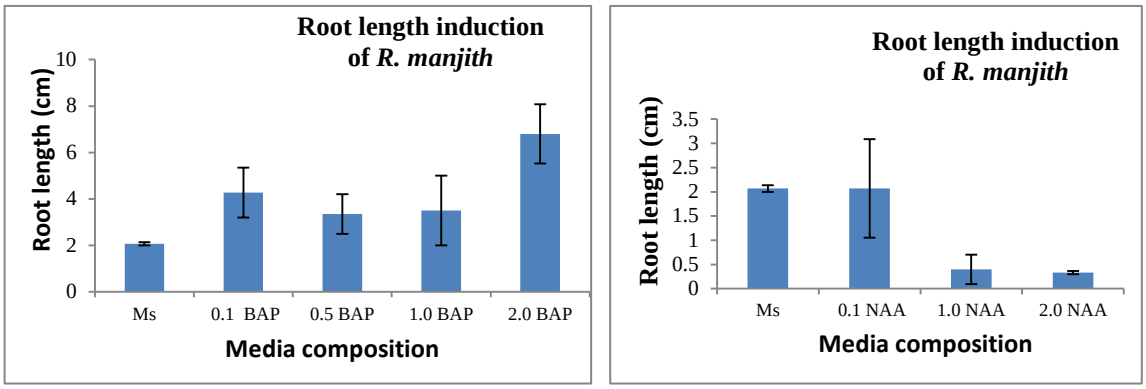


Figure 10: Mean root length in BAP and NAA hormone treatment of *R. manjith*

In the hormone combination treatment, the highest mean root length observed was in the MS media containing 0.5 mg/L BAP + 1.0 mg/L NAA with 1.55±1.25 cm (P value > 0.05). Some treatments did not respond to the root induction. No roots were observed in MS media containing 0.1 mg/L BAP + 0.1 mg/L NAA, 0.1 mg/L BAP + 1.0 mg/L NAA, and others (P value < 0.05) (Fig. 11; Appendix-I).

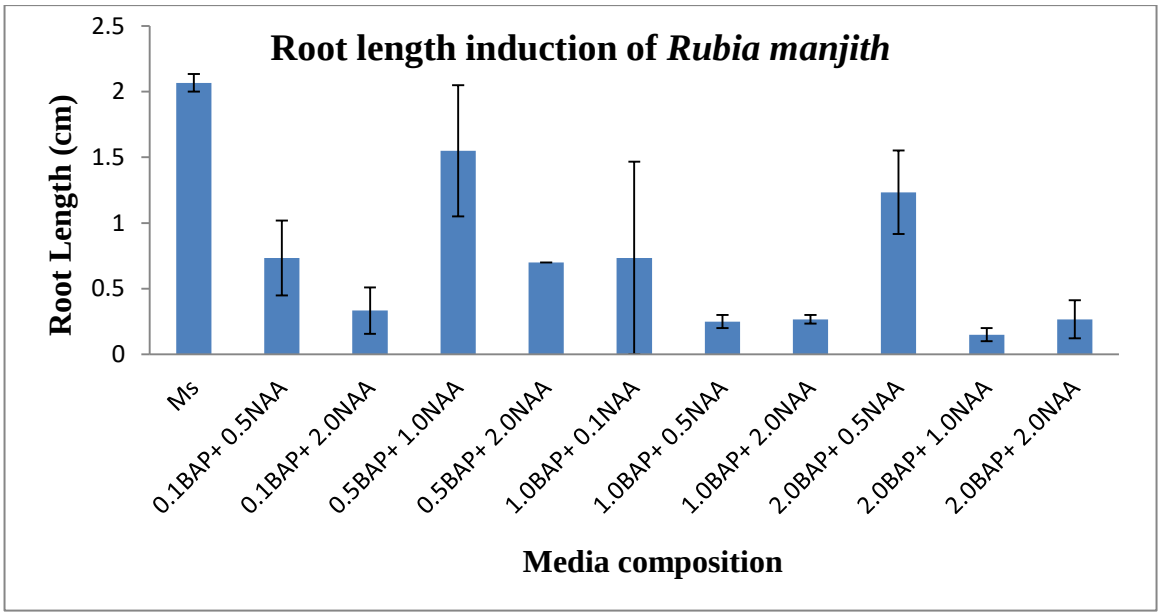


Figure 11: Mean shoot length in BAP and NAA combination treatment of *R. manjith*

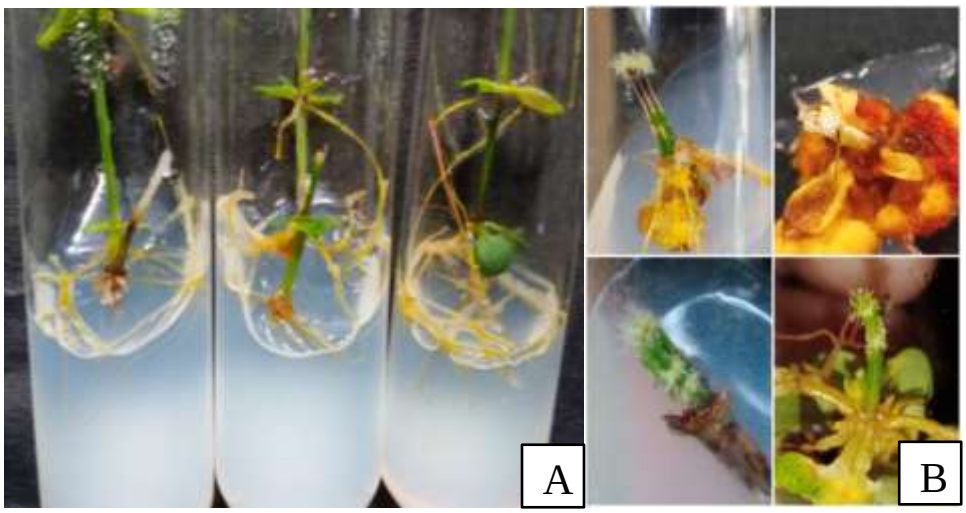


Figure 12: A: Induction of roots in the shoot; **B:** Fuzzy tufts, seen in the upper part of the node

4.1.3. Callus induction in *Rubia manjith*

In the present study, the mass of callus was observed in different parts like the leaf, node and root of *R. manjith*, on MS media with BAP and NAA within 8 weeks. The nodal portion including the leaves was subcultured therefore, callus was formed on those leaves. The node and leaves began to swell after one week of inoculation, while the callus on the roots appeared four to five weeks after the development of the roots. Depending on the hormone concentration and combination, the amount of callus developed differentially. The color of the callus also varied from dark orange to orange to yellow. The particular indicator was given to the callus condition based on its approximate size. The presence of a callus was rated as -, +, ++, +++ for no callus, ≈0.1x0.5 cm, ≈05x1.0 cm, and more than 1.0x1.5 cm, respectively.

In the single hormone treatment, the highest mean callus (+++) was observed in the MS media containing 2.0 mg/L NAA, whereas there was no callus in the MS media and MS media containing 1.0 mg/L BAP. In the hormone combination treatment, the highest mean callus (+++) was observed in 0.1 mg/L BAP + 1.0 mg/L NAA, 0.1 mg/L BAP + 2.0 mg/L NAA and 1.0 mg/L BAP + 1.0 mg/L NAA. Moderate mean callus (++) was obtained in the MS media containing 0.1 mg/L BAP + 0.5 mg/L NAA, 0.5 mg/L BAP + 2.0 mg/L NAA and so on. There was no callus observed in 2.0 mg/L BAP+ 1.0 mg/L NAA (P value < 0.05) (Table 1).

Table 1: Induction of callus in different parts of *Rubia manjith*

Treatment (mg/L)	Callus range	Response mechanism
MS Media	-	*
0.1 BAP	+	R
0.5 BAP	+	R
1.0 BAP	-	*
2.0 BAP	+	R
0.1 NAA	+	N-L
0.5 NAA	+	N-L
1.0 NAA	+	N-L-R
2.0 NAA	+++	L-R
0.1BAP + 0.1NAA	+	N-L
0.1BAP + 0.5NAA	++	N-L-R
0.1BAP + 1.0NAA	+++	N
0.1BAP + 2.0NAA	+++	N-L-R
0.5BAP + 0.1NAA	+	N-L
0.5BAP + 0.5NAA	+	N-L
0.5BAP + 1.0NAA	+	N-L-R
0.5BAP + 2.0NAA	++	L-R
1.0BAP + 0.1NAA	+	L-R
1.0BAP + 0.5NAA	+	N
1.0BAP + 1.0NAA	+++	N-L
1.0BAP + 2.0NAA	++	N-L
2.0BAP + 0.1NAA	+	N
2.0BAP + 0.5NAA	++	N-R
2.0BAP + 1.0NAA	-	*
2.0BAP + 2.0NAA	++	N-L-R

[Callus observed in different parts. ***:** No callus; **L:** Leaves (residual); **N:** Node; **R:** Grown roots; **N-L:** Node along with leaves (residual); **L-R:** Leaves (residual) along with grown roots; **N-R:** Node with grown roots; **N-L-R:** node along with leaves (residual) and grown roots]

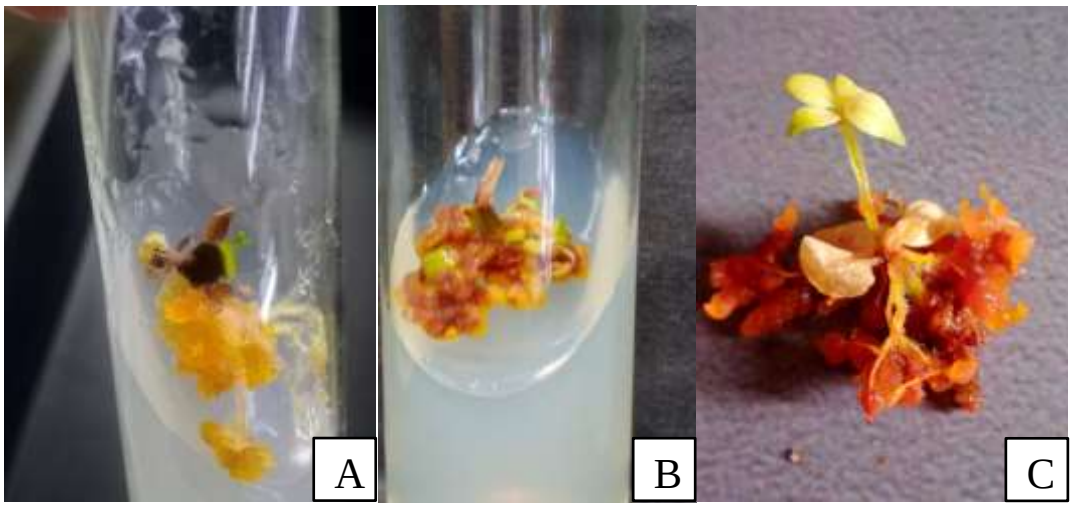


Figure 13: Callus induction in *R. manjith.* **A:** Yellow callus in grown roots; **B:** Yellow to dark orange callus in cultured node and leaves; **C:** Orange to dark orange callus in the grown roots.

4.2. Phytochemical analysis

4.2.1. Quantitative phytochemical analysis

4.2.1.1. Total phenolic content

The total phenolic content assay was used to perform a quantitative phytochemical analysis of *R. manjith*. The phenolic content present in the crude extract of root, stem and leaf of *R. manjith* was calibrated using gallic acid. The total phenolic content is expressed in terms of microgram of gallic acid equivalent per milligram of dry extract (µg GAE/mg). The gallic acid calibration curve is shown in Fig. 14.

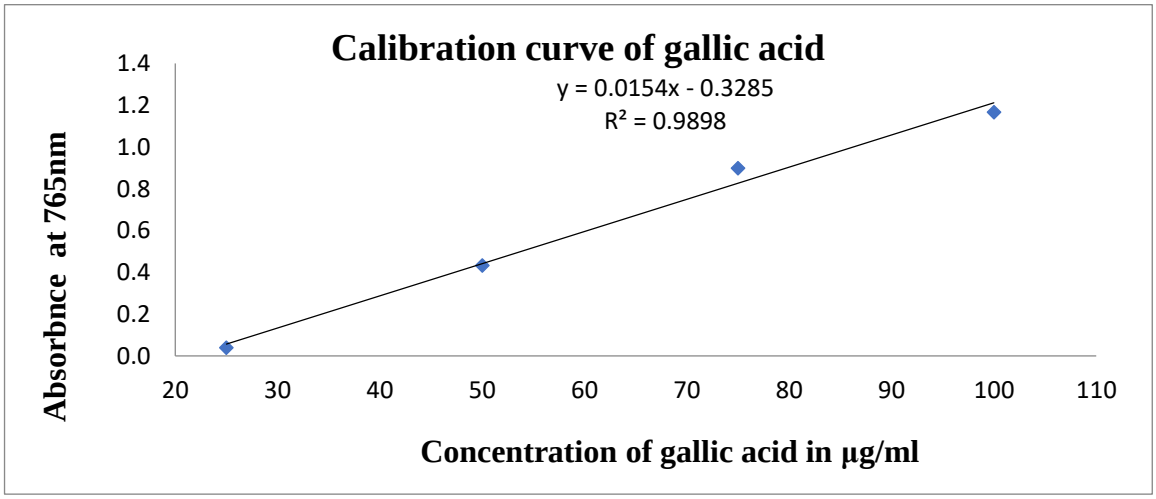


Figure 14: Calibration curve of gallic acid

Overall, the dichloromethane fraction showed high total phenolic content in *R. manjith*. The highest phenolic content was present in the root extract in dichloromethane with 77.5±0.01 µg GAE/mg followed by the stem extract in dichloromethane with 70±0.01 µg GAE/mg.

The lowest total phenolic content was found in root extract in methanol with 31±0.01 µg GAE/mg followed by stem extract in hexane with 36±0.02 µg GAE/mg. The total phenolic content of various samples is shown in Fig. 15.

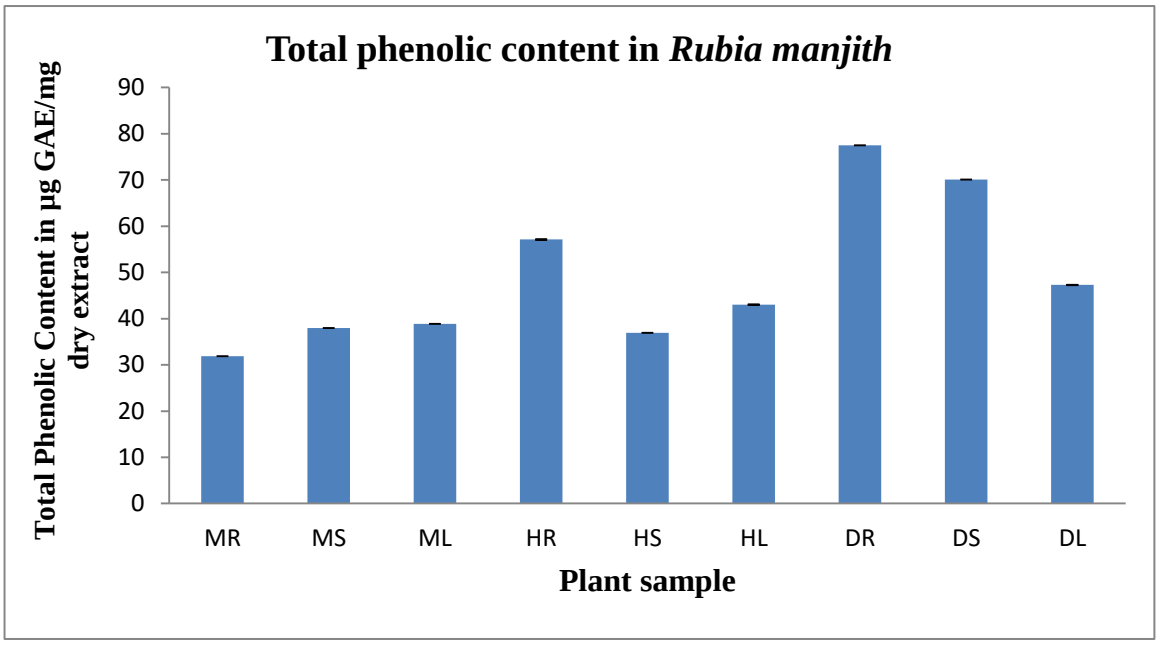


Figure 15: Total phenolic content in *R. manjith*

4.3. Biological analysis

4.3.1. Antioxidant activity

The antioxidant properties were determined by using the DPPH free radical scavenging capacity of the root, stem and leaves of *R. manjith* in three different crude extracts, i.e. methanol, hexane and dichloromethane. The scavenging activities of plant extracts of *R. manjith* against DPPH radicals were in the following order: HR > DR > MS > ML > DS > DL > MR > HS > HL. Radical scavenging activity of root extract in hexane, root extract in dichloromethane and stem extract in methanol was found to be highest 83.65±0.79 %, 72.2±0.37% and 58.15±1.23% respectively, at 125 µg/mL concentration (Fig. 16).

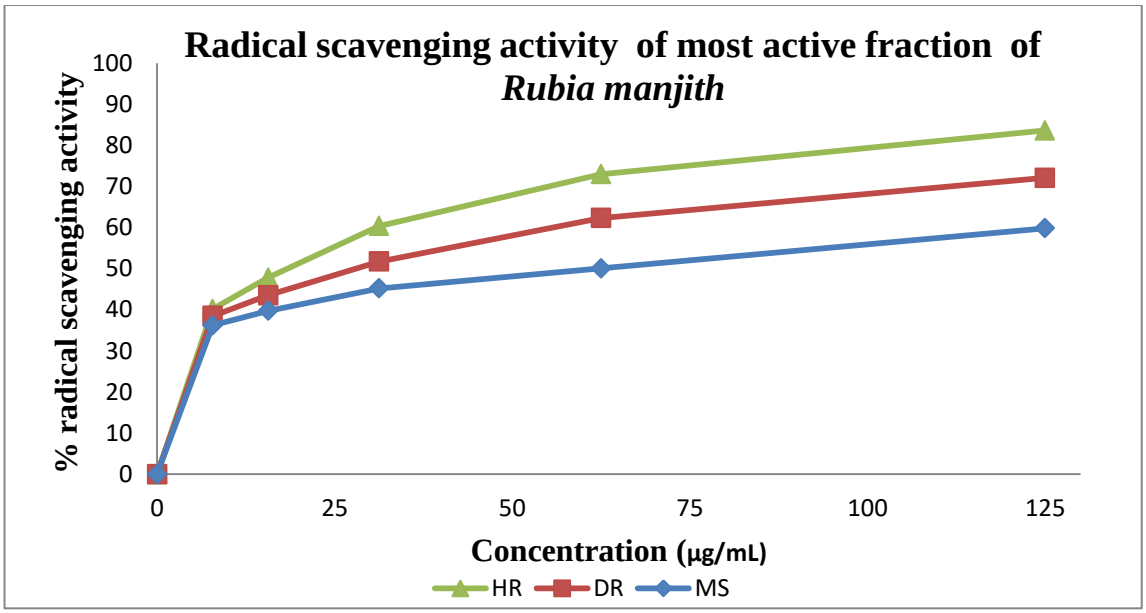


Figure 16: Percentage DPPH Radical scavenging activity of extract of *R. manjith*

The highest antioxidant activity was shown by root extract in hexane having the lowest IC_{50} value, i.e. 16.92±2.97 µg/mL followed by root extract in dichloromethane having an IC_{50} value 35.17±1.42 µg/mL which is statistically lower than that of standard ascorbic acid (AA) (IC_{50}: 38.21 µg/mL). Likewise, the stem extract in methanol has an IC_{50} value 46.49±0.85 µg/mL. The methanol leaf extract showed moderate antioxidant activity with an IC_{50} value 100.26±10.75 µg/mL. The lowest antioxidant activity was shown by the leaf extract in hexane having the highest IC_{50} value 671.13±17.35 µg/mL (Fig. 17).

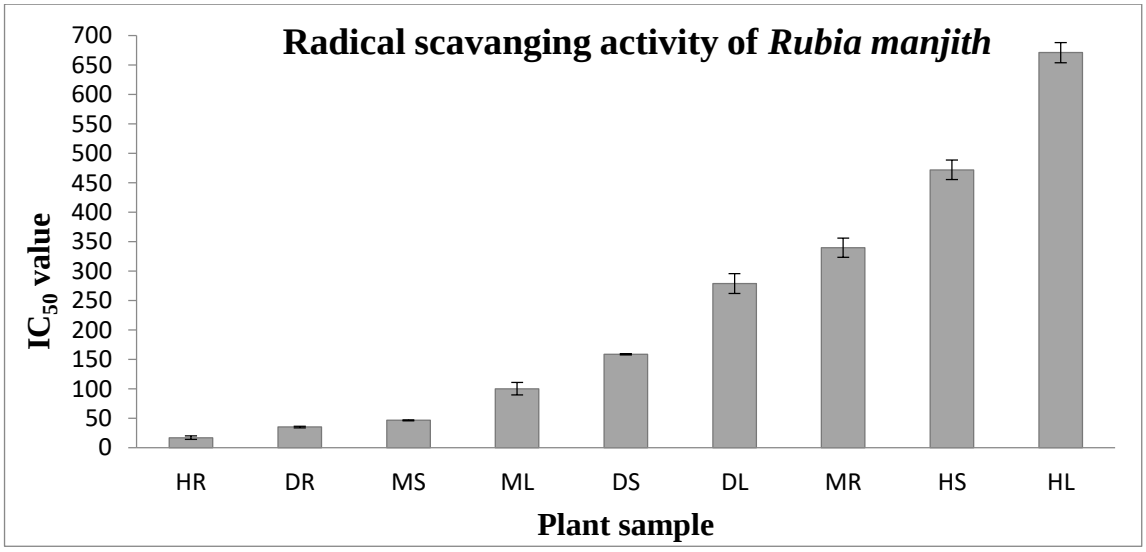


Figure 17: IC_{50} values of extract from various samples of *R. manjith*

4.3.2. Toxic activity

The toxic activity of various crude extracts of different plant parts of *R. manjith* against Brine shrimp (*Artemia salina*) was evaluated using Brine shrimp lethality assay and LC_{50} values were determined (Fig. 18). The crude extract concentration was used up to 8 mg/mL. All the observation was done after 24 hours of incubation. All the nauplii survived in the control. The result indicated that the hexane extract of the stem of *R. manjith* had the most potent toxicity with an LC_{50} value 194.99±34.58 µg/mL followed by the hexane extract of the leaf with an LC_{50} value 253.44±4.08 µg/mL. The dichloromethane extract of leaf had the least toxicity with an LC_{50} value 1189.37±117.96 µg/mL. However, none of the methanolic crude extracts showed toxic effects on Brine shrimp.

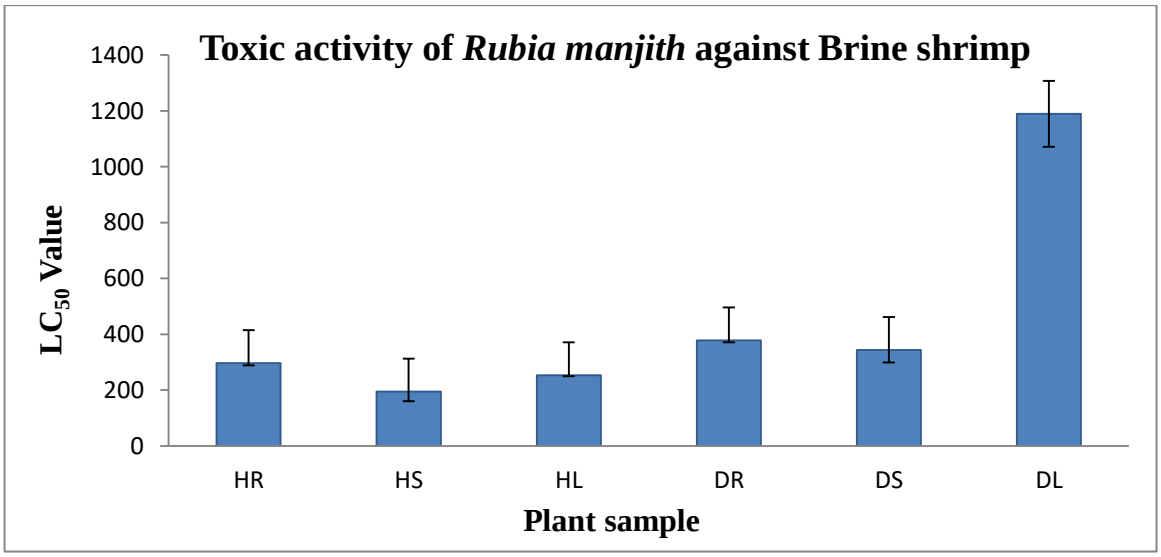


Figure 18: IC_{50} values of toxic activity of various samples

5. DISCUSSION

5.1. Plant tissue culture

Since there is no available work on this plant and hence we claim this work to be the first attempt of it's kind of this plant. In this study, *R. manjith* was micropropagated by inoculating single nodes in MS media supplemented by plant growth regulators, BAP and NAA to formulate an efficient protocol. Current results indicated that MS media without any plant growth regulators gave better results (5.33±0.33 shoots and 8.00±1.00 roots per explant) in shoot and root number induction. A similar result was observed in the study in the same genus, *R. akane,* where the highest shoot (4.5 shoots) and root numbers (9.14 roots) were produced within 8 weeks in the MS media without any cytokinins (Hapsari and Ermayanti, 2020). They used shoot tip and nodal explant and found that MS media with 0.5mg/L kinetin gave the highest number of leaves, whereas 0.5mg/L BAP induced the highest number of internodes, giving elongation to the length. In this study the highest shoots; 8.36 cm were produced in MS media without any hormone, whereas in our study the length in MS media was observed higher than this result which is 14.13±1.12 cm. Armin *et al.,* (2011), also discovered that MS media without BAP and NAA was better than those growth hormones in root and shoot induction using the node of the *Solanum tuberosum.*

Galo (2019) also suggested that the MS media is suitable for the shoot induction of *Stevia* and node is suitable than the other parts. A similar result was seen in *Colocasia esculenta* (L.), where MS media gave better shoot and root induction than MS media with BAP and TDZ (Alam and Kadir, 2022). In the *in-vitro* seed germination and plant multiplication of rice varieties, MS media without any hormonal supplement were found to be best (Puhan and Siddiq, 2013). According to Aktar *et al.,* (2008), the combination of macronutrients, micronutrients, vitamins, and organic compounds is extremely suitable for a broad range of plant species, which is also applied to *R. manjith* regarding root and shoot induction.

Likewise, the highest shoot length induction was observed at single BAP treatment, which was also best for the highest root length induction. The highest mean shoot length observed was 14.33±1.54 cm in 0.1 mg/L BAP and the highest mean root length observed was 6.80±1.27 in 2.0 mg/L BAP. Ghatge *et al.,* (2011) and Radha *et al.,* (2011) also

supported the idea that BAP alone at a certain optimum concentration of 3 and 2 mg/L BAP, respectively, can perform better in *R. cordifolia.* They also observed that in MS media with 3mg/L BAP and 0.02 mg/L IAA, the maximum shoot induction was 88% among the cultures and the mean shoot lengths were 3.25 cm and 3.7 cm, respectively.

In addition, Vivekanandan *et al.,* (2014) also supported the finding that BAP is very essential for shoot induction. They found that the combination of 4 mg/L BAP and 5 mg/L AdS produced a significant shoot induction response (60%) with a mean length of 5.4±0.3 cm in *R. cordifolia*.

For the formation of roots, NAA is often used as an auxin in tissue culture. The presence of NAA in MS media is effective for root formation (George and Sherrington, 1984). In our study, besides MS media, the highest mean root number (7.67±0.33) was observed in 2.0 mg/L NAA. A similar result was observed in the *R. cordifolia* plant (Vivekanandan *et al.*, 2014). Most roots per shoot were seen in 2 mg/L IBA, where 60% of the responses had mean roots of 8.0±02.

In various studies, 1 mg/L IBA as auxin showed the highest root induction in *R. cordifolia*. For instance, Radha *et al.,* (2011) reported a 98% response in 8.9 mean roots with 6.4±0.3 cm, and Khadke *et al*., (2013) had a similar observation with a 93.7% response in 4.9±0.7 mean root with a mean length 4.7±0.2 cm.
Our study showed that 2.0 mg/L BAP is suitable for the long root induction (6.80±1.27) of *R. manjith.* Yuniastuti *et al.,* (2018), also mentioned that the higher concentration of BAP can play a role in long root lengths, which supports our finding. BAP is believed to have a positive impact on root elongation activity and cytokine concentrations, which promote cell division in the root. Durrani *et al.,* (2010) cultured spinach using BAP and NAA and found that maximum root length was observed on 10^{-3} M BAP.

In our study, there was no callus in any of the cultures on the control treatment (MS media). Callogenesis and growth of the callus were poorly supported by single BAP and single NAA treatments, whereas the combination of BAP and NAA treatments were comparatively better in the development of the callus. The highest calluses were induced

in the MS media containing 2.0 mg/L NAA, 0.1 BAP + 1.0 NAA, 0.1 BAP + 2.0 NAA, 0.1 BAP + 0.1 NAA and 1.0 BAP + 1.0 NAA. This result was supported by Radha *et al.,* (2011), who used a shoot tip and node and split nodal half of *R. cordifolia* and found that none of the explants gave calluses in MS media. Also, a compact red callus was observed at the cut ends of the node in the MS media containing BAP and NAA. According to Labade (2009), combination of 2 mg/L NAA and 0.5 mg/L BAP in MS media was best for the initiation and proliferation of calli in *R. cordifolia*. Khadke *et al*., (2013) reported a significant amount of callus on MS medium with the combination of 2,4-D and NAA, which also supports our finding that the combination of cytokinin and auxin is suitable for callus induction.

5.2. Phytochemical and biological analysis

5.2.1. Phytochemical analysis

Plant phenolics are the main group of compounds that work as primary antioxidants (Karaman *et al.,* 2010). According to Singleton *et al.,* (1999), the Folin-Ciocalteu method has been used and favored over others in most cases. The blue colored solution is formed when FC reagent reacts with active extract and the more concentrated color intensity indicates a higher total phenol content. We detected a significant amount of phenols in the dichloromethane fraction of the root and stem (77.5±0.01 µg GAE/mg and 70±0.01 µg GAE/mg respectively) followed by the hexane fraction of the root (57.11±0.05 µg GAE/mg) in *R. manjith*. Besides the dichloromethane fraction in stem, two of them showed very powerful free radical scavenging activity.

In the stem of *Wendlandia tinctoria* of Rubiaceae, the highest Phenol content was present in the dichloromethane fraction with a phenol content of 289.87 ± 0.47 mg of GAE/g of dried extract followed by methanol with 286.91 ± 0.28 mg of GAE/g of dried extract and lowest phenol content was present in the hexane fraction with 37.07 ± 0.11 mg of GAE/g of dried extract (Farzana *et al.,* 2022). The dichloromethane fraction in our analysis also had the highest total phenol content, which is similar to this finding.

In the study of Humbare *et al.,* (2022) the highest TPC was obtained in root extract with in the methanolic fraction having 43.34 ± 0.27 µg GAE/gm phenols among ethanol and aqueous extract which was in contrast to our study. In our study root methanol had the

lowest TPC among all the crude extracts *R. manjith* which was 31.85065± 0.03 µg GAE/gm phenol.

Total phenolic compounds comprise different compounds such as simple phenols, phenolic acids, tannins, lignins, flavonoids, glycosides, anthocynins, hydroxycinnamates, and proanthocyanidin oligomers (Al-Farsi *et al.,* 2005; Das and Gezizi, 2018). Phenolics compounds are responsible for the majority of the antioxidant activity in plants or plant-based products (Okpuzar *et al.,* 2009). Additionally, the total phenolic compounds show a wide range of beneficial properties, including anti-inflammatory, anti-cancer, anti-microbial, and cardio-protective properties. Phenolic compounds are used to treat neurological illnesses, hypertension, metabolic issues, and incendiary infections. They can also inhibit enzymes linked to the development of human diseases. (Das and Gezici, 2018; Barros, 2020; Rahman *et al.,* 2021).

5.2.2. Biological analysis

5.2.2.1. Antioxidant activity

Antioxidants can be considered those substances which can donate hydrogen or electron to DPPH that is a nitrogen-centered free radical. Those substances are also called radical scavengers because of their ability to scavenge free radicals and inhibit oxidative reactions. Radical scavenging activities are crucial to eliminate and prevent free radicals in various diseases. After the interaction with DPPH, the discoloration of violet color depends on the reducing power of substances that indicates the radical scavenging potential (Singh *et al.,* 2002; Ratty *et al.,* 1988). Besides the abundant components like phenolic and flavonoid compounds, other phytochemicals for instance terpenoids, alkaloids, and fatty-acid derivatives also plays vital role in antioxidant activities (Grabmann, 2005).

For the determination of the antioxidant potential of *R. manjith*, DPPH radical scavenging activity was assessed in all of the crude extracts. The result revealed that root extract of hexane is a very powerful antioxidant agent, particularly when compared to the IC_{50} value of quercetin. According to Molyneux (2004), if the IC_{50} value is less than 50, the antioxidant activity is considered to be "very powerful", "strong" if 50-100 ppm,

"moderate" if 101-150 ppm, and "weak" if 150-200 ppm. This indicates that the root extract of hexane, the root extract of dichloromethane and the stem extract of methanol are very powerful antioxidant (IC_{50} value 16.92±2.97 µg/mL, 35.17±1.42 µg/mL, 46.49±0.85 µg/mL respectively) whereas the leaf extract of methanol is strong (100.26±10.75 µg/mL) and the stem extract of dichloromethane is moderate antioxidant agent (158.64±1.05 µg/mL). These findings showed the hexane and dichloromethane fractions of *R. manjith* have very strong antioxidant properties, even in crude form.

In the recent study of the stem of *Wendlandia tinctoria* of Rubiaceae (Farzana, 2022), all three solvents hexane, dichloromethane and methanol was used among which dichloromethane had lowest IC_{50} value with 18.83 ± 0.22 µg/mL followed by methanol with IC_{50} value 20.09 ± 0.22µg/mL and highest was of hexane i.e. 53.26 ± 0.05µg/mL. In addition, dichloromethane had the highest TPC value while hexane fraction had lowest. So, there was positive correlation between the total phenolic content and the free radical scavenging activity of the stem. In our study, dichloromethane extract of root had the highest total phenol content and it showed second highest free radical scavenging activity indicating the positive correlation to the total phenolic content.

Our research revealed that the hexane fraction of the root exhibited a powerful antioxidant capacity in *R. manjith,* and that the value is also significantly greater when compared to the closely related species *R. cordifolia.* Some other studies also had the similar results showing high antioxidant activity in hexane, for instance, in *Gynura procumbens,* the highest antioxidant activity through the DPPH assay was observed in hexane fraction than in the ethanol fraction whose IC_{50} values were 78 µg/mL and 419 µg/mL respectively (Purwantiningsih *et al.,* 2019). In the research of Garut Orange leaves (*Citrus reticulate*), antioxidant activity was tested in ethanol, n-hexane, ethyl acetate and water fractions by using DPPH assay. The highest antioxidant activity was obtained in ethanol extract (42.925 ppm) followed by the n-hexane fraction (46.146ppm) while the lowest was in the water fraction (55.662 ppm) (Fadhlillah *et al.,* 2019).

In the recent study of *R. cordifolia* (Humbare *et al.,* 2022), the effect of using polyvinylpolypyrrolidone (PVPP) in the DPPH assay was examined. It was found that

the extract maintains its capacity to act as an antioxidant even after the removal of phenols and flavonoids. This shows that other secondary metabolites other than phenolic ones may also have contributed to the antioxidant activity in the *Rubia* species.

Some of the investigations demonstrated that lipophilic compounds such as carotenoids, some of the polyphenols and flavonoids show antioxidant activity (Huang and Wang, 2004; Maeda *et al.,* 2008). According to the qualitative analysis of the stem of *R. manjith* (NPRL/DPR, 2014), phytochemicals glycoside, steroid, terpenoid, tannin, saponin, flavonoid, volatile oil, reducing molecule, polyurenoid, polyoses, emodin, fatty acid, and carotenoid were present. However, alkaloids were not found in that study, similar to the finding of Sisubalan (2016) in the root of *R. cordifolia*. In addition, analysis of lipophilic antioxidants in the leaves of *Kaempferia parviflora* was done and quantified using LC–MRM–MS. It was found that carotenoids, tocopherols, phytosterols, fatty acids, and vitamin K were responsible for the antioxidant activity (Song *et al.,* 2021). So there is a possibility of presence
of those non-polar compounds in the hexane fraction that are responsible for the higher antioxidant activity in *R. manjith* as well.

Methanol extracts of stem and leaf showed strong and moderate antioxidant activity, respectively, in our study. Earlier research showed that *Rubia's* antioxidant properties may be due to the presence of anthraquinones (Yen *et al.,* 2000; Cai *et al.,* 2004) and most of the anthraquinones found in plants or plant extracts are glycoside (Blomeke *et al.,* 1992), which are polar in nature. The antioxidant activity of *R. manjith* shown by stem and leaf in methanol might be due to the presence of polar anthraquinones, phenolics and flavonoids in it.

In comparison to the leaves and stem, the methanolic root extract of *R. cordifolia* produced more phytochemicals (Chandrashekar *et al.,* 2018; Humbare *et al.,* 2022). Root and stem extracts showed higher antioxidant activity than leaves in our study as well. The lowest IC_{50} obtained in *R. cordifolia* was on the methanolic root, which was 79.1 ± 1.92 µg/mL among the ethanol and aqueous fractions (Humbare *et al.,* 2022). In the previous study, the leaf of *R. cordifolia* showed high antioxidant activity by DPPH

assay in ethanol, n- butanol, methanol, ethyl acetate and hexane, respectively (Khan *et al.*, 2021). This pattern is similar to our study in the context of leaves.

Potent antioxidant compounds can play an important role in therapeutic properties as they can reduce oxidative stress in the body. Those compounds are employed in the treatment of wrinkle formation, rheumatoid arthritis, inflammation, hypertension and can play a significant role in neuroprotective, cardioprotective, anticarcinogenicity, antimutagenicity, antiallergenicity, antibiotics, immunity booster and antiaging activities (Abbas & Monireh, 2008; Bhattacharyya, 2014; Anwar *et al.*, 2018).

5.2.2.2. Toxic activity

A substance is called toxic if it inhibits important metabolic processes that may cause abnormal behavior or death (Fatope, 1995). According to Meyer *et al.,* (1982) an LC_{50} value< 1000 µg/mL is considered as bioactive in the toxicity analysis of plant extract in standard brine shrimp lethality assay. The highest toxicity was observed in hexane extract of the stem with lowest LC_{50} value 194.99±34.58 µg/mL. All of the hexane and dichloromethane extract (besides dicholormethane extract of leaf, LC_{50} value 1189.37 µg/mL) of *R. manjith* have less than 400µg/mL LC_{50} values which indicates that the dichloromethane and hexane fractions of this plant are toxic whereas methanolic fraction in nontoxic.

Similar to our findings, polar solvent extract from fractionation did not exhibit toxicity, while non-polar solvent extract from fractionation showed toxic activities in the Brine Shrimp lethality assay of *R. cordifolia*. The experiment was done in the chloroform, ethyl acetate, methanol fraction and aqueous extract. It was found that aqueous extract had high toxicity with an LC_{50} value of 75.81 µg/mL, chloroform extracts had 718.40 µg/mL and methanol had more than 1000 µg/mL while ethyl acetate showed no toxicity (Ghwanga, 2019). The study of *Rosmarinus officinalis* (Rosemary) also had the similar result that the hexane and dichloromethane extract showed high toxicity than that of methanol and aqueous extract (Kabubii, 2015).

Different bioactive components are extracted by using various solvents. Wax, fats and fixed oils are extracted using hexane, whereas alkaloids, aglycones, and volatile oils by

dichloromethane which is more polar than hexane (Cowan, 1999). The report of DPR also mentioned that 0.34% essential oil was extracted from the closest species, *R. cordifolia* (NPRL/DPR, 2014). Essential oils comprise varieties of volatile compounds that often have a strong odor and perform a wide range of ecological functions (Teixeira *et al.,* 2013). Those compounds can be used as anticancer agents as well as use against different toxicity such as hepatotoxicity, nephrotoxicity, neurotoxicity, ototoxicity, carcinogen, etc (Dixit, 2019).

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

For the shoot and root induction of *R. manjith*, MS media without any growth hormone and single BAP treatments were found to be most favored by the plant. MS media without any treatment is suitable for the rapid multiplication of the species (5.33±0.33 shoots), while 0.1 mg/L BAP was most effective to induce long shoots (14.33±1.54 cm), followed by MS media (14.13±1.13 cm). The highest mean root number was observed in the MS media (8.0±1.0 roots), and 2.0 mg/L BAP was suitable for the highest mean root length (6.08±1.27 cm). In addition, the combination of both cytokinin and auxin seems to be favored regarding callogenesis. Therefore, considering that the use of auxin and cytokinin promotes the growth of *R. manjith*, the nutrients in MS media are sufficient for optimum growth of this species, particularly in the shoot and root induction.

Quantitative phytochemical analysis of *R. manjith* shows a significant amount of phenol content in the crude extract. The highest phenol content was recorded in the dichloromethane fraction: root (77.5±0.01 µg GAE/mg) and stem (70±0.01 µg GAE/mg). Interestingly, the highest antioxidant activity was performed by hexane extract of root (16.92±2.97 µg/mL) though it had relatively low phenol content (57.11039±0.01 µg GAE/mg). The non-polar character of this solvent may account for the low phenol content of the hexane fraction. The higher antioxidant activity of hexane may be attributed by the carotenoid pigments, terpenoids, fatty acids, vitamins, flavonoids, and certain phenols extracted in that fraction. However, the dichloromethane fraction of the root showed a positive correlation of antioxidant activity with the total phenol content, indicating that the phenol content in that fraction is attributed to the high antioxidant activity (35.17±1.42 µg/mL).

Comparatively, all of the hexane fractions showed the highest toxic activity in the brine shrimp assay. Hexane extracts of stem and leaf show highest toxicity activity in *R. manjith* (LC_{50} 194.99±34.58 µg/mL and 253.44±4.08 µg/mL respectively). Our study suggested that essential oils of *R. manjith* which are more non-polar in nature, were extracted in the hexane fraction and could have played a role in the high mortality of brine shrimps. These active compounds may act as potential anticancer agents.

6.2. Recommendation

Since the present study is the first work of its kind in this plant, further research is suggested to verify the present research results. This initial work on an important Nepalese medicinal plant has shown a wide range of research possibilities in the future regarding its medicinal potential in the treatment of different elements, as suggested by the present work. These are preliminary experiments and the result shown by the extract of *Rubia manjith* in this study is in crude form, so a comprehensive experiment on the active components can be done thoroughly. Further work on the phytochemical analysis of this pant may ensure the discovery of novel compounds useful for various purposes. Besides all that, this important plant asset of Nepal needs to be conserved by all means (*in-situ* or *ex-situ*) for the maintenance of the ecosystem and for its utilization by future generations.

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8. APPENDIX

APPENDIX-I: Induction of shoot, root and callus in *Rubia manjith*

Media Composition	Shoot number	Shoot Length	Root number	Root length	Callus
MS Media	5.33±0.33	14.13±1.12	8.00±1.00	2.07±0.07	-
0.1 BAP	4.33±0.33	14.33±1.54	6.00±1.15	4.27±1.07	+
0.5 BAP	3.66±0.33	8.00±1.00	3.5±0.50	3.35±0.85	+
1.0 BAP	4.33±0.33	7.35±3.15	4.00±2.00	3.50±1.50	-
2.0 BAP	2.66±0.33	7.30±1.43	3.67±1.76	6.80±1.27	+
0.1 NAA	0.67±0.33	3.10±2.95	4.00±0.58	2.07±1.02	+
0.5 NAA	1.33±0.33	0.10±0.00	0.00±0.00	0.00±0.00	+
1.0 NAA	1.67±0.33	0.93±0.19	2.67±1.76	0.40±0.31	+
2.0 NAA	0.67±0.67	0.03±0.03	7.67±0.33	0.33±0.03	+++
0.1BAP + 0.1NAA	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	+
0.1BAP + 0.5NAA	1.00±0.58	0.63±0.58	2.33±0.67	0.73±0.28	++
0.1BAP + 1.0NAA	1.00±0.00	0.10±0.00	0.00±0.00	0.00±0.00	+++
0.1BAP + 2.0NAA	0.67±0.67	0.10±0.10	2.00±1.150	0.33±0.18	+++
0.5BAP + 0.1NAA	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	+
0.5BAP + 0.5NAA	0.33±0.33	0.03±0.03	0.00±0.00	0.00±0.00	+
0.5BAP + 1.0NAA	1.00±0.00	0.15±0.05	4.50±1.50	1.55±1.25	+
0.5BAP + 2.0NAA	2.00±0.00	0.40±0.00	4.00±0.00	0.70±0.00	++
1.0BAP + 0.1NAA	1.00±0.00	2.23±1.08	0.67±0.67	0.73±0.73	+

1.0BAP + 0.5NAA	1.50±0.41	0.10±0.00	2.00±0.00	0.25±0.05	+
1.0BAP + 1.0NAA	1.00±0.58	0.37±0.32	0.00±0.00	0.00±0.00	+++
1.0BAP + 2.0NAA	0.33±0.33	0.03±0.03	4.33±1.86	0.27±0.03	++
2.0BAP + 0.1NAA	1.50±0.50	0.20±0.10	0.00±0.00	0.00±0.00	+
2.0BAP + 0.5NAA	1.33±0.33	1.60±0.67	4.67±0.88	1.23±0.32	++
2.0BAP + 1.0NAA	2.00±0.00	11.0±0.50	3.50±0.50	0.15±0.05	-
2.0BAP + 2.0NAA	1.00±0.00	0.30±0.20	3.33±2.03	0.27±0.15	++

APPENDIX-II: Photo plates

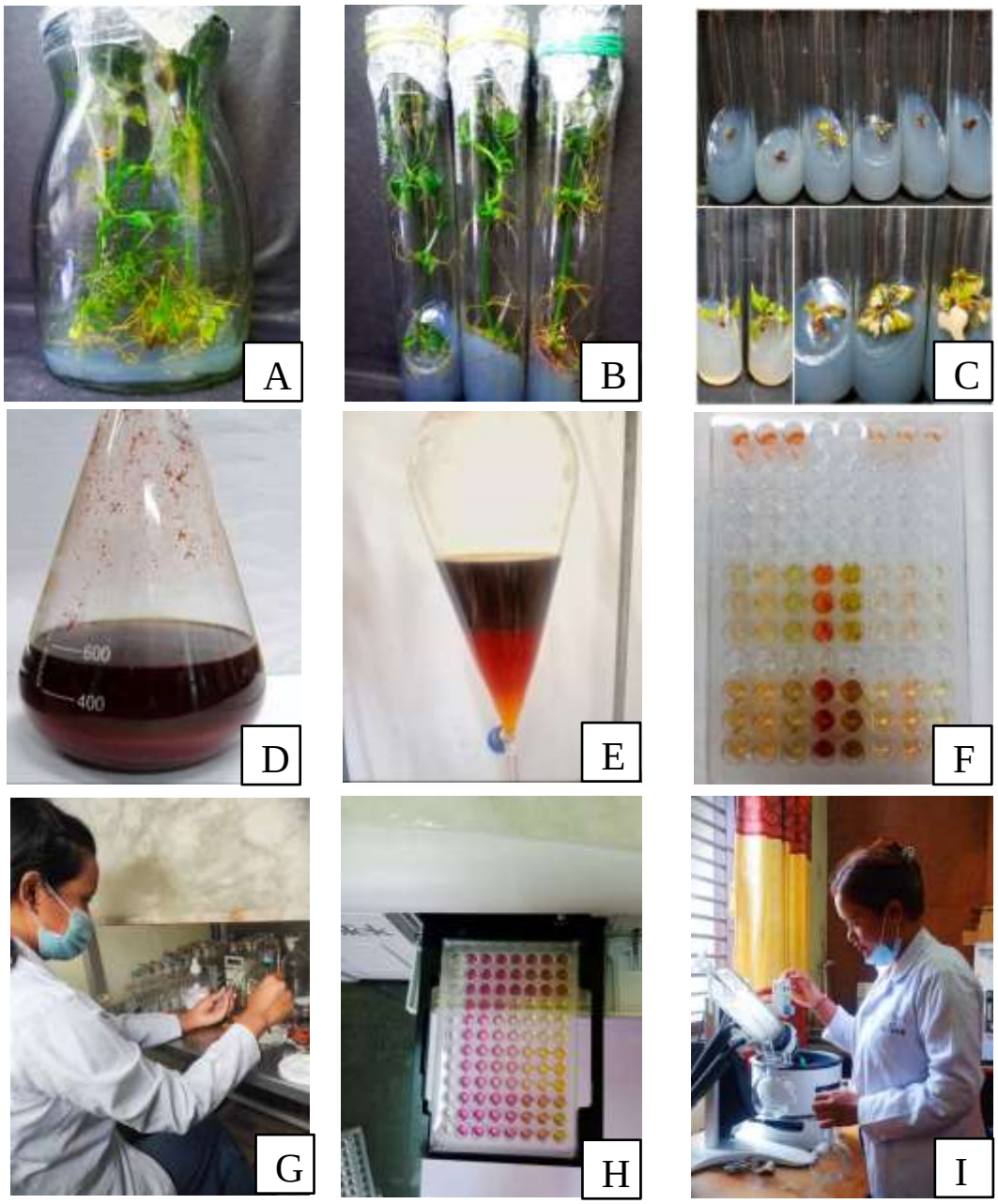

Photo plates 1: A: Sub cultured plants of the *R. manjith* in a jar; **B:** Plants in MS media having secondary roots and shoots on second and third nodes after 8 weeks; **C:** Explant showing no response in different hormonal treatment; **D:** Soaking dried root powder in methanol; **E:** Fractionation of solvent using a separating funnel; **F:** Brine shrimp lethality experiment in a sterilized 96-well plate; **G:** Culturing single node inside the laminar air flow chamber; **H:** measuring absorbance of samples for antioxidant activity using micro-plate reader; **I:** Evaporating solvent in the rotatory evaporator.