Comparative Phytochemical Screening and Biological Activities of *In vitro* and *In vivo Vanda tessellata* (Roxb.) Lodd. ex G. Don and *V. testacea* Rchb. f.

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RECOMMENDATION

This is to certify that the dissertation work entitled **"Comparative Phytochemical Screening and Biological Activities of *In vitro* and *In vivo Vanda tessellata* (Roxb.) Lodd. ex G. Don and *V. testacea* Rchb. f."** submitted by **Mr. Tej Lal Chaudhary** was accomplished under my supervision. The candidate has carried out the original research and to the best of my knowledge the work has not been submitted anywhere for any academic purpose. I hereby recommend for the approval of this dissertation as a partial fulfillment of the requirements of Master's Degree in Botany at Tribhuvan University.

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LETTER OF APPROVAL

The M. Sc. Dissertation entitled **"Comparative Phytochemical Screening and Biological Activities of *In vitro* and *In vivo Vanda tessellata* (Roxb.) Lodd. ex G. Don and *V. testacea* Rchb. f."** submitted at the Central Department of Botany, Tribhuvan University by **Mr. Tej Lal Chaudhary** for the partial fulfilment of his Master's degree in Botany, has been approved.

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The dissertation entitled "**Comparative Phytochemical Screening and Biological Activities of *In vitro* and *In vivo Vanda tessellata* (Roxb.) Lodd. ex G. Don and *V. testacea* Rchb. f**." which is being submitted to the institute of Science and Technology (IoST), Tribhuvan University, for the completion of Master's Degree in Botany is genuine work carried out by me under the supervision of **Prof. Dr. Bijaya Pant,** Professor of Central Department of Botany. I further declare that the work reported in this research has not been previously deposited either in part or in full for the merit of any degree, in this or any other institute or University.

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Tej Lal Chaudhary

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ABSTRACT

V. tessellata and *V. testacea* are most commonly used medicinal orchid. This study aims to develop a sustainable alternative through plant tissue culture technique, by comparing the phytochemicals and biological activities of *in vitro* raised samples with their wild counterparts. Tissue culture of *V. tessellata* and *V. testacea* was carried out through asymbiotic seed germination on Murashige and Skoog (MS) medium. The methanolic extracts of wild (root and leaf) and *in vitro* (protocorm and plantlets) samples were qualitatively screened for alkaloids, phenols, flavonoids, saponins, tannins, phliobatannins, terpenoids, steroids and anthocyanins, and quantitatively screened for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) using FC-reagent and $AlCl_3$ colorimetric test. Also, the extracts were examined for antioxidant, antibacterial and cytotoxic activity by using DPPH, agar well diffusion and MTT assay respectively. The best results for seed germination and protocorm formation of *V. tessellata* and *V. testacea* were observed in full strength MS medium with 10% coconut water (FMSCW) and quarter strength MS medium with 10% coconut water (QMSCW) respectively. The highest number of shoots (10.5±0.65) of *V. tessellata* was observed in FMSCW fortified with 1.5 mg/L BAP and 0.5 mg/L NAA and longest shoot length (2.98±0.18cm) was recorded in FMSCW fortified with 1 mg/L BAP and 0.5 mg/L NAA. In *V. testacea*, highest number of shoots (10.75±0.48) was found in FMSCW and 0.5 mg/L BAP and the longest shoot length (3.1±0.24 cm) was obtained in FMSCW. Phytochemical screening of *V. tessellata* and *V. testacea* revealed the presence of alkaloids, phenols, flavonoids, saponins, tannins, phliobatannins, terpenoids, and steroids in both wild and *in vitro* raised samples. The highest amount of TPC (223.69±0.33 mg GAE/gm) and TFC (87.93±0.43 mg QE/gm), lowest IC_{50} value (109.79±0.57 µg/mL) for antioxidant and the highest zone of inhibition was obtained in wild leaf extract of *V. testacea* followed by *V. tessellata* leaf extract. All samples showed very high IC_{50} values against HeLa and MCF7 cell lines indicating inactive or very weak cytotoxic activity. In case of normal cell, instead of inhibition cell were proliferated. The wild leaf extract of *V. testacea* showed lower AC_{50} value (484.94±5.31 µg/mL) for normal cell line than *in vitro* raised samples. In conclusion, an efficient protocol for *in vitro* propagation for phytochemical extraction of *V. tessellata* and *V. testacea* was established. The *V. testacea* sample showed better phytochemicals and bioactivity than the *V. tessellata* sample. The wild *V. tessellata* and

V. testacea plant extracts had higher medicinal potential due to higher TPC, TFC, antioxidant, and antibacterial activity, while *in vitro* raised plantlets can still be used as an alternative source for bioactive compounds for medicinal and other applications as well as for conservation and sustainable utilization of the species.

ABBREVIATIONS AND ACRONYMS

°C = degree centigrade

µg = microgram

µL = microliter

µM = Micro molar

AC_{50} = Half-Maximal Activating Concentration

DPPH = 1, 1-Diphenyl-2Picrylhydrazyl

GAE = Gallic Acid Equivalent

IC_{50} = Half-Maximal Inhibitory Concentration

mg = milligram

mL= milliliter

Mm = millimolar

MS = Murashige and Skoog (1962)

MTT = 3- [4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide

NB = Nutrient Broth

SNA = Soya Nutrient Agar

QE = Quercetin Equivalent

ROS = Reactive Oxygen Species

rpm = revolutions per minute

RSA = Radical Scavenging Activity

SE = Standard error

TFC = Total Flavonoid Content

TPC = Total Phenolic Content

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CHAPTER ONE: INTRODUCTION

1.1 Background

The search for medicine from natural resources has been ongoing since ancient times, with medicinal herbs being used for various ailments throughout human history. Traditional knowledge of these plants has been passed down through generations and is now being utilized in modern plant research (Santic *et al*., 2017). With many diseases developing resistance to existing drugs, the investigation for novel products is crucial. Natural remedies have the potential to be a part of basic healthcare, and many herbal ingredients are currently in clinical trials. However, scientific confirmation and recording of these traditional medicinal plants are essential, as they may contain biologically active chemicals that can treat a wide range of diseases without the side effects of existing drugs.

Among thousands of medicinal plants orchids are also utilized as natural remedies, nutritional and aesthetic value by many traditions and ethnicities across the globe. Since ancient times, orchids have been utilized in various areas of the globe in indigenous healthcare practices (Pant *et al.*, 2013). They are regarded as a valuable source of therapeutic herbal remedies. Medicinal orchids are rich in phytochemicals such as alkaloids, phenols, flavonoids, bibenzyl compounds, phenanthrenes, etc. (Gutierrez, 2010). These plants are treasure trove of novel therapeutic and pharmacological compounds that might aid in the development of new drugs.

The geographical location and topography of Nepal contribute to a massive biological diversity. Orchidaceae is Nepal's one of the dominant flowering plant family, with 108 genera and 502 taxa, including 20 endemics, 252 epiphytic, 184 terrestrial, and 14 saprophytic orchids, with some orchids having dual nature (Rajbhandari, 2014; Rajbhandari *et al.*, 2016 Raskoti and Ale, 2019). In Nepal, orchids are commonly known by the vernacular names ‘Sunagava’ or ‘Sunakhari’ and ‘Chadigava.’ They grow in a variety of habitats from 60-5200 meters above sea level (Rokaya *et al.*, 2013).

Orchids are a perennial herb with modified sepals, petals, and united stamen and carpels that distinguish them from other plants. Their stem lacks persistent woody structure and may form pseudobulbs (mostly in sympodial orchids). Orchids typically grow in either

a monopodial or sympodial pattern. In monopodial orchids, the stem begins to grow vertically from a single bud and continues to develop year after year, but in sympodial orchids, the stem stops growing vertically after flowering and produces lateral branches. In terrestrial orchids, roots can be rhizomatous, corm-like, or tuberous, whereas aerial roots are seen in epiphytic orchids. Stems are leafy and divided into nodes and internodes. Leaves range one to many, glabrous or very rarely hairy, alternate or occasionally opposite, frequently fleshy. Inflorescence erect to pendent raceme. Orchids have nectarless blooms and rewardless pollination, low fruit-to-flower ratios, numerous but minute seeds that lack endosperm for optimal energy utilization (Tremblay *et al.,* 2005). Orchids require a symbiotic or asymbiotic relationship with fungus for seed germination. The orchid embryo grows into a protocorm after seed germination before producing a plantlet. The protocorm of an orchid is a unique structure that is designed to form a symbiotic relationship with a compatible fungus. The protocorm divides indefinitely to generate the shoot apical meristem, which eventually develops into a plantlet.

Vanda is one of the genus of Orchidaceae which have been used for millennia to cure different aliments and now belongs to one of the endangered category. Therefore, it is necessary to conserve and analyze its valuable assets and tissue culture is one of the most reliable, efficient and practical approach for conservation of endangered species. Nowadays, plant tissue culture evolved as new discipline for the production of phytochemicals in commercial scale.

1.2 Botanical description of Genus *Vanda* Jones ex R. Br.

Vanda comprises approximately 73 species endemic to Asia's tropical and subtropical areas (Gardiner *et al*., 2013) where Nepal harbors five species of it (Rokaya *et al*., 2013). The generic name is coined from the Sanskrit term ‘Vandak’ which means plants of epiphytic or parasitic habit referring to *Vanda roxburghii* Jones ex R. Br. the now changed into *Vanda tessellata* (Roxb.) Hook. ex G. Don (Chhetri *et al.,* 2013).

Vanda is an epiphytic but sometimes lithophytic or terrestrial orchid with monopodial growth habit. Plants are 20 cm to 2 m tall, with often brilliantly coloured, sometimes fragrant, long lasting flowers. There is large variation in floral shape, size, and color and especially in labellum structure. Most of the *Vanda* species such as *V. tessellata* have multiple blooming season in a year and the flowers are long lasting. *Vanda* is one

of the five most valuable horticultural orchid of the world (Gardiner *et al*., 2013). Most of the species of this genus are medicinal.

1.3 Importance of *Vanda*

Vanda orchids are admired for their lovely, alluring and frequently fragrant colorful flowers, but they also have many other applications and advantages, including cosmetic purposes (anti-aging, moisturizing), medical uses (arthritis, bone dislocation, anti-inflammatory), culinary uses (herbal tea, immune booster) and several other significant economic and ecological values. Most of the species of *Vanda* are used for horticulture and medicinal purposes.

Many people are attracted to *Vanda* because of its vibrant color, massive, long-lasting, fragrant, and glossy blossoms, and several blossoming times throughout the year. Cross compatibility exists with other vandaceous orchid genera such as *Ascocentrum, Aerides, Rhyncostylis, Neofinetia, Renanthera* and even *Phalaenopsis* (De, 2002). Because of these traits, orchid breeders are very concerned in this species. Thousands of *Vanda* hybrids have been generated till date. These hybrids have desirable traits such as diverse color palettes, fragrant blooms, long-lasting blossoms, numerous inflorescences, compact growth habits and cold tolerance. Due to these exceptional characteristics, *Vanda* dominated the American and European markets too.

Along with its horticultural value *Vanda* have been used to treat varieties of aliments by different ethnicities. This orchid have from Vedic period as a component of Rasna Panchaka Quatha, an Ayurvedic formulation for rheumatic disorders and arthritis (Mukhtar and Kalsi, 2017). Several modern researches have been carried out in different species of *Vanda* such as in *Vanda tessellata* (Vijaykumar 2013; Subin and Williams 2018), *V. cristata* (Chand *et al.,* 2016), *V. coerulea* (Nag and Kumaria 2018), *V. spathulata* (Konduri *et al.,* 2022), etc. and these research suggest that this genus is very much potential as antioxidant. Research on different species of *Vanda* such as *V. coerulea* (Priya *et al.,* 2011), *V. spathulata* (Aparna *et al.,* 2021), *V. tessellata* (Behera *et al.,* 2013; Bhattacharjee *et al.,* 2015; Ramana, 2021), *V. testacea* (Kaushik, 2019) are reported to have different inhibitory potential against the various strains of microbes. Even some species of *Vanda* such as *Vanda spathulata* (Rani and Nandagopalan, 2022), *V. bensonii* (Jimoh *et al.,* 2022) are found to have some cytotoxic activity against various cancerous cell lines.

1.4 Conservation status of *Vanda*

Whole Orchidaceae family have been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Genus *Vanda* is an epiphytic tropical orchid with small distribution range. This genus is under severe threat due to rapid habitat destruction, agriculture, road construction, rapid urbanization and uncontrolled collection for illegal trade mainly for horticultural and medicinal purpose. Because of over-exploitation and a lack of awareness, *Vanda* species are becoming increasingly rare and endangered. In the context of Nepalese *Vanda*, there are 5 species of *Vanda* which are stunning and rich in therapeutic properties, among which one species *V. tessellata* is included in the IUCN red list in least concern (LC) category. In global contest, among 73 *Vanda* species the IUCN Red List includes five species, including *V. tessellata* (least concern), *V. javierae* (endangered), *V. scanders* (endangered), *V. spathulata* (vulnerable) and *V. hindsii* (least concern) (IUCN Red List of Threatened Species).

1.5 *Vanda tessellata* (Roxb.) Hook. ex G. Don

1.5.1 Botanical description

Synonyms: *Vanda roxburghii* var. *wrightiana* Rchb.f., *Aerides tessellata* (Roxb.) Wight ex Lindl., *Epidendrum tessellatum* Roxb., *Cymbidium tessellatum* (Roxb.) Sw.

Common name: Checkered Vanda, The lattice-like patterned flower Vanda, Grey orchid

V. tessellata is a perennial epiphytic orchid, 30-60cm height, stem stout, covered with sheath and scandent by the stout aerial roots, leaves are succulent, inflorescence raceme, flower 5-12, sepals yellow tessellated with brown lines, petals yellow with brown spots shorter than the sepals, lip purple, column white 0.9 cm and ribbed capsule 7.5–9 cm long.

V. tessellata is a monopodial orchid distributed in tropics of India, Nepal, China, Bangladesh, Sri Lanka, Myanmar, etc. at an elevation around 200-600 m (Rokaya *et al*., 2013). The plant blooms twice a year at early summer (April-May) and early winter (October-November) (De *et al*., 2015).

Figure 1. 1: Photographs of *V. tessellata.* (A) Whole plant in natural habitat; (B) Flower.

1.5.2 Medicinal importance of *V. tessellata*

This species has ethno medicinal value used for several years as a component of Rasna Panchaka Quatha, an Ayurvedic remedy to cure rheumatic disorders and arthritis (Mukhtar and Kalsi, 2017). The leaves of this species have been used traditionally to relief ear ache (Tiwari and Yadav (2003). Its roots are used as an essential ingredient in Ayurvedic medication 'The Rasna,' a bitter and hot formulation used to treat dyspepsia, respiratory problems, joint pains, abdominal disorders, hiccough, and tremors. It is a laxative and liver and brain tonic in the 'Unani' school of medicine, and it is effective in bronchitis, piles, toothache, boils, inflammations, and bone fractures (Hossain, 2009). Its roots are also reported to be useful to treat STDs, rheumatism and neurological problems (Dash *et al.* 2008).

Chemically this species is reported to be the treasure house of various secondary metabolites including terpenoids, flavonoids, phenols, alkaloids, tannins, phalbotanin, saponins, steroids, anthocyanins, anthraquinones, cardiac glycosides, coumarins, flavonones, and glycosides. (Arya *et al.,* 2012; Chowdhury *et al.,* 2014; Bhattacharjee *et al.,* 2015; Islam *et al*., 2016; Biswas and Sinha 2020).

It is also known to have various pharmaceutical activities such as antioxidant activity, antimicrobial activity, cytotoxic activity, anti-diabetic activity, etc. (Vijaykumar, 2013; Behera *et al.*, 2013).

1.6 *Vanda testacea* (Lindl.) Rchb. f.

1.6.1 Botanical description

Synonyms: *Vanda parviflora* Lindl., *Aerides testacea* Lindl., *Aerides wightiana* Lindl., *Vanda vitellina* Kraenzl.

Common name: Small flowered Vanda, Yellow Vanda

V. testacea is also a perennial epiphytic orchid, 25cm height, stem stout, covered with sheath and scandent by the stout aerial roots. Leaves 9-16 x 1 cm, strap-shaped, keeled and unequally lobed at apex. Flowers yellow, in 10-15 cm long, few-branched erect, raceme; dorsal sepal obovate-oblong or spathulate, obtuse, 5-veined; lateral sepals elliptic-subacute, 5-veined; petals spathulate, obtuse, 5-veined; lip pink, margins finely close-dentate, with 2 parallel fleshy ridges; spur cylindrical and capsule ribbed 4-6 cm long.

V. testacea is a monopodial orchid distributed in moist deciduous forests, also in the plains of India, Nepal, Sri Lanka, Myanmar, Burma, Thailand, etc. at an elevation around 200-2200 m and the plant blooms during May-June (Rokaya *et al*., 2013).

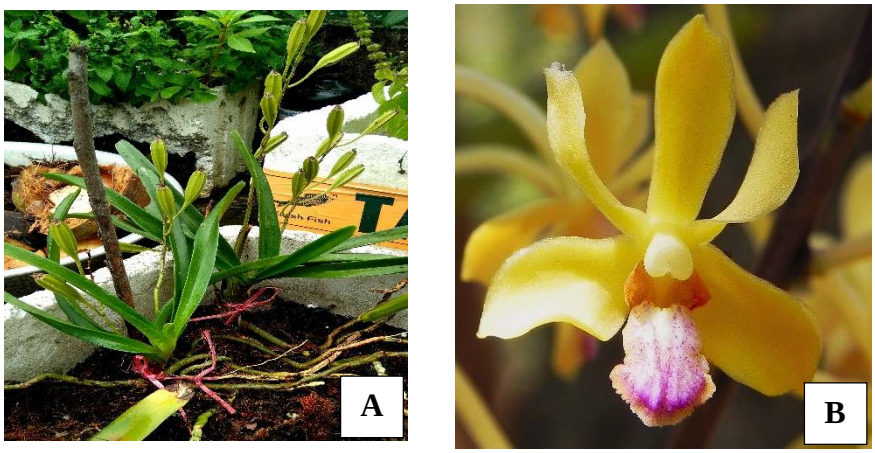


Figure 1. 2: Photographs of *V. testacea.* (A) Whole plant; (B) Flower.

1.6.1 Medicinal importance of *V. testacea*

This species is the least explored species in terms of ethnomedicinal, phytochemical and biological activities. So exploration of this species is essential. This species has been used to treat ear aches, asthma, rheumatism, piles, bronchitis, inflammation, nervous disorder (Chauhan 1990; Dash *et al.*, 2008). Phyotochemical screening only

reported the presence of flavonoids, reducing sugar, terpenoids and tannin in *V. testacea* (Maridass *et al.*, 2008). Examination of biological activity is lacking.

1.7 Tissue culture

Plant tissue culture is the aseptic *in vitro* cultivation of plant cells, tissues or organs (shoot tips, root tips, nodes, seeds, embryos, even a single cells or protoplasts) on defined nutrient media. Plant tissue culture relies on the concept of totipotency; that is, the ability of a living plant cell to produce an entire new plant. Tissue culture is a very effective way of producing large numbers of plantlets rapidly throughout the year. Micro propagated plants are observed to establish more quickly, grow more vigorously and are healthy, have a shorter and more uniform production cycle, and produce higher yields than conventional propagules. Tissue culture techniques have facilitated in the large-scale production of both highly threatened and economically important species. For *ex situ* plant conservation, tissue culture is becoming a new and reliable approach for multiplication of highly threatened taxa. It is most suited to conservation of very rare species that have poor seed set, low germination or problematic storage requirements. For the long-term conservation of orchid species, tissue culture technology has proved to be the most efficient and practical approach.

Now a day's, plant tissue culture becomes alternative tool for rapid plant propagation and controlled production of vital phytochemicals in medicinal plants. With advancements in tissue culture and genetic engineering, specifically transformation technology, there are now new opportunities for producing large quantities of pharmaceuticals, nutraceuticals, and other beneficial substances.

1.8 Objectives

1.8.1 General objectives

In vitro propagation of *V. tessellata* and *V. testacea* for their comparative phytochemical and biological study with wild plant.

1.8.2 Specific objectives

1. To develop efficient tissue culture protocol of *V. tessellata* and *V. testacea* for production of phytochemical.

2. To screen phytochemicals of wild and *in vitro* cultured *V. tessellata* and *V. testacea.*
3. To quantify the total phenolic and flavonoid content of wild and *in vitro V. tessellata* and *V. testacea.*
4. To test antioxidant, antimicrobial and cytotoxic activity of wild and *in vitro V. tessellata* and *V. testacea.*

1.9 Rationale of the study

V. tessellata and *V. testacea* are monopodial orchids with well-known therapeutic properties that are used to treat a variety of diseases by various ethnic groups as well as in Ayurvedic formulations. These two species are well renowned for their appealing bloom colors, and are in high demand for hybridization in the horticulture industry. Most of the need were fulfilled by unsustainable and illegal collection from wild and conventional cultivation and propagation techniques resulting in a severe threat to both species. So, urgent conservation strategies are required to address this issue. Therefore, in order to protect, both species have been included in CITES-II, and *V. tessellata* has been listed in the IUCN red list as well. However, merely listing them is insufficient, and new technological approaches are required to conserve and sustainably use these valuable plants. A new and practical biotechnological approach for plant conservation is plant tissue culture. Nowadays, *in vitro* propagation has emerged as a new discipline for the production of bioactive metabolites, which is also tremendously helpful in conserving biodiversity.

Comparative phytochemical and biological study of plant tissue cultured and wild *V. tessellata* and *V. testacea* will be very useful for sustainable utilization of valuable plants, biodiversity conservation as well as this research may also be useful for the validation of ethnobotanical knowledge in some extent.

1.10 Limitations

1. Only crude extracts were used for phytochemical and biological study.
2. Quantitative test was done only for total phenol and total flavonoid quantification while remaining group of compounds were tested qualitatively.
3. Only zone of inhibition assay was done for antimicrobial study.

CHAPTER-TWO: LITERATURE REVIEW

2.1 Plant tissue culture

Plant tissue culture is based on Scheilden's (1838) and Schwann's (1839) discovery that many plant cells have the power to regenerate a whole plant, a phenomenon known as cellular totipotency. The first experiment with *in vitro* plant tissue culture was made in 1902 by the German plant physiologist Gottlieb Haberlandt, who is known as the father of plant tissue culture.

Knudson (1922) germinated orchid seeds asymbiotically in *in vitro* conditions. This was the first feasible approach for *in vitro* multiplication of any plant in pure (axenic) culture. Then orchid tissue culture was revolutionized as a result of this discovery. Morel (1960), the pioneer of orchid micropropagation, successfully carried out vegetative multiplication of orchids using *Cymbidium* meristem culture for virus-free production of orchids. Murashige and Skoog (1962) developed Murashige and Skoog (MS) medium which is now the most frequently used medium for varieties of plants. After that, many progress towards the orchid tissue culture took place.

Now a days, exceptional progress has been achieved in the field of plant tissue culture and has become a multidisciplinary field of science. Conservation biology, agriculture, plant physiology, phytochemistry, genetic engineering, physiology, pharmacology, and many other fields are now interconnected.

2.2 Plant tissue culture in genus *Vanda*

In addition to rising demand for *Vanda*, the genus has faced a number of issues ranging from illegal harvesting to habitat deterioration. *In vitro* mass propagation is a promising strategy for assuring long-term horticulture business and conservation. To address the demand, various studies on mass propagation of this genus have been conducted.

Decruse (2003) studied shoot multiplication from the foliar meristem of *Vanda spatulata* and found that Mitra nutrient medium supplemented with a combination of BAP (66.6 µM) and IAA (28.5 µM) was the best combination for shoot proliferation and Mitra nutrient medium supplemented with a combination of banana pulp (75g/L) and IAA (5.7 µM) was used for rooting.

Smoke saturated water (SSW) was tested for *in vitro* seed germination and micropropagation of *V. parviflora* by Malabadi *et al.,* (2008), who found that Mitra basal medium supplemented with 10% SSW was very effective. *In vitro* propagation of *V. testacea* from *in vitro* grown foliar explants was studied by Kaur and Bhutani (2009), and they discovered that the optimum media for propagating plantlets was Mitra (M) medium enriched with 1 mg/L BAP and 1 mg/L NAA combined with 2% activated charcoal.

Rahman *et al.,* (2009) investigated *in vitro* micropropagation of *V. tessellata* from shoot tip explants and discovered that MS media supplemented with 1 mg/L BAP and 1.5 mg/L NAA was the optimal medium for shoot induction and proliferation. Prakash *et al.,* (2012) investigated the influence of medium on seed germination of *V. tessellata* and determined that MS>Kundson C>VW>RT. Prakash *et al.,* (2013) discovered that pH 5.5 is optimal for seed germination of *V. tessellata*. Bhattacharjee and Islam (2014) investigated the application of PGRs on *V. tessellata* multiple shoot proliferation on three distinct culture media: PM, MS, and B5 and discovered MS medium enriched with 1 mg/L BAP and NAA was the most effective for multiple shoot induction. Islam *et al.,* (2014) observed that MS medium with 0.1 mg/L NAA and 15% CW lowers the time required for seed germination of *V. roxburghii*.

Roy *et al.,* (2011) tested for mass propagation of *V. coerulea* in four different nutrient media and found that Phytamax basal medium was the best medium for seed germination, Phytamax medium fortified with 5.36 µM NAA and 3.80 µM BAP for maximum protocorm proliferation and 3g/L activated charcoal for the healthy plantlet production.

Jualang *et al.,* (2014) discovered that KC medium was superior to MS media for *V. dearei* seed germination, root and shoot proliferation, and elongation. They also discovered that 1% sucrose improved seed germination and that 20% CW improved protocorm development and soot growth.

Obsuwan and Thepsithar (2014) investigated on the effects of organic supplements on stimulating *in vitro* growth of *Vanda* Tokyo Blue and Mokara seedlings and discovered that Vacin and Went (VW) medium supplemented with Gros Michel banana was the

best for shoot proliferation, VW medium with CW for shoot elongation and VW medium with Namwa banana for rooting.

Sebastinraj *et al.,* (2014) studied seed germination of *V. testacea* in three different medium; MS, Kundson C, and VW media, and concluded that 1/2 MS was superior to the other two media for seed germination, HMS with 0.2 mg/L TDZ for mass proliferation, and 0.2 mg/L IBA for rooting. Thirugnanasampandan *et al.*, (2014) investigated the *in vitro* seed germination and synthetic seed development of *V. testacea* and discovered that the best seed germination medium was 1/2 B5 medium supplemented with 1mg/L GA3, while synthetic seed were prepared by using 5% sodium alginate and 100 mM calcium chloride.

Sachin (2015) investigated the effect of temperature and pH on the *in vitro* protocorm development of *V.tessellata* in basal MS medium and concluded 20°C and 5.5 pH were most efficient for protocorm formation.

From leaf and stem explants in MS medium supplemented with 0.5 mg/L BAP and 1 mg/L KN, Bhattacharjee and Islam (2017) developed a significant number of somatic embryos of *V. tessellata*. They also discovered that MS medium supplemented with 1.5 mg/L BAP and 1 mg/L NAA was best for shoot proliferation and elongation, while 0.5 mg/L IAA and 0.5 mg/L NAA was best for root proliferation and elongation.

Budisantoso *et al.,* (2017) used several combinations of 2,4-D to induce callus of *Vanda* species from leaf explants and discovered that the 2 ppm concentration was the most effective.

Nag and Kumaria (2018) investigated the *in vitro* propagation of the medicinally endangered orchid *V. coerulea* utilizing several medium (MS, KC, B5, and VW). They discovered that B5 was the optimum medium for seed germination, and B5 with 3µM thidiazuron increased the overall growth and development of *V. coerulea* plantlets.

Madhavi and Shankar (2019) studied *in vitro* seed germination of *V. tessellata* and found that ½ MS medium supplemented with banana powder (10 gm/L), tomato powder (10 gm/L) and 15% CW was most effective.

Maharjan *et al.,* (2019) experimented *in vitro* propagation of *V. pumila* and concluded that ½ MS containing 1 mg/L KN and 10% CW was the best for shoot proliferation, ½ MS containing 2 mg/L BAP and 10% CW for shoot elongation and ½ MS containing 0.5 mg/L IAA for root multiplication and elongation.

The New Dogashima Medium (NDM) fortified with 0.5 mg/L BAP was shown to be the best for mass proliferation of protocorm like bodies (PLBs) and overall development of *V. tricolor* by Rineksane *et al.,* (2020).

Malla *et al*., (2020), propagated *V. tessellata* from protocorms in different strength of MS media where QMS media was found to be most effective. For shoot multiplication, different concentration of coconut water, GA_3, BAP and NAA supplemented QMS media with 3% and 6% sucrose was examined which revealed QMS with 0.5 mg/L, BAP was most effective for shoot formation, QMS with 1.5 mg/L BAP + 6% sucrose for shoot-bud development, QMS with 0.5 mg/L NAA for root formation and QMS with 1.5 mg/L GA_3 for higher number of leaf formation and 0.5 mg/L GA_3 for leaf elongation.

On Mitra orchid medium, Kaur (2021) evaluated the micropropagation of *V. testacea* from undehisced and dehisced capsules and discovered that 1 m/L NAA was superior for promoting quick seed germination and 20% CW for overall development.

Manokari *et al.,* (2021) reported that seed germination of *V. tessellata* was best on MS medium supplemented with 1.5 mg/L IBA, protocorm and shoot proliferation.

In conclusion, the *Vanda* genus faces numerous challenges, including habitat deterioration and illegal harvesting, as demand for these plants rises. However, *in vitro* mass propagation has the potential to address these challenges and help ensure long-term horticultural business and conservation. Multiple studies have been conducted on the mass propagation of various species within the genus *Vanda*, with varying optimal propagation media, including Mitra, Phytamax, KC, and MS media. The optimal conditions for seed germination and plantlet growth were determined to include specific combinations of plant growth regulators, pH, and temperature. These studies highlight the potential of *in vitro* mass propagation to support both conservation efforts and commercial horticulture of the *Vanda* genus. Nowadays, plant cell culture for

production of secondary metabolites became a competitive area of research involving different scientific disciplines, including pharmacognosy (Biswas and Singh, 2019).

2.3 Ethnobotany, Phytochemistry and Biological activities of *Vanda*

2.3.1 Traditional use of *Vanda*

Some Indian scriptures mention the usage of orchids under the name of *Vanda* for its medicinal and aphrodisiac effects as early as the Vedic period (200 B.C. to 600 B.CV. *V. tessellata* is a component of Rasna Panchaka Quatha, an Ayurvedic remedy used to cure rheumatic disorders and arthritis (Mukhtar and Kalsi, 2017). Under certain ancient Sanskrit manuscripts, *V.roxburghii* leaf was applied externally for conditions including rheumatism, ear infections, fractures and nervous system disorders (Hossain, 2011).

The root of *V. tessellata* is a component in the Ayurvedic medication 'The Rasna,' which is bitter, hot, alexiteric, antipyretic, and used to treat dyspepsia, bronchitis, rheumatic pains, abdominal disorders, hiccough, and tremors. It is a laxative and tonic to the liver and brain in the 'Unani' school of medicine, and it is effective in bronchitis, piles, toothache, boils, inflammations, and bone fractures (Hossain, 2009).

Vanda species are being used in the folk medicine in various part of the Asia, mainly in India, Nepal, China and Bangladesh (Dash *et al.*, 2008; Hossain, 2009). *V. tessellata* plant has been employed in indigenous medicinal systems such as Ayurveda and local traditional medical practices to treat a variety of diseases (Mukhtar and Kalsi, 2017). Dash *et al.* (2008) investigated the ethnobotanical knowledge of the Dongria Kandha tribe of Orissa and reported the use of *V. tessellata* root to treat STDs and root paste to treat rheumatism and neurological problems, as well as *V. testacea* leaf paste to treat earaches and root decoction to treat asthma. Singh (2008) investigated the value of several helpful plants in ethnobotany and reported around 100 plants, including the usage of *V. tessellata* Had-jod plant paste in bone fracture.

Tiwari and Yadav (2003) described the usage of *V. tessellata* leaf decoction for ear ache relief. The roots, leaves, and flowers of *V. tessellata* are used to treat a number of inflammatory disorders, ear ache, fever, rheumatism, acute inflammation, nerve issues, bronchitis, indigestion, fever, impotence and barrenness, antidote for scorpion stings and as a laxative and liver tonic (Gutierrez, 2010; De *et al.*, 2015; Shengji and Zhiwei, 2018).

In conclusion, ethnobotany of the genus *Vanda* is a broad and complicated discipline that encompasses a wide range of medical usage of these plants by indigenous groups in Asia, Australia, and the Pacific Islands from time immemorial to treat variety of ailments.

2.3.2 Phytochemistry of *Vanda*

Genus *Vanda* is highly used by different ethnic communities as a medicine from ancient time. This genus is also used by different traditional school of medicine such as Unani, Ayurveda. Although being widely used genus, only very few research have been carried out for screening, separation, identification and structure determination of biologically active compounds.

Maridass *et al.,* (2008) carried out qualitative analysis for five phytochemicals in 61 orchid species and reported the presence of flavonoids, cyanogenic glycosides, terpenoids and tannin in *V. tessellata* and flavonoids, reducing sugar, terpenoids and tannin in *V. testacea.* Terpenoids, flavonoids, phenols, alkaloids, tannins, phalbotanin, saponins, steroids, anthocyanins, anthraquinones, cardiac glycosides, coumarins, flavonones, and glycosides all have been reported from *V. tessellata* (Arya *et al.,* 2012; Chowdhury *et al.,* 2014; Bhattacharjee *et al.,* 2015; Islam *et al*., 2016; Biswas and Sinha 2020). Total phenolic and flavonoid content of *V. tessellata* was also determined (Uddin *et al*., 2015a; Prakash *et al.,* 2018).

Nayak *et al.,* (2021) screened leaf, root and stem of *V. tessellata* for the detection of primary metabolite and reported the presence of carbohydrate, protein and lipid in leaf, carbohydrate, protein, lipid and amino acid in root and carbohydrate, protein, sucrose, starch and lipid in stem.

Chand *et al.,* (2016) investigated the presence of alkaloids, flavonoids, steroids, terpenoids, and tannins in *V. cristata* as well as they also quantify the amount of total phenolic and flavonoid content. Nag and Kumaria (2018) also quantified the amount of total phenolic and flavonoid content present in *V. coerulea.*

Ramana (2021) screened leaf of *V. tessellata* for the detection of bioactive compounds through GC-MS and detected Dimethyl sulfide, Acetic acid, anhydride and Propanoic acid, 2-oxo-, ethyl ester from methanolic extract and 2-Butynoic acid, Ethanol, Acetic

acid, methyl ester, dl-Malic disodium salt, [1,3,4] Oxadiazole, anhydride and Hydrogen azide from ethyl acetate extract.

Eucomic acid and its derivatives, phenanthrene derivatives, glucosides, anthocyanins and other phenolic chemicals, together with a few steroid derivatives are reported in *Vanda* species. Eucomic acid and its derivatives (vandaterosides I, II, and III) were discovered in the stems of *V. teres*, according to Simmler *et al.,* (2011). Additionally, the vandaterosides I and II from the aerial portions of *V. teres* were reported by Cakova *et al.,* (2015). There are several phenanthropyran derivatives that have been extracted and identified, including tessallatin from the entire plant of *V. tessellata* (Anuradha and Rao, 1998a), parviflorin from *V. parviflora* (Anuradha and Rao, 1998b), flavidin, and imbricatin from stems of *V. coerulea* (Simmer *et al.,* 2010). In a similar manner, the entire plants of *V. tessalata* were used to extract the phenanthropyrone derivative oxo-tessallatin (Anuradha *et al.,* 2008). Coelonin and methoxycoelonin, two phenanthrene derivatives, were also discovered in the stems of *V. coerulea* (Simmler *et al.,* 2010). Gigantol, a bibenzyl derivative, was discovered in the stems of *V. coerulea* (Simmler *et al.,* 2010). Tris [4-(β-D-glucopyranosyloxy) benzyl] citrate (parishin) and 4-β-D-glucopyr-anosyloxy) benzyl alcohol was identified from *V. parishii* (Dahmén and Leander, 1976). From the flowers of numerous *Vanda* hybrids, Tatsuzawa *et al.,* (2004), Junka *et al.,* (2011), and Junka *et al.,* (2012) have reported a variety of anthocyanins. In addition, two novel phenolic derivative 3, 4, 5-trihydroxybenzoicacid (gallic acid) and flavonoid derivative 2, 5-dimethoxy-6, 8-dihydroxy-Isoflavone have been characterized from the leaf of *V. tessellata* (Prakash and Bais, 2016). Other compounds such as Heptacosane, Octacosanol, 2, 7, 7-Trimethyl bicycle [2.2.1] heptanes were also characterized from *V. tessellata* (Subramoniam *et al.*, 2013).

It is plainly clear from the available scientific literature that *Vanda* is a source of active chemical compounds with a variety of biological activities. Overall, *Vanda* phytochemistry is a dynamic field of study, with the potential to produce valuable insights and possibly beneficial natural chemicals for disease prevention and therapy.

2.3.3 Biological activities of *Vanda*

Biological activity is stated as the occurrence of certain effects after exposure to a specific chemical; such as changes in metabolism or physiological responses (Karas *et*

al., 2017). There are numerous biological activities such as antioxidant, antimicrobial, anti-diabetic, anticancer, and so on, that can be examined both *in vivo* and *in vitro* (Mariod and Tahir, 2022).

2.3.3.1 Antioxidant activity

Radicals, ions, or molecules with unpaired electrons that can sustain their own existence are considered reactive oxygen species. They are generated as a byproduct of both enzymatic and non-enzymatic reactions that occur naturally as part of the human body's fundamental metabolic activities, but exposure to physical agents including X-rays, ozone, nicotine, air pollution and some industrial chemicals can also produce them. Nearly every molecule present in live cells, including DNA, membrane lipids, proteins and carbohydrates, is reactive with ROS because they are so reactive (Liou and Storz, 2010). Oxidative stress is caused by changes in the redox state and the depletion of antioxidants by exposure to oxidants. This leads to hundreds of illnesses, including asthma, diabetes, inflammation, cancer, cardiovascular disease, renal disorders, mental disorders depressive disorders, atherosclerosis, liver cirrhosis, immunosuppression, nephrotoxicity, etc. (Suzuki *et al.,* 1998; Bayr, 2005; Prakash *et, al.,* 2018; Kumar *et al.,* 2021).

Nearly all organisms have endogenous enzymatic and non-enzymatic antioxidant defense and repair systems, but under conditions of extreme stress, these systems are ineffective at preventing oxidative damage; as a result, exogenous antioxidant supplementation is advised to meet the body's adequate needs. Antioxidants are constituents which hinder the auto-oxidation of fats and oils by delivering hydrogen to free radicals formed in the course of autoxidation initiation and proliferation phases. Antioxidants act as free radical scavengers, reducing the harmful effects of free radicals on organisms (Taghvaei and Jafari, 2015, Simic, 1988). Chemicals, whether synthetic or natural, that prevent, reduce, or postpone the oxidative damage and protect human, animal, and plant cells from the destructive effects of free radicals such as reactive oxygen species (ROS), reactive nitrogen species are known as antioxidants (Karak, 2019).

A diverse spectrum of naturally occurring antioxidants have been found in medicinal plants, each with its unique components, chemical and physical characteristics, and mechanism of action (Bhatt *et al.,* 2013). Among them, phenolics and flavonoids have

been discovered to be strong antioxidants that consistently protect through scavenging a wide range of reactive oxygen species (Grabmann, 2005; Pirzadah *et al.*, 2017). Ascorbic acid, carotenoids, anthocyanins, vitamins, lignans, stilbenes, tannins, gallic acid, and other bioactive chemicals are also naturally occurring antioxidants (Kumar *et al*., 2021).

The genus *Vanda* has drawn special attention in the realm of medicinal plants because of its possible antioxidant action. The antioxidant capacity of *Vanda* orchids has been the subject of several investigations, with encouraging findings.

Two *in vitro* free radical scavenging techniques; DPPH and NO were used to test the antioxidant activity of a petroleum ether extract of *Vanda tessellata* Roxb by Vijaykumar (2013). The extract inhibited NO significantly 61.50±1.48% at 200µg/mL but had less DPPH free radical scavenging activity 52.07±0.58 at 400µg/mL. This finding imply that the extract may have antioxidant action.

Uddin *et al.,* (2015a) used three approaches to assess the antioxidant activity of four fractions of the chloroform extract from the plant *V. roxburghii*: ferric-reducing antioxidant power (FRAP), DPPH scavenging activity and hydroxyl free radical scavenging activity. They discovered that the petroleum ether fraction has the highest antioxidant activity with $_{IC50}$ 35.02 ± 1.56 (DPPH), 23.91 ± 1.58 (OH) and concluded that *V. roxburghii* possesses strong antioxidant activity, most likely due to its polyphenolic components, which have antioxidant qualities.

Chand *et al.,* (2016) evaluated antioxidant activity of selected wild orchid of Nepal using DPPH assay and reported 98.23µg/mL and 79.69µg/mL IC 50 of leaf and stem extract of *V. cristata.*

Subin and Williams (2018) assessed the antioxidant activity of *V. tessellata* ethanol extract using three methods: DPPH scavenging activity, hydroxyl radical scavenging activity, and nitric oxide radical-scavenging activity. They discovered that the extract has substantial radical scavenging activity with $_{IC50}$ 149.054µg/mL, 116.71µg/mL and 152.62µg/mL by using DPPH, OH and NO radical-scavenging method respectively. Soni *et al.,* (2018) also analyzed the antioxidant activity of epiphytic orchids and reported 80.65% DPPH inhibition by *V. tessellata*. Both research concluded that *V.*

tessellata has good antioxidant potential and might be utilized to treat a variety of diseases.

Similarly, DPPH radical scavenging activity and hydroxyl radical scavenging activity were used by Subin *et al.,* (2018) to examine the antioxidant activity of benzene, ethanol, acetone, and chloroform extracts of *V. roxburghii* leaves. They discovered that the ethanol extract performed best in both experiments; 27.28% ± 0.196 (25µL) to 61.91% ± 1.196 (100µL) in DPPH and m 18.12% ± 0.461 (100µL) to 58.5% ± 0.475 (500µL) in OH radical scavenging method, proving that it possesses potent antioxidant action.

Nag and Kumaria (2018) investigated the influence of chitosan on the growth, development and phytochemical production of *V. coerulea*. They discovered that chitosan at 4 mg/L in the B5 medium boosted the accumulation of secondary metabolites such as phenolics, alkaloids, and flavonoids while also improving the plant's antioxidant capacity. They found that chitosan may have activated metabolic pathways in *V. coerulea*, resulting in greater synthesis of these phytochemicals and an increase in the plant's overall therapeutic value.

Konduri *et al.,* (2022) conducted an *in vitro* evaluation of the antioxidant activity of petroleum ether, ethyl acetate, and methanolic extracts of *V. spathulata* flowers using a DPPH assay. They found that the methanolic extract had very strong antioxidant activity, with an $_{IC50}$ value of 38.39 µg/mL.

Overall, the available research reveals that *Vanda* orchids have substantial antioxidant activity, which may offer health advantages. More study is needed, however, to completely understand the processes behind this action and to pinpoint the precise health advantages of these plants.

2.3.3.2 Antimicrobial activity

The ability of a material to kill or hinder the development of bacteria is referred to as antibacterial activity. This is a significant feature for medications used to treat or prevent bacterial infections. Antibacterial activity may be found in a wide range of substances, including antibiotics and biologically active compounds. Antibiotics, the miracle medications of the twentieth century, serve an important role in the treatment

of bacterial illnesses, but its illogical and improper usage has led in the rise of resistant microbial diversity (Gupta and Birdi, 2017). The emergence of resistant bacteria is biological adaptation process in which application of an antimicrobial agent that suppresses susceptible organisms and chooses the resistant ones (Sibanda and Okoh, 2007). Antibiotic-resistant bacteria, often known as "superbugs," are becoming more widespread. These bacteria can cause infections that are difficult or impossible to cure with current medicines, resulting in multidrug-resistant illnesses. The challenge of bacterial resistance is increasing, and the future use of antibiotic agents remains questionable. This is why it is critical to keep researching for novel and efficient strategies to tackle these illnesses. Steps should be implemented to minimize this issue and restrict over use of antimicrobial drugs, conduct studies that clarify the genetic causes of resistance, and undertake investigations to develop novel antibiotics, both synthesized and natural.

Plants have always been a great source of herbal formulations for sustaining human wellbeing, particularly in the recent years more extensive investigations have been carried out for natural remedies. Medicinal plants have the potential to be a significant field of research in the development of innovative antibacterial medicines. Antimicrobial characteristics of medicinal plants have been employed in traditional medicine for generations, and some current research has revealed that several of them have the potential to be beneficial in the treatment of bacterial infections. These medicinal plants may contain active chemicals that restrict bacterial growth or kill them completely, making them interesting candidates for the creation of novel antibacterial medications. In contrast, plant-derived antimicrobial medications have no/less adverse effects and can be used to treat a variety of infectious diseases (Rahman *et al.,* 2013).

The use of plants for natural remedies is gaining popularity, and *Vanda* orchids have received special attention in this regard. A number *Vanda* species are being examined for their antibacterial potential such as *V. coerulea* (Priya *et al.,* 2011; Win *et al.*, 2020), *V.cristata* (Marasini and Joshi 2012), *V. spathulata* (Aparna *et al.,* 2021), *V. tessellata* (Gupta and Katewa, 2012; Behera *et al.,* 2013; Chaitanya *et al.,* 2013; Gupta and Katewa, 2014; Bhattacharjee *et al.*, 2015; Patra *et al.,* 2015; Ramana *et al.*, 2020a; Ramana *et al.*, 2020b; Ramana, 2021), *V. testacea* (Kaushik, 2019; Ramana *et al.*, 2020b) against various bacterial strains and reported various degree of antibacterial activity.

Vanda have been found to have broad-spectrum antimicrobial properties, making it a candidate for bio-prospecting for antibiotic and antifungal drugs. Studies have shown that different species of *Vanda* have varying range and level of antimicrobial activity against various strains of bacteria and fungi. Many *Vanda* species have demonstrated moderate to good antibacterial and antifungal activity including *V. coerulea* against bacteria: *Bacillus cereus, Streptococcus faecalis, S. pneumonia, Staphylococcus aureus, Escherichia coli, Salmonella typhi, Proteus vulgaris, Enterobacter aerogenes* (Priya *et al.*, 2011); *V. cristata* against bacteria: *S. aureus, Klebsiella pneumonia* and fungi: *Candida albicans, Mucor* sp. (Marasini and Joshi 2012); *V. tessellata* against bacteria: *S. aureus, B. subtilis, V. cholerae, E. coli, B. pneumonia, K. pneumonia, P. mirabilis* and fungi: *Penicillium* sp., *Rhizopus* sp., *Aspergillus niger, C. albicans* (Gupta and Katewa, 2012; Bhattacharjee *et al.*, 2015); *V. testacea* against bacteria: *B. subtilis, E. coli, K. pneumonia, S. typhi, S. aureus* (Kaushik, 2019).

The *Vanda* extract contain various chemical compounds, including tannins, phenols, alkaloids, flavonoids, terpenoids, glycosides, and steroids, (Arya *et al.*, 2012; Chowdhury *et al.*, 2014) which have been proven to have antibacterial capabilities, with flavonoids being particularly efficient against a wide spectrum of microbes. It is assumed that their efficacy arises from their ability to attach with proteins and bacterial cell walls, as well as potentially disrupt microbial membranes. Furthermore, the inclusion of saponins, alkaloids, and tannins in plant extracts increased their antibacterial efficacy against pathogenic microbes. Plant phenols and polyphenols are known to be harmful to microbes (Bhattacharjee *et al.*, 2015). Similarly, Gupta (2016) discovered that a novel compound isolated from *V. tessellata*, 3-ethoxy-10,17-dimethyltetradecahydro-1H-cyclopenta[a] phenanthren-17(2H)-one, had potent antimicrobial activity against *E. coli, P. mirabilis*, and *B. subtilis*. It also had moderate antifungal activity against *C. albicans* and *A. niger*.

In conclusion, it is crucial to conduct research on new approaches for combating bacterial infections due to the increasing threat of antibiotic resistance. The utilization of medicinal plants, like *Vanda* orchids, which have demonstrated potential as antibacterial medications, is one promising field of research. Although further research is required to properly explore these plant's potential as complementary medicine,

initial findings suggest that they could be an important tool in the fight against antibiotic-resistant bacteria.

2.3.3.3 Cytotoxic activity

Cancer is a condition in which the ability of one or more cells to die is lost, resulting in uncontrolled cell growth and the creation of either a solid mass of cells known as a tumor or a liquid cancer (i.e. blood or bone marrow-related cancer) (Nussbaumer *et al.*, 2011). Cell viability is defined as the amount of healthy cells in a sample, and cell proliferation is an important sign for understanding the mechanisms of action of certain genes, proteins, and pathways involved in cell survival or death following exposure to harmful chemicals (Adan *et al.*, 2016).

In 2020, 19.3 million new instances of cancer and nearly 10.0 million deaths from cancer were recorded globally, with a 20% lifetime chance of having cancer and a 10% lifetime risk of dying from cancer; one in every five people will develop cancer and one in every ten will die from the illness (Ferlay *et al.*, 2021). According to GLOBOCAN, there were 20,502 new cancer cases and 13,629 fatalities in 2020 in Nepal.

The treatment choices that are currently accessible have seen tremendous improvement over the past few decades, and a significant number of effective chemotherapeutic anticancer drugs have been found and effectively applied in clinical settings. Drug screening usually employs cell cytotoxic tests to determine if the test compounds exhibit direct cytotoxic effects or have an impact on cell growth.

There is a long history of using plant-based remedies to cure cancer. Many anticancer medications that are now being used in clinical trials were developed as a consequence of plant-based drug discovery. Vinca alkaloids like vinblastine and vincristine were extracted from *Catharanthus roseus* began a new era of plant materials being used as anti-cancer treatments. Paclitaxel was found in *Taxus brevifolia* is one more example of how successful natural product medication discovery has been (Shoeb, 2006). Number of orchid species have been examined for their cytotoxic potential. A lot of the compounds have been isolated from the orchids that are found to have significant anti-cancer activity, for instance, Moscatilin, Denbinobin, Erianin, Dendrochrysanene, Fimbriatone, Cirrohopetalanthrin have been isolated from different orchids (Ma *et al.*, 1998; Heo *et al.*, 2007; Peng *et al.*, 2007; Wu *et al.*, 2006; Chen *et al.*, 2007).

Most of the *Dendrobium* species were studied for cytotoxic activity and very low to strong cytotoxic activity results were reported against various cell lines such as *Dendrobium moniliforme* against human cervical carcinoma (HeLa) and human glioblastoma (U251) cell lines (Paudel *et al.,* 2018); *D. crepidatum* against HeLa and U251 cell lines (Paudel *et al.,* 2019); *D. amoenum* against HeLa and U251 cell lines (Paudel and Pant, 2017); *D. longicornu* against HeLa and U251 cell lines (Paudel *et al.,* 2017); *D. loddigesii* against HT, JEG-3, JAR, BeWo, HEp3B, HA22T, HEpG2, HCC36, NCI-Hut 125, CH27 LC-1, H2981, Calu-1, AZ521, NUGC-3, KATO-III and AGS cell lines (Ho and Chen, 2003).

Whereas limited research were carried out on *Vanda* spp. *Vanda spathulata* was tested against MCF 7 cell line (Rani and Nandagopalan, 2022); *V. tessellata* against Human hepatoma (Hep G2) and Murine skin melanoma (B16-F10) cell lines, Brine shrimp (*Artemia salina*) (Chowdhury *et al.,* 2014; Uddin *et al.,* 2015b; Ramana *et al.,* 2020a; Nayak *et al.,* 2021); *V. tetacea* (Ramana *et al.,* 2020b); *V. bensonii* against NCI-H460 cell line (Jimoh *et al.,* 2022); *Vanda cristata* against HeLa and U251 cell lines (Joshi *et al.,* 2020).

Most of the findings on *V. tessellata* and *V. testacea* suggest very low cytotoxicity or inactivity against various cell lines as well as in brine shrimp lethality tests. Ramana *et al.,* (2020a) and Ramana *et al.,* (2020b) reported inactive cytotoxic result of *V. tessellata* and *V. testacea* repectively. Chowdhury *et al.,* (2014) and Uddin *et al.,* (2015b) also reported very low cytotoxicity against brine shrimp (*Artemia salina*) in *V. tessellata*. Whereas, Nayak *et al.,* (2021) found very low cytotoxicity of methanol extract of *V. tessellata.*

In contrary, some research reported cytotoxic activity of various species of *Vanda.* Nayak *et al.,* (2021) discovered very strong activity of ethanol and acetone extract of *V. tessellata* against brine shrimp. Joshi *et al.,* (2020) also found significant cytotoxic activity of *V. cristata* against HeLa and U251 cell line. Jimoh *et al.,* (2022) reported promising results in *V. bensonii* against NCI-H460 cell line. Rani and Nandagopalan, (2022) also reported significant cytotoxic activity of *V. spathulata* against MCF 7 cell line.

Some of the *Vanda* species were found to have no any cytotoxic activity against normal cell. Cauchard *et al.,* (2010) reported normal human fibroblasts (NHF) cell proliferation by *V.coerulea*, Simmler *et al.,* (2010) discovered that *V.coerulea* did not have any cytotoxic effects on human normal epidermal keratinocytes (HaCaT). Similarly, Simmler *et al.,* (2011) observed that *V.teres* bioactive compounds did not exhibit any cytotoxicity towards the human immortalized keratinocyte cell line (HaCaT).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Research design

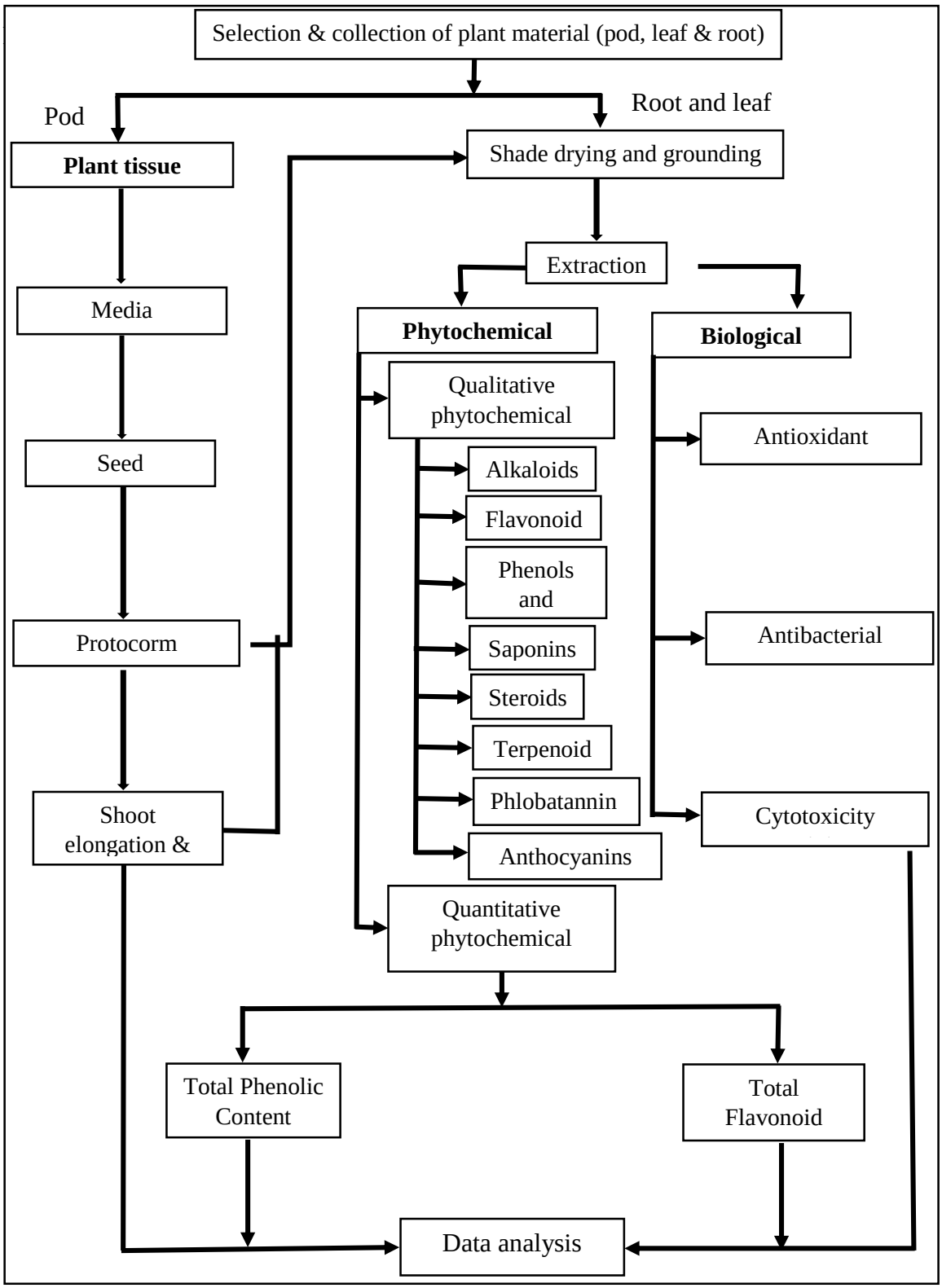


Figure 3. 1: Flow chart of research design.

3.2 *In vitro* propagation

3.2.1 Collection and identification of plant materials

Plant material collection permission was taken from Department of Forest, Babarmahal, Kathmandu before the plant collection. Root, leaf and mature pods/capsules of *Vanda tessellata* and *V. testacea* were collected from Ghodaghodi municipality-05, Kailali, Nepal without causing complete damage to the orchid plant. *V. tessellata* sample was collected from the host plant *Syzygium cumini* (L.) Skeels whereas *V. testacea* sample was collected from the host plant *Schleichera oleosa* (Lour.) for *in vitro* propagation. Root, leaf and pods were kept in zipper lock plastic bag, tagged. Root and leaf were shade dried and pods were stored in refrigerator at 4ºC before inoculation. Plants were identified by using different relevant literatures and herbarium of TUCH and crossed checked with the expert.

3.2.2 Sterilization techniques

The sterilization process was achieved with six actions: dry sterilization (hot air oven), stem sterilization (autoclave), chemical sterilization (disinfectants and alcohols), filter sterilization (HEPA filter), radiation sterilization (UV light) and flame sterilization.

Dry sterilization: Glassware and metallic instruments were sterilized by using hot air oven at 160°C for ½ hour.

Stem sterilization: Nutrient medium, distilled water and tissue culture instrument such as forceps, petri plates were sterilized by autoclaving at 121°C with pressure 15 psi for 15 minutes.

Chemical sterilization: Chemicals such as tween twenty, sodium hypochlorite/ mercuric chloride and 70% ethanol were used for surface sterilization of explant. 70% ethanol were also used for sterilizing the hands and working surface.

Filter and radiation sterilization: Aseptic condition inside the laminar air flow cabinet was achieved through HEPA filter and UV light.

Flame sterilization: This method was used for sterilization of instrument that were continuously used during tissue culture inside the air flow cabinet.

3.2.3 Preparation of MS medium

The MS medium of Murashige and Skoog (1962) is very widely used in different culture system. So, this medium was selected for the experiment. This medium was

prepared using standard stock solutions of macronutrients, micronutrients, iron and vitamins. The p^H was maintained at 5.7±0.1 by adjusting with 0.1N NaOH or 0.1N HCl. sucrose (3%), agar (0.8%) and myoinositol (0.01%) was added to the medium. MS medium with different plant growth regulators (PGRs) such as BAP, Kn, GA_3, NAA individually, and in combination were used according to need. Coconut water (CW: 10 % v/v) was also incorporated in the medium according to need. The medium was sterilized at 121ºC, 15 psi for 15 min in an autoclave. The required apparatus such as beaker, forceps, scissors, razor blade, and petri-plates were also autoclaved.

3.2.4 Surface sterilization of plant materials

The collected capsules were thoroughly washed under running tap water to remove soil and unwanted particles. Then, capsules were dipped in tween-20 for 15-20minutes and then washed in running tap water until all the tween-20 washed off. The capsules were transferred to laminar air flow; where they were surface sterilized with 1% sodium hypochlorite or 0.1M mercuric chloride solution for 15 minutes followed by rinsing with sterile distilled water. Then, the capsules were dipped in 70% ethanol for 2 minutes and flamed slightly.

3.2.5 Inoculation of seeds

The surface sterilized capsules of *V. tessellata* and *V.testacea* were put on sterile petridish containing sterile filter paper for soaking the surface moisture of orchid capsules. Then each capsule was cut longitudinally with a sterile surgical blade and the seeds were scooped out and inoculated on the surface of full, half and quarter strength of the MS medium supplemented with or without different hormones and coconut water.

The entire experiment was performed in minimum 4 replicates in aseptic condition under laminar air flow cabinet. All the cultures were maintained at 25±2ºC temperature, 60-70% relative humidity and continuous dark. After 2-3 weeks the cultures were transferred to light conditions, 16/8 hours (light/dark) photoperiods using white fluorescent tubes. The observation on the initiation of seed germination and different protocorm development stages were observed and the data was documented.

After protocorm formation, around half of the well grown protcorms were harvested and shade dried for phytochemical extraction and remaining protocorms were further sub-cultured for shoot induction.

3.2.6 Shoot Induction

The protocorms were sub-cultured on full MS, half MS and quarter MS media supplemented with or without different PGRs and coconut water (CW). The basal medium was supplemented with various types and concentrations of PGRs such as BAP, NAA, Kn and GA_3 individually, and in combination were used. Two sets of media were prepared one set was only treated with PGRs and another set was treated PGRs along with coconut water (CW: 10 % v/v). The entire experiment was performed in minimum 4 replicates in aseptic condition under laminar air flow cabinet. The culture condition was maintained at 25±2ºC temperature, 60-70% relative humidity and 16/8 hours (light/dark) photoperiods using white fluorescent tubes. Growth and development of protocorm to seedling was measured in month basis. Well grown plantlets were harvested and shade dried for phytochemical extraction.

3.3 Phytochemical and biological assay

3.3.1 Preparation of plant materials

Wild and *in vitro* raised plant materials were cleaned to remove unwanted materials and cut into small pieces for rapid drying. The wild plant samples as well as *in vitro* raised samples were shade dried until constant weight and finely ground using electric grinder. All the plant material were tagged with specific letters such as VTL (for *V. tessellata* leaf), VTR (for *V. tessellata* root), VTP (for *V. tessellata* protocorm), VTW (for *V. tessellata in vitro* raised plantlets), VTL2 (for *V. testacea* leaf), VTR2 (for *V. testacea* root), VTP2 (for *V. testacea* protocorm), and VTW2 (for *V. testacea in vitro* raised plantlets). The ground powder was stored in air tight container.

3.3.2 Extraction

Simple maceration method was used for the extraction of phytochemicals. In this method the powder was dissolved in 90% methanol 1:10 (w/v) for 48 hour and filtered twice through filter paper. The filtrate was concentrated and dried under the reduced pressure in rotatory evaporator by evaporating the solvent. Extracts were stored in refrigerator at 4°C for further use.

3.3.3 Phytochemical assay

3.3.3.1 Qualitative phytochemical assay

The methanolic extract of plant samples were subjected to preliminary phytochemical screening to detect the presence of major phytochemicals constituents following Savithramma *et al.,* (2011) and Shaikh and Patil (2020).

3.3.3.1.1 Test for alkaloids

The crude extract was dissolved in methanol and then mixed with few drops of Wagner's reagent. Appearance of reddish brown precipitate indicated the presence of alkaloids.

3.3.3.1.2 Test for flavonoids

Alkaline reagent test was done in which small amount of crude extract was mixed with 2mL of 2% NaOH which resulted in formation of intense yellow color that becomes colorless on addition of few drops of dilute HCl. Hence it indicated the presence of flavonoids.

3.3.3.1.3 Test for phenols and tannins

The crude extract was mixed with about 2mL of ferric chloride ($FeCl_3$) solution. An appearance of dark blue-green or black color indicated the presence of phenols and tannins.

3.3.3.1.4 Test for saponins

The crude extract was mixed with about 5mL of distilled water and it was shaken vigorously. Formation of stable foam indicated the presence of saponins.

3.3.3.1.5 Test for steroids

2mL of chloroform ($CHCl_3$) was added to the crude extract followed by careful addition of 2mL conc. H_2SO_4 and it was shaken gently. An appearance of reddish brown color ring indicated the presence of steroids.

3.3.3.1.6 Test for terpenoids

The crude extract was treated with few drops of copper acetate (prepared by dissolving copper acetate monohydrate in warm water until a solution forms blue color) solution. The formation of emerald green color indicated the presence of terpenoids.

3.3.3.1.7 Test for phlobatannins

2mL crude extract was treated with equal volume of 1% HCl and boiled. Appearance of red precipitate indicates the presence of phlobatannins.

3.3.3.1.8 Test for anthocyanins

2mL plant extract was treated with equal volume of 2N HCl and few drops of ammonia was added. Appearence of pink-red solution which turns blue-violet after addition of ammonia indicates presence of anthocyanins.

3.3.4.2 Quantitative phytochemical assay

3.3.4.2.1 Total phenolic content

Total phenolic content in methanolic extract of wild and *in vitro V. tessellata* and *V. testacea* was determined using Folin-Ciocalteau (F-C reagent) phenol reagent colorimetric method by following Zhang *et al.*, (2006) protocol with slight modification. For this, in each well of the 96 well plate, 75 µL of distilled water was added, followed by 25 µL of either sample(1mg/mL) or standard (25-200mg/mL) and 25 µL of F–C reagent (diluted 1: 1 (v/v) with distilled water). The blank or control was prepared by replacing the same volume of plant sample by absolute methanol. Then all the reaction mixture was mixed by repeated pipetting. After the solution was mixed, left for 6 min and then 100 µL of 1M Na_2CO_3 was added to each well. The solutions were mixed again and the plates were covered and left in the dark for 90 min. The absorbance at 765 nm was measured with a micro-plate reader (Azure biosystems Micro-plate Spectrophotometer). Each standard and sample solution was taken in triplicate. Gallic acid was used as a standard at 25, 50 100 and 200 µg/mL to obtain a calibration curve. Total phenolic content was expressed in terms of milligram of Gallic acid equivalent per gram of plant extract (mg GAE/g).

3.3.4.2.2 Total flavonoid content

Total flavonoid content in methanolic extract of wild and *in vitro V. tessellata* and *V. testacea* was determined using Aluminium Chloride ($AlCl_3$) colorimetric method by following Chang *et al.,* (2002) protocol with slight modification. At first 1 mg/mL of plant extract solution were prepared then from 1 mg/mL stocks 25µL of the plant extract was taken 96 well plate followed by 75µl of 10% $AlCl_3$ and 5µL (1M) potassium acetate. Then the solutions were diluted by adding 140 µL distill water and incubated at room temperature for 30 minutes. The blank was prepared by replacing same volume of absolute methanol. The absorbance at 415 nm was measured with a micro-plate reader (Azure biosystems Micro-plate Spectrophotometer). The calibration curve was obtained by using Quercetin as a standard at 25, 50 100 and 200 µg/mL. The total flavonoid content was expressed in terms of milligram of Quercetin equivalent per gram of plant extract (mg QE/g).

3.3.5 Biological assay

3.3.5.1 Antioxidant activity

To check the antioxidant activity through free radical scavenging potential by the test extract, the change in optical density of DPPH radicals was monitored. The antioxidant activity of crude extract of wild and *in vitro V. tessellata* and *V. testacea* was determined by following Mansour *et al.,* (2016) protocol with slight modification. The stock solution of each extract and ascorbic acid (Standard or positive control) was prepared (1 mg/mL) using methanol. Then, a serial dilution was carried out to obtain a solution at concentrations of 200, 100, 50 and 25µg/mL. A fresh DPPH solution of 0.2mM was prepared by dissolving 7.88mg of DPPH powder (molecular weight 394.32 gm/mol) in 100mL of methanol and kept away from direct light. Then, a volume of 50 μL of each sample (25–200 µg/mL) was mixed with 50 μL of DPPH in a 96-well plate in triplicate. The blank or control was prepared by replacing the same volume of plant sample by absolute methanol. After 30 minutes of incubation at room temperature the absorbance was measured at 517 nm using a micro-plate reader (Azure biosystems Micro-plate Spectrophotometer). A lower absorbance value indicates higher antioxidant activity of the sample.

The free radical scavenging activity of the plant samples were calculated in percentage using the following formula;

$$\%\text{Radical Scavenging activity (RSA)} = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} \times 100\%$$

Standard graph was plotted taking concentration on X-axis and percentage radical scavenging activity on Y-axis based on the standard curve. The IC_{50} value was also calculated by using linear equation of the curve obtained;

$$Y = mX + C$$

$$IC_{50} = (50 - C)/m$$

Where X=concentration, Y=%RSA, m and C are the coefficient and constant of the linear equation respectively.

3.3.5.2 Antimicrobial activity

In this assay only antibacterial activity was performed.

3.3.5.2.1 Bacterial strains

Five ATCC strains were selected for antimicrobial assay which were provided by National Public Health Laboratory (NPHL), Teku, Kathmandu, Nepal.

Gram positive bacterial Strains: *Staphylococcus aureus* (ATCC-25923) and *Enterococcus faecalis* (ATCC-29213)

Gram negative bacterial Strains: *Escherichia coli* (ATCC-25923), *Acinetobactor baumanii* (ATCC-19606) and *Shigella sonnei* (ATCC-25931)

3.3.5.2.2 Media preparation

Nutrient Broth (NB)

Nutrient Broth is a general-purpose medium used for cultivation of broad variety of fastidious and non-fastidious microorganisms with non-exacting nutritional requirement. For the preparation of NB media, 6.5 gram of NB powder (HI-media laboratories Pvt. Ltd, Mumbai) was mixed with distill water to make a final volume of 500 mL. The media was then sterilized by autoclaving at 15lbs pressure and 121°C for

15 minutes which was then cooled under laminar airflow hood. The prepared media was used for suspension type of bacterial culture and kept in aseptic condition for further use.

Soya Nutrient Agar (SNA) media

Soya Nutrient Agar (SNA) media was prepared by dissolving 30 gram of SNA powder (HI-media laboratories Pvt. Ltd, Mumbai) with distill water to make final volume of 1 liter. The media was then sterilized by autoclaving at 15 lbs. pressure and 121°C for 15 minutes. Then it was allowed to cool for about 1 hour in laminar airflow hood. The media was then transferred to the sterilized petri plates by pouring about 20 mL of media on each plates of 9 cm diameter. Then the media was allowed to cool and placed in aseptic condition for further use.

3.3.5.2.3 Revival of bacterial strains

All the bacterial strains were streaked on Soya Nutrient Agar (SNA) plates and incubated at 37°C for 24 hour to revive the bacteria as well as to obtain pure strain.

3.3.5.2.4 Standard culture inoculum preparation

For inoculum preparation, different strains of pure bacterial strain were revived by transferring aseptically to Nutrient broth for suspension culture and was kept overnight in a shaking incubator at 37ºC of temperature for its growth. This culture was then just touched in a test tube containing Nutrient broth with the help of cotton ear bud and the turbidity of bacterial suspension was adjusted at the 0.5 McFarland standards for the antibacterial test.

3.3.5.2.5 Antibacterial activity

Antimicrobial activity was determined by well diffusion method on the basis of Athanassiadis *et al.*, (2009) with slight modification. Each bacterial strains from the 0.5 McFarland bacterial inoculum were streaked on Soya Nutrient Agar (SNA) plates with a sterile cotton swab. Five wells with a diameter of 6 mm were prepared. 20 µL of different concentration of plant extract (25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/m), DMSO (the negative control) was added in each well. At the center standard antibiotics gentamycin/ (the positive control). The plates were incubated at 37°C for 24

h. After incubation, the clear zone of inhibition (diameter) around the point of application of each sample solution was measured in millimeter (mm).

3.3.5.3 Cytotoxic activity

3.3.5.3.1 Cancer cell line

In the present study, two human cancer cell line (HeLa and MCF7) and one normal cell line were used to assess *in vitro* cytotoxicity assay. All the experiments have been done in Annapurna Research Center, Maitighar, Kathmandu, Nepal.

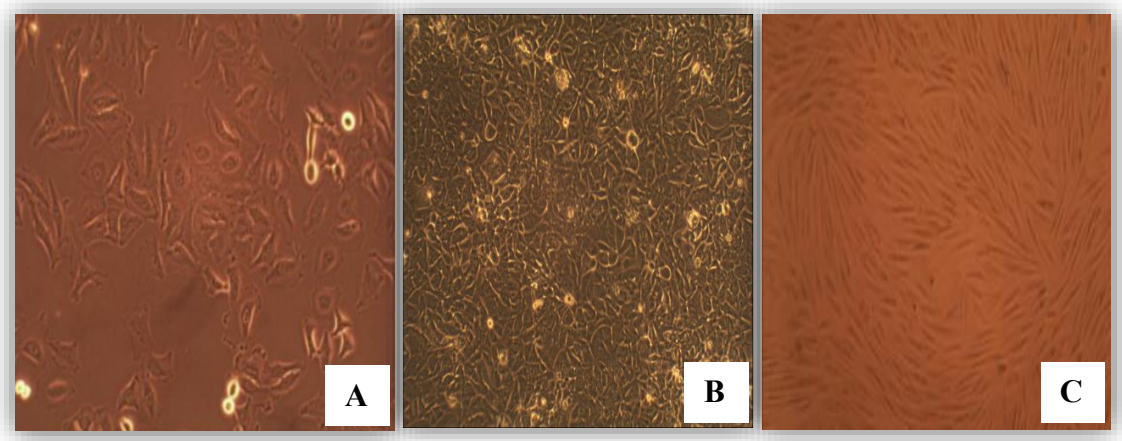


Figure 3. 2: Human cell lines. (A): HeLa cell line; (B): MCF7 cell line & (C):Normal cell line.

3.3.5.3.2 Cell culture

All cell line were cultured in EMEM medium supplemented with 10% FBS and 1% antibiotics and 1% L-Glutamine maintained at 37°C with 5% CO_2 in a CO_2 incubator.

3.3.5.3.3 Resuscitation of cell

Frozen cells were thawed and added to a 5 mL (EMEM) medium. The cell suspension was centrifuged at 2500 rpm for 3 minutes. The supernatant was removed and cells were suspended in a medium. The cell suspension is then transferred into T-flask (25 cm^2) and incubated at 37°C with 5% CO_2 in a CO_2 incubator.

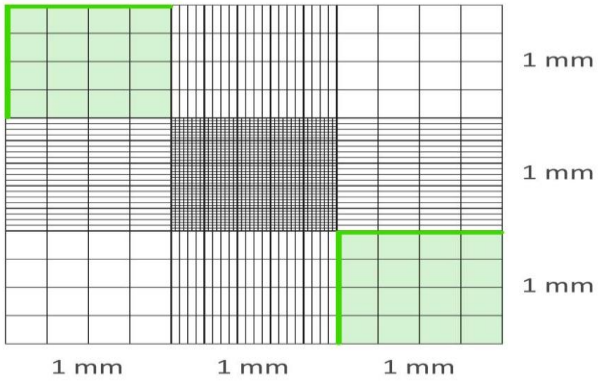
3.3.5.3.4 Subculture of cells

Once cells were attached and more than 80 % confluence was reached, the cells were sub-cultured. Briefly, the medium was removed and the cell layer is rinsed with PBS to remove the traces of medium. 2 mL trypsin is added to the flask and then incubated 5 to 10 min for the detachment of the cells. Then detached cells were centrifuged and

pellet formed is suspended in the fresh medium and cell suspension was transferred to another T flasks. The flasks were incubated at 37°C with 5% CO_2. The medium was changed when necessary, in 2 to 3 days.

3.3.5.3.5 Cell counting

Cells were counted using Haemocytometer. For cell counting the medium was removed from the flask and the cells were washed with PBS. The cells were trypsinized with a 2 mL Trypsin-EDTA solution for detachment. The cell suspension was placed in a falcon tube and centrifuged and the supernatant was removed and 20 µL cell suspension was applied in the counting chamber. The cells of each of the four corners and Central Square were counted. The cells per milliliter were calculated using the formula below.



$$\textbf{Cells/mL} = \frac{\text{Total no of counted cells in 5 squares}}{5} \times 10^4 \times \text{Dilution factor (D.f.)}$$

3.3.5.3.6 MTT assay

Cytotoxic activity of extracts were evaluated in 96-well plates by using the standard MTT (3- [4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay with slight modification. For this purpose, HeLa (Cervicle cancer cell line), MCF7 (Breast cancer cell line) and normal cell line were cultured in Minimum Essential Medium Eagle (EMEM), supplemented with 10% of fetal bovine serum (FBS), 1% of penicillin/streptomycin as antibiotic and 1% L-Glutamine, in T flasks, and kept in 5% CO_2 incubator at 37°C in CO_2 incubator (Mossman, 1983). The cells were seeded in 96 well plate ($1x10^4$ cells/well) 100µL of medium and incubated 5% CO_2 incubator at 37°C for 24 hours. Following the attachment and cell confluence, the cells was treated with different concentrations (50, 100, 200, 400µg/mL) of the plant extracts for 48 hours. The assay was performed in triplicates. Following 48-hour

incubation supernatant was removed and 150 µL of medium with 50 µL of MTT will be added to each of the well. Following 4-hour incubation a purple formazan product was produced. DMSO (0.1%) was added (100 µL) followed by incubation for another 15 minutes at room temperature to dissolve formazan. The absorbance was measured with a micro plate reader (Azure biosystems micro-plate spectrophotometer) at 595nm. The percentage of the cytotoxic activity and cell proliferation was calculated.

$$\% \text{ cytotoxic activity} = \frac{Abs1-Abs2}{Abs1} X100$$

$$\% \text{ cell proliferative activity} = \frac{Abs2-Abs1}{Abs1} X100$$

Where,

Abs1 is the absorbance of control

Abs2 is the absorbance of treated sample.

After calculating the percentage cytotoxic and percentage cell proliferative activity, IC_{50} and AC_{50} were calculated. Both IC_{50} and AC_{50} were calculated using a linear regression analysis of dose-response curves.

Here, IC_{50} (Half-Maximal Inhibitory Concentration) is a measure of the effectiveness of an extract in inhibiting the growth of HeLa and MCF7cells, typically used to measure the potency of extracts. It is the concentration of a substance that is required to inhibit the growth of 50% of the cells in a culture. The lower the IC_{50} value, the more potent the extract.

Similarly, AC_{50} (Half-Maximal Activating Concentration) is a similar measure, but it is used to measure the effectiveness of an extract in activating a cell proliferation. The AC_{50} is the concentration of an extract that is required to activate the cell proliferation in 50% of the normal cells.

3.4 Data analysis

All the data were analyzed by using Microsoft excel 2007 and SPSS 25 software. The experiments were carried out in triplicates and values were presented in the form of Mean±SE and the data was subject to analysis of variance (ANOVA) followed by Tukey test.

CHAPTER FOUR: RESULTS

4.1 Plant tissue culture

4.1.1 *In vitro* seed germination of *V. tessellata*

Seeds from eight-month-old capsules were used as explants for *in vitro* seed germination of *V. tessellata*. The study measured four different parameters: initiation of germination, protocorm formation, shoot initiation, and germination percentage as shown in Figure 4.1.

The media composition MS fortified with10% CW (MSCW) had the most favorable results overall. The initiation of germination occurred in the shortest amount of time at 3.25±0.25 weeks, followed by MS, HMS fortified with10% CW (HMSCW) and QMS fortified with10%CW (QMSCW) all at 4.25 weeks. Protocorm formation was likewise quickest with MSCW at 5.25±0.25 weeks, followed by MS, HMSCW and QMSCW all at 6.25 weeks. Shoot initiation was fastest with MSCW at 7.5±0.29 weeks, followed by MS, MS with 0.5mg/L BAP+0.5 mg/L NAA, which had the same time requirement at 7.75 weeks. Furthermore, MSCW had germination percentage 85±2.04%, followed by MS and HMSCW with germination percentage 73.75±2.40% and 72±2.38% respectively.

The media composition QMS with 0.5 mg/L BAP and 0.5 mg/L NAA was the weak among all the media compositions used in the experiment. It took the longest time for initiation of germination (6±0.41 weeks), protocorm formation (7.75±0.25 weeks) and shoot initiation (10.25±0.48 weeks). HMS with 0.5 mg/L BAP and 0.5 mg/L NAA had the second-worst results for these stages of germination. Both QMS with 0.5 mg/L BAP and 0.5 mg/L NAA and HMS with 0.5 mg/L BAP and 0.5 mg/L NAA had the lowest germination percentage (58.75%) among all the media compositions, followed by MS with 0.5 mg/L BAP and 0.5 mg/L NAA and QMS with 61.25±3.15% and 63.75±2.40% respectively.

Full strength MS media was better than half and quarter strength MS for the seed germination of *V. tessellata*. Similarly, basal media shows better response than plant growth hormone supplemented media.

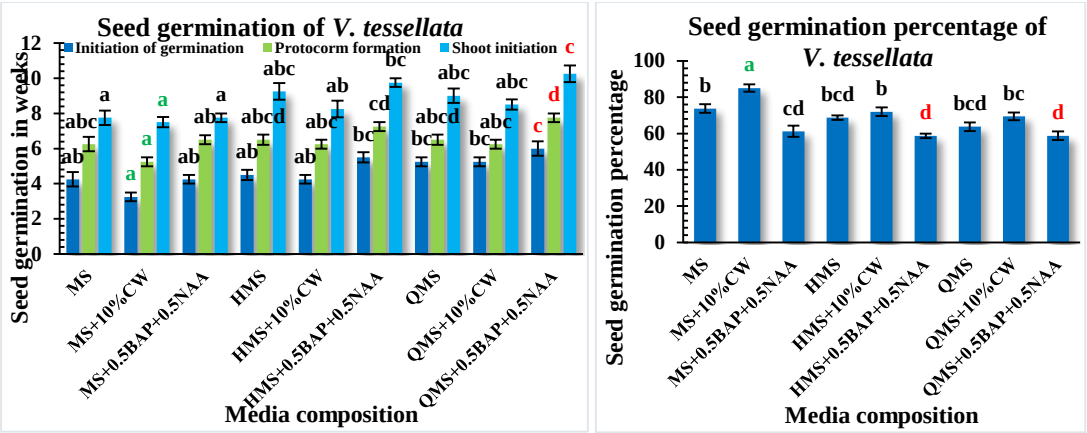


Figure 4. 1: Different stages of *in vitro* seed germination and germination percentage of *V. tessellata*. (Data are presented in the mean of four replicates ± standard error (SE). In each bar, the same letters denoted are not significantly different at $p<0.05$.)

Overall, the media composition MSCW had the best values for all four parameters measured, indicating that it may be the most effective for *in vitro* seed germination of *V. tessellata.*

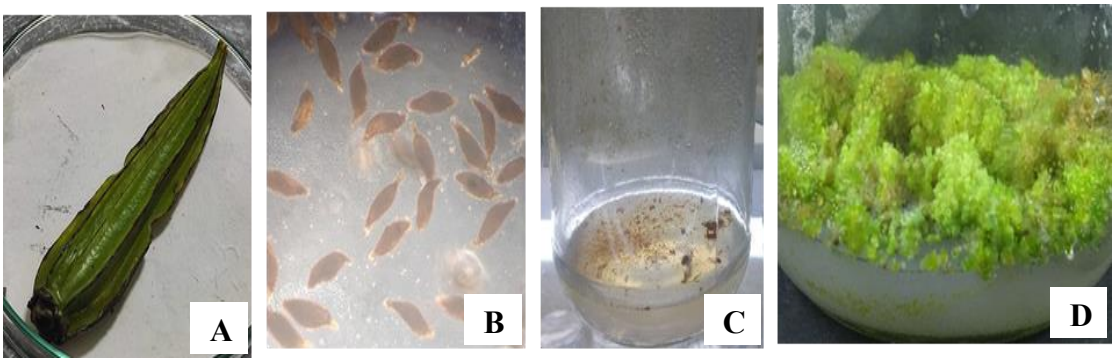


Figure 4. 2: Different stages of *in vitro* seed germination of *V. tessellata*. (A): Mature seed pod; (B): Seed under microscope; (C): Inoculated seed in culture jar; (D): Germinated seed in MSCW.

4.1.2 *In vitro* seed germination of *V. testacea*

Similar experiments and parameters were used for seed germination of *V. testacea* as stated above and is shown in Figure 4.3.

The media composition QMSCW had the most favorable results overall. The initiation of germination occurred in the shortest amount of time at 3.75±0.25 weeks, followed by QMS and HMSCW both at 5±0.41 weeks. Protocorm formation was likewise quickest with QMSCW at 5.25±0.48 weeks, followed by QMS and HMSCW both at

6.25±0.48 weeks. Shoot initiation was fastest with MSCW at 8.25±0.48 weeks, followed by MS with1 mg/L BAP and 0.5 mg/L NAA at 11±0.41 weeks. Furthermore, the QMSCW had germination percentage 86±2.39%, followed by HMSCW with germination percentage 66.25±2.39%.

The media composition MS with 1mg/L BAP and 0.5mg/L NAA was the worst among all the media compositions used in the experiment for the shoot initiation, protocorm formation and germination percentage. It took the longest time for initiation of germination (7.5±0.29 weeks), protocorm formation (10±0.41 weeks). Whereas, QMS was the most time consuming media composition for shoot initiation (13.25±0.48 weeks). HMS with 1mg/L BAP and 0.5 mg/L NAA had the second-slow results for the shoot initiation, protocorm formation and germination percentage. MS with 1mg/L BAP and 0.5mg/L NAA had the lowest germination percentage (23.75±2.39) among all the media compositions, followed by MS with 1 mg/L BAP and 0.5 mg/L NAA (26.25±2.39%).

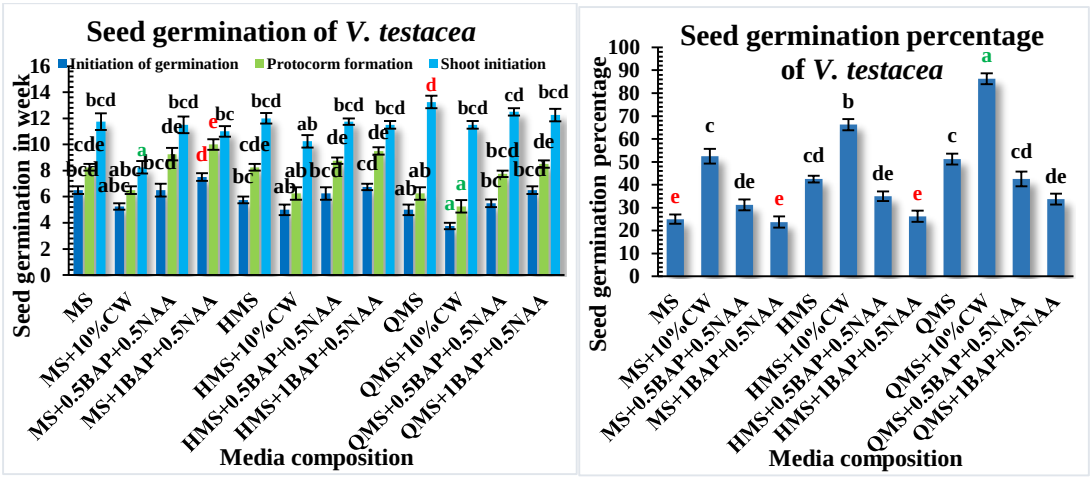


Figure 4. 3: Different stages of *in vitro* seed germination and germination percentage of *V. testacea*. (Data are presented in the mean of four replicates ± standard error (SE). In each bar, the same letters denoted are not significantly different at $p<0.05$.)

Quarter strength MS media was better than full and half strength MS for the seed germination of *V. testacea*. Similarly, basal media showed better response than plant growth hormone supplemented media. Overall, the media composition QMSCW had the best results for initiation of germination, protocorm formation and germination percentage whereas, shoot initiation was best in MS with 1 mg/L BAP and 0.5 mg/L

NAA. This result indicate that QMSCW may be the most effective for *in vitro* seed germination of *V. testacea.*

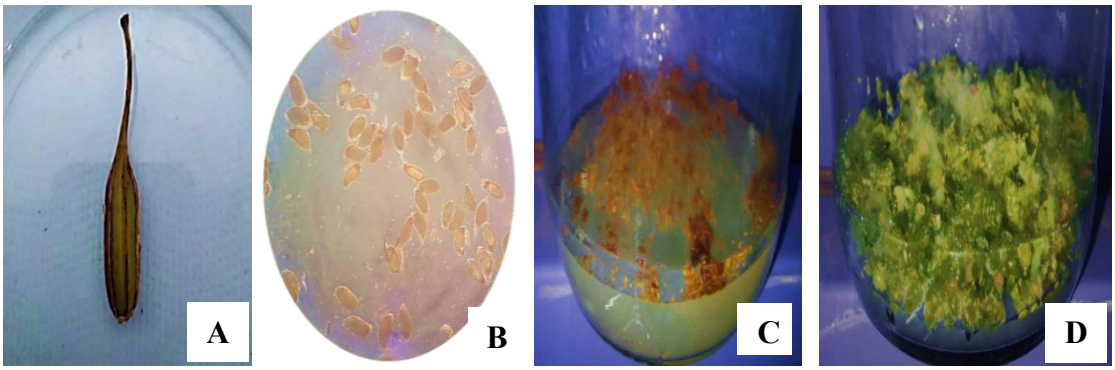


Figure 4. 4: Different stages of *in vitro* seed germination of *V. testacea*. (A): Mature seed pod; (B): Seed under microscope; (C): Inoculated seed in culture jar; (D): Germinated seed in MSCW.

4.1.3 Shoot elongation and proliferation of *V. tessellata*

Different media compositions were used for the shoot elongation and proliferation of *V. tessellata,* but the efficiency of HMS and QMS media was found to be inferior compared to FMS and the use of Kn, and GA_3 was also not as effective as BAP, thus these combinations were relegated to the appendix-I. So, the media compositions for body part only include MS, MSCW, and various combinations of MSCW with different concentrations of plant growth regulators BAP and NAA. The results are presented in terms of the mean shoot number, shoot length, root number, and root length, as well as the standard error and the letters indicating the level of statistical significance at $p<0.05$ as shown in Figure 4.5 and 4.6.

The results show that the media composition had a significant effect on the shoot elongation and proliferation of *V. tessellata*. The highest mean shoot number (10.5±0.65) was observed in the MSCW with 1.5 mg/L BAP and 0.5 mg/L NAA treatment and shoot length (2.98±0.18) was observed in MSCW with 1 mg/L BAP and 0.5 mg/L NAA treatment, while the lowest mean shoot number (4.5±0.65) and shoot length (0.48±0.09) were observed in the MSCW with 3 mg/L BAP treatment. The addition of BAP and NAA to the MSCW media generally increased the shoot number and length.

The root number and length were also affected by the media composition, but to a lesser extent as compared to the shoot number and length. The highest mean root number

(3.25±0.49) and root length (1.55±0.28) were observed in the MSCW with 1 mg/L BAP and 0.5 mg/L NAA treatment, while the lowest root elongation and proliferation was observed in the MSCW with 0.5 mg/L BAP treatment.

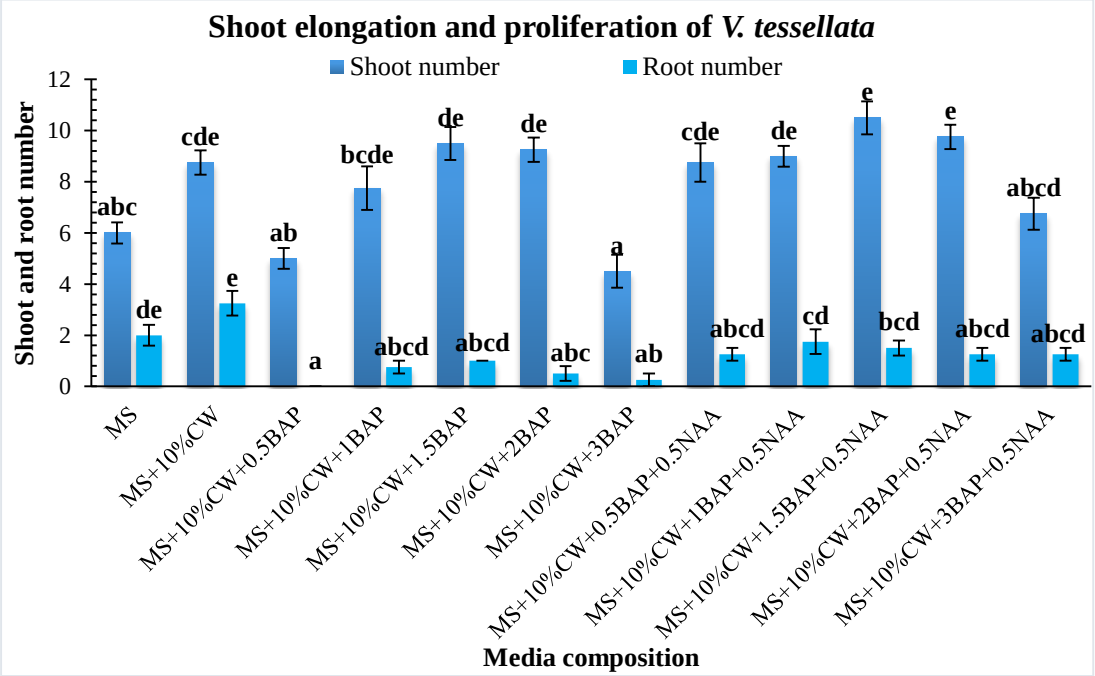


Figure 4. 5: Shoot and root number of *V. tessellata.*

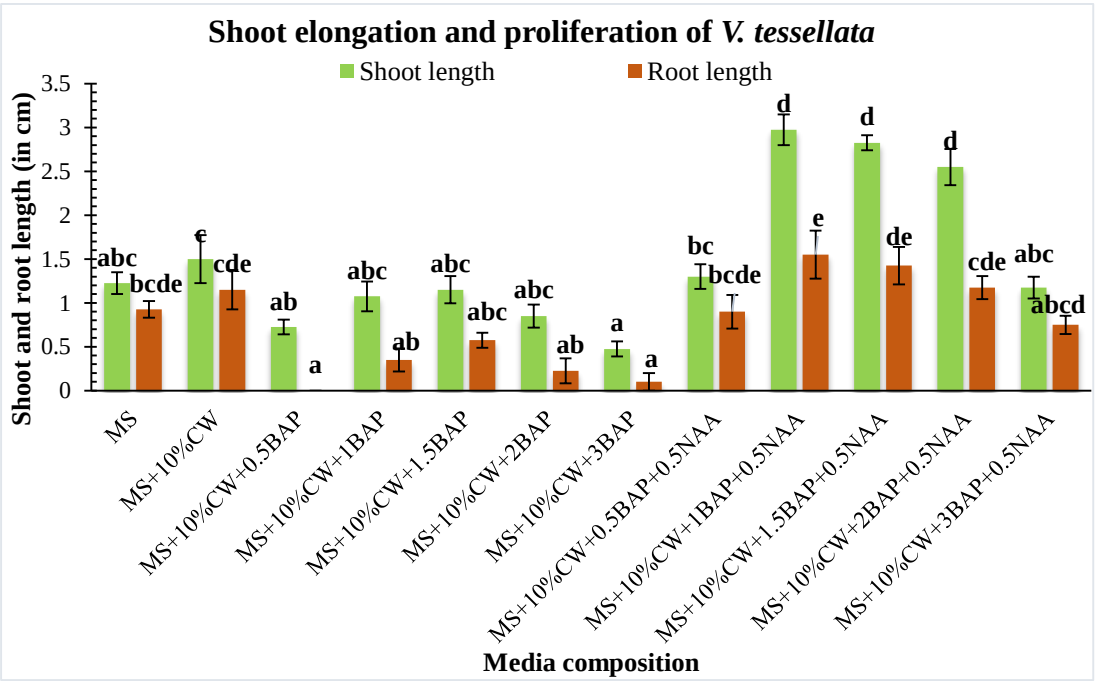


Figure 4. 6: Shoot and root length (in cm) of *V. tessellata.*

Overall, the results indicate that the optimal media composition for shoot elongation and proliferation of *V. tessellata* is MSCW with 1 mg/L BAP and 0.5 mg/L NAA, which resulted in the highest mean shoot number, shoot length and root length.

Figure 4. 7: Different stages of shoot elongation and proliferation of *V. tessellata* in MSCW with 1 mg/L BAP and 0.5 mg/L NAA medium

4.1.4 Shoot elongation and proliferation of *V. testacea*

Different media compositions were used for the shoot elongation and proliferation of *V. testacea.* But the efficiency of HMS and QMS media was found to be inferior compared to FMS and the use of Kn, and GA_3 was also not as effective as BAP, thus these combinations were relegated to the appendix-II. So, the media compositions for body part only include MS, MSCW, and various combinations of MSCW with different concentrations of plant growth regulators BAP and NAA. The results are presented in terms of the mean shoot number, shoot length, root number, and root length, as well as the standard error and the letters indicating the level of statistical significance at $p<0.05$ as shown in Figure 4.8 and 4.9.

The results show that the media composition had a significant effect on the shoot elongation and proliferation of *V. testacea.* The highest mean shoot number (10.75±0.48) was observed in the MSCW with 0.5 mg/L BAP treatment and shoot length (3.1±0.24) was observed in MSCW treatment, while the lowest mean shoot number (4.5±0.29) and shoot length (0.48±0.05) were observed in the MSCW with 3 mg/L BAP treatment.

The root number and length were also affected by the media composition, but to a lesser extent than the shoot number and length. The highest mean root number (2.25±0.48)

was observed in the MSCW and root length (1.15±0.16) was observed in the MSCW with 1 mg/L BAP and 0.5 mg/L NAA treatment, while the lowest mean root number (0±0) and root length (0±0) was observed in the MSCW with 3 mg/L BAP treatment.

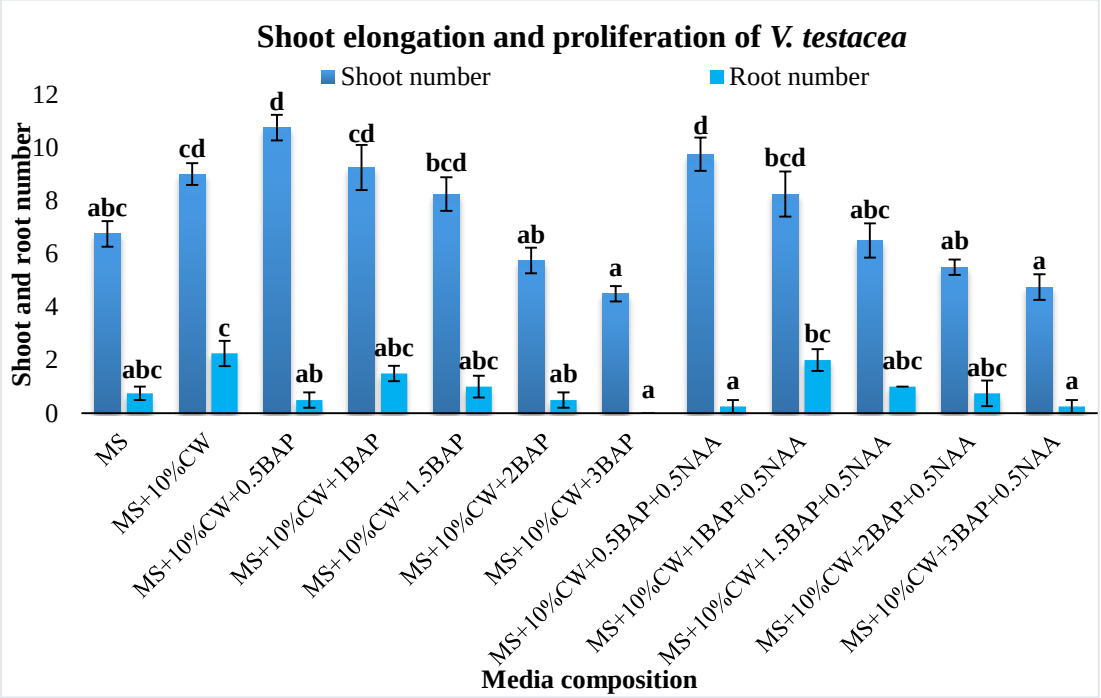


Figure 4. 8: Shoot and root number of *V.tesacea.*

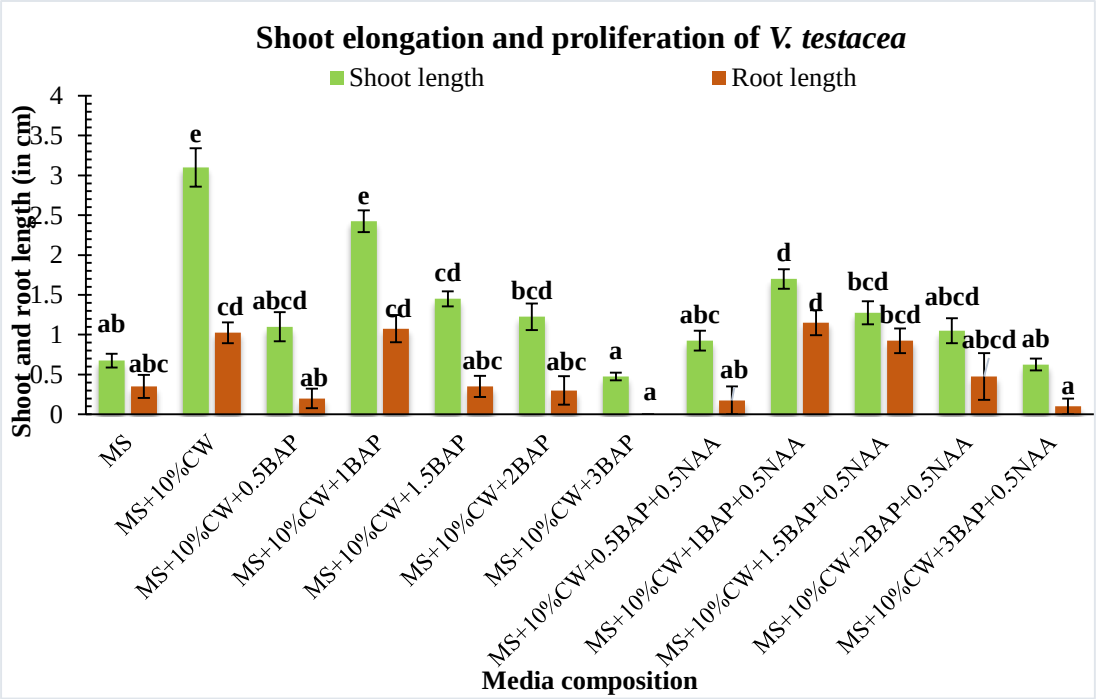


Figure 4. 9: Shoot and root length (in cm) of *V.tesacea.*

Overall, the findings show that MSCW is the best media composition for *V. testacea* shoot elongation and proliferation since it resulted into the highest mean shoot number, shoot length, and root number.

Figure 4. 10: Different stages of shoot elongation and proliferation of *V.tesacea* in MSCW medium.

4.2 Phytochemical assay

4.2.1 Qualitative phytochemical assay

Qualitative phytochemical screening of wild and *in vitro* raised samples of *V. tessellata* and *V. testacea* is tabulated in Table 4.1. All extracts tested had phytochemical groups such as alkaloids, flavonoids, phenols and tannins, saponins, steroids, terpenoids and phlobatannins. But none of the samples had anthocyanins.

It is important to note that all of the samples from the wild and i*n vitro* raised specimens of *V. tessellata* and *V. testacea* had identical findings in the phytochemical screening, despite being taken from various plant parts, including leaves, roots, protocorms, and *in vitro* raised whole plants.

Table 4. 1: Qualitative phytochemical screening of wild and *in vitro* raised samples of *V. tessellata* and *V. testacea*

Extracts	Alkaloids	Flavonoids	Phenols and tennins	Saponins	Steroids	Terpenoids	Phlobatannins	Anthocyanins
VTL	+	+	+	+	+	+	+	-
VTR	+	+	+	+	+	+	+	-
VTP	+	+	+	+	+	+	+	-
VTW	+	+	+	+	+	+	+	-
VTL2	+	+	+	+	+	+	+	-

VTR2	+	+	+		+	+	+	+	-
VTP2	+	+	+		+	+	+	+	-
VTW2	+	+	+		+	+	+	+	-

Legend: + indicate presence and – indicates absence**.**

4.2.2 Quantitative phytochemical assay

4.2.2.1 Total phenolic content

Gallic acid was used to calibrate the equivalent amount of phenolic content present in the crude methanolic extract of different plant sample of *V. tessellata* and *V. testacea*. The calibration curve of gallic acid is presented in Figure 4.11.

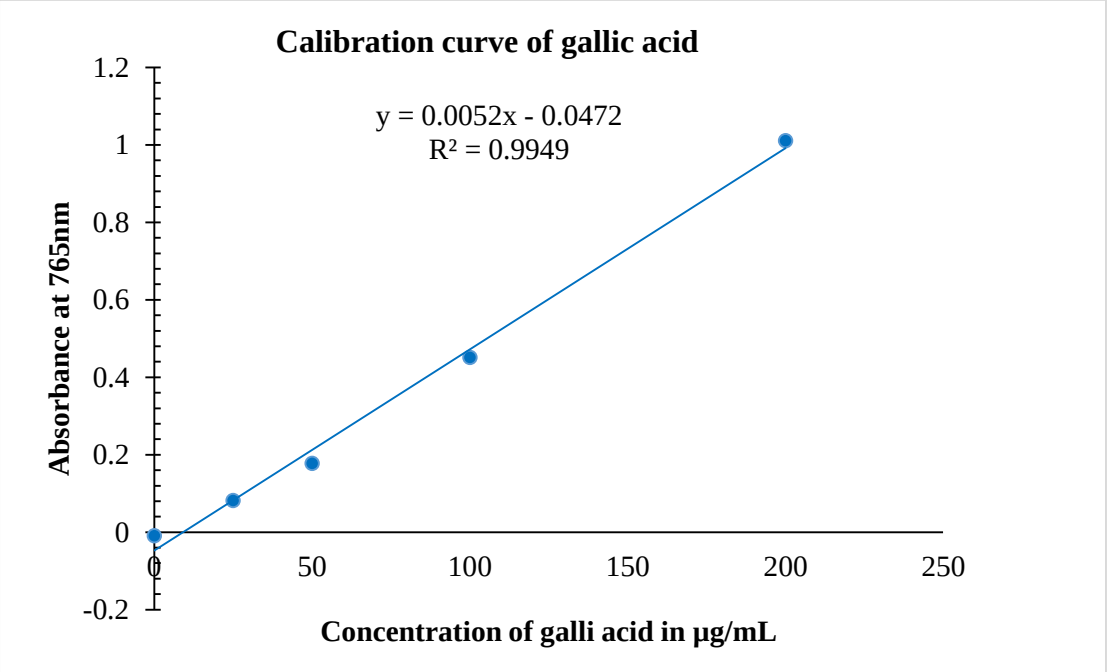


Figure 4. 11: Calibration curve of gallic acid.

The total phenolic content was expressed in terms of milligram of gallic acid equivalent per gram of plant extract (mg GAE/g). The total phenolic content of both plant was determined in different plant samples. The results showed the highest total phenolic content in *V. testacea* leaf (VTL2) with 223.69±0.33 mg GAE/gm, followed by *V. tessellata* leaf (VTL) with 198.76±3.34 mg GAE/gm. The lowest total phenolic content was found in *V. tessellata* protocorm (VTP) with 108.12±0.29 mg GAE/gm followed by *V. testacea* protocorm (VTP) with 126.06±12.92 mg GAE/gm.

In general, the leaves of both plant had the highest total phenolic content, followed by the roots. The protocorms had the lowest total phenolic content. The *in vitro* whole plants of both plant had intermediate total phenolic content. Total phenolic content of *V. tessellata* and *V. testacea* is presented in Figure 4.12.

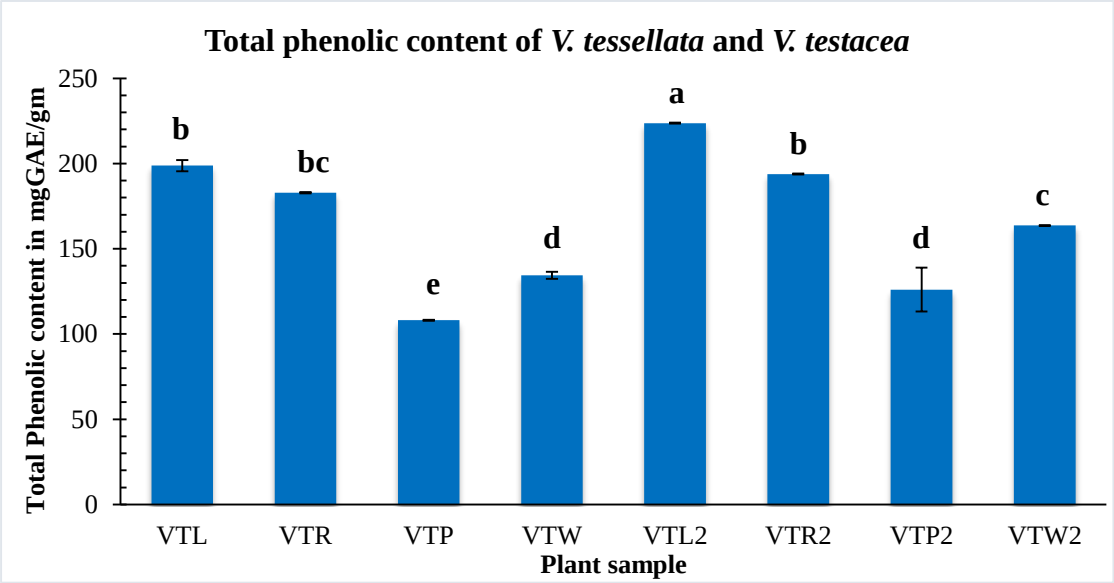


Figure 4. 12: Total phenolic content in selected species of *V. tessellata* and *V. testacea.*

4.2.2.2 Total flavonoid content

Quercetin was used to calibrate the equivalent amount of flavonoid content present in the crude methanolic extract of different plant sample of *V. tessellata* and *V. testacea*. The calibration curve of quercetin is presented in Figure 4.13.

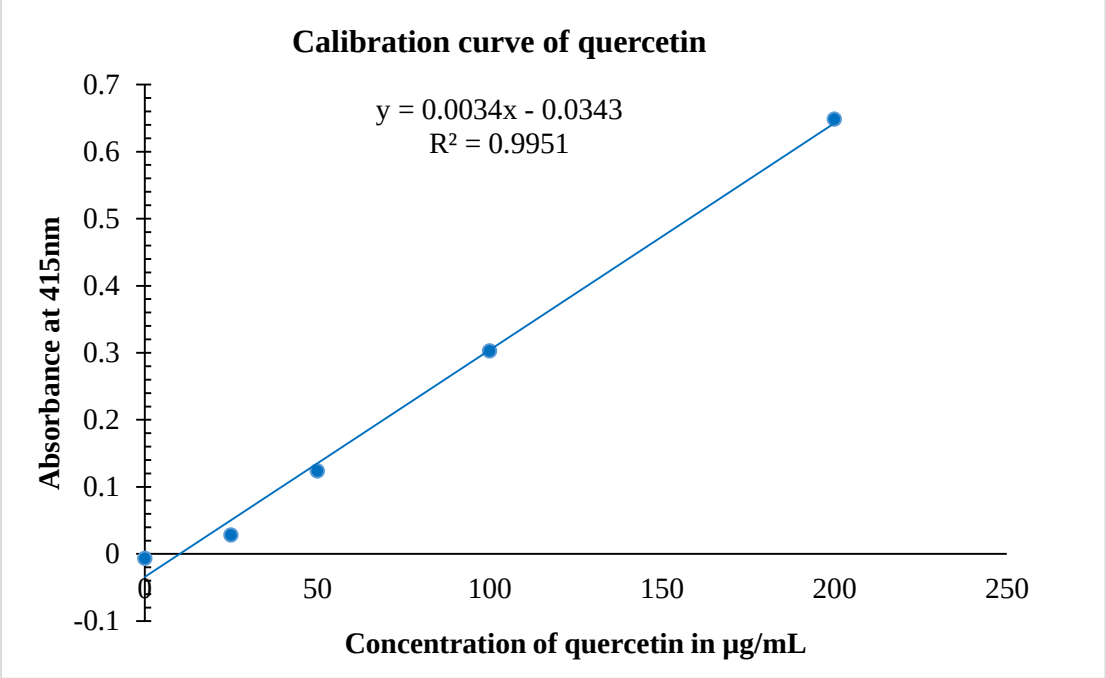


Figure 4. 13: Calibration curve of quercetin.

The total flavonoid content was expressed in terms of milligram of quercetin equivalent per gram of plant extract (mg QE/g). The total flavonoid content of both plant was determined in different plant samples. The results showed the highest total flavonoid content in *V. testacea* leaf (VTL2) with 87.93±0.43 mg QE/gm, followed by *V. tessellata* leaf (VTL) with 69.40±0.20 mg QE/gm. The lowest total flavonoid content was found in *V. tessellata* protocorm (VTP) with 34.89±0.43 mg QE/gm followed by *V. testacea* protocorm (VTP) with 38.72±0.80 mg QE/gm.

In general, the leaves of both plant had the highest total flavonoid content, followed by the roots. The protocorms had the lowest total flavonoid content. The *in vitro* whole plants of both plant had intermediate total flavonoid content. Total flavonoid content of *V. tessellata* and *V. testacea* is presented in Figure 4.14.

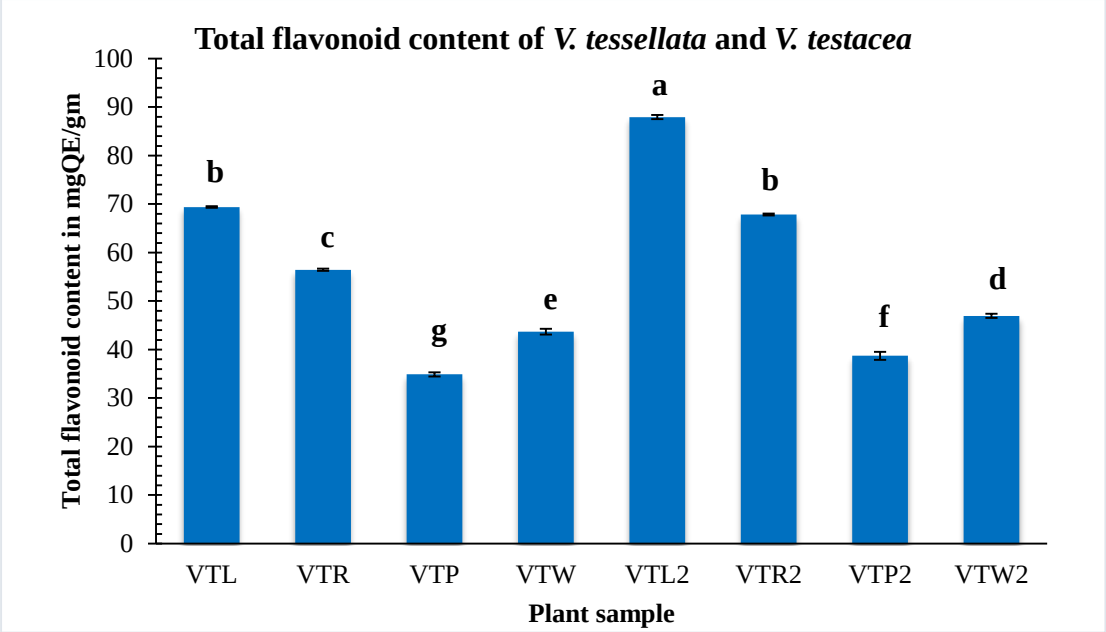


Figure 4. 14: Total flavonoid content in selected species of *V. tessellata* and *V. testacea.*

4.3 Biological assay

4.3.1 Antioxidant activity

The antioxidant activity was determined by using the DPPH free radical scavenging capacity of methanolic crude extracts of different plant samples of *V. tessellata* and *V. testacea*. Radical scavenging activity of leaf of *V. testacea* was found to be highest (83.84±0.33%) followed by root of *V. testacea* (74.81±0.33%) and leaf of *V. tessellata* (72.73±0.46%) and lowest was found in protocorm of *V. tessellata* (42.86±0.08%) followed by protocorm of *V. testacea* (49.53±0.38%) at concentration of 200µg/mL Overall, wild sample shows higher radical scavanging activity than *in vitro* raised samples. Similarly, the *V. testacea* samples have higher average percentages of DPPH radical scavenging activity than *V. tessellata* samples. The leaf samples have the highest average percentages among all samples.

Overall, the DPPH radical scavenging activity increases with increasing concentration for all samples, with the highest average percentage observed at a concentration of 200µg/mL for most samples. Percentage DPPH radical scavenging activity of methanolic extract of different sample *V. tessellata* and *V. testacea* is given in Figure 4.15 whereas percentage DPPH radical scavenging activity of ascorbic acid is presented in appendix-III.

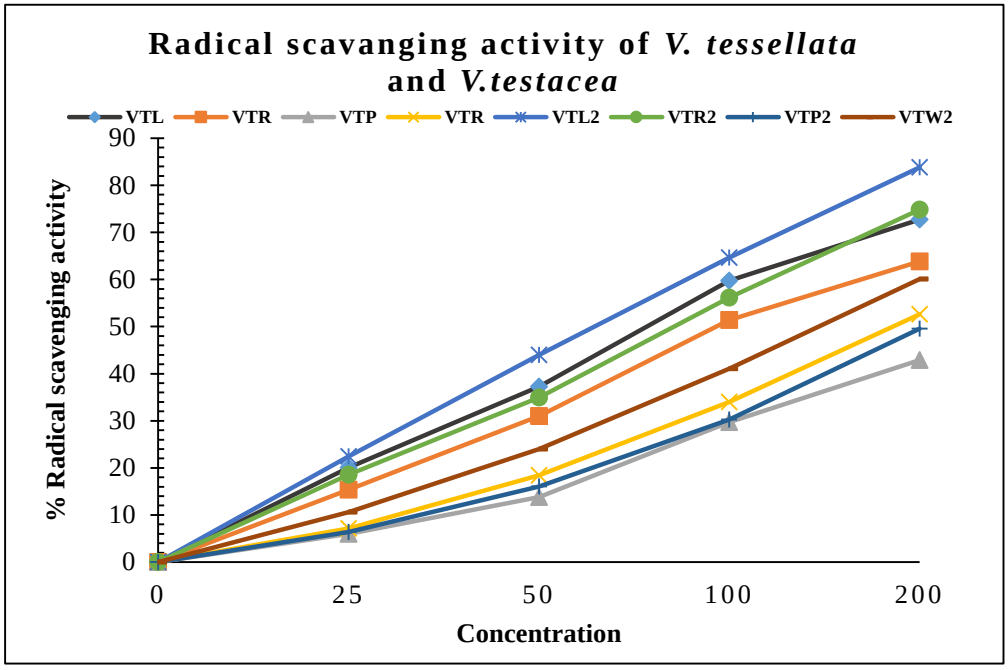


Figure 4. 15: Percentage DPPH Radical scavenging activity of methanolic extract of different sample *V. tessellata* and *V. testacea*.

Among all the samples, *V. testacea* leaf has the highest antioxidant activity with an IC_{50} value of 92.78±0.97 µg/mL followed by leaf sample of *V. tessellata* with an IC_{50} value of 109.79±0.57 µg/mL, indicating potential antioxidant activity. The root samples of both plants also have moderate antioxidant activity.

The protocorm and *in vitro* whole plant samples showed lower antioxidant activity compared to the leaf and root samples. IC_{50} values for antioxidant activity of methanolic crude extracts of various samples of *V. tessellata* and *V. testacea* is presented in Figure 4.16.

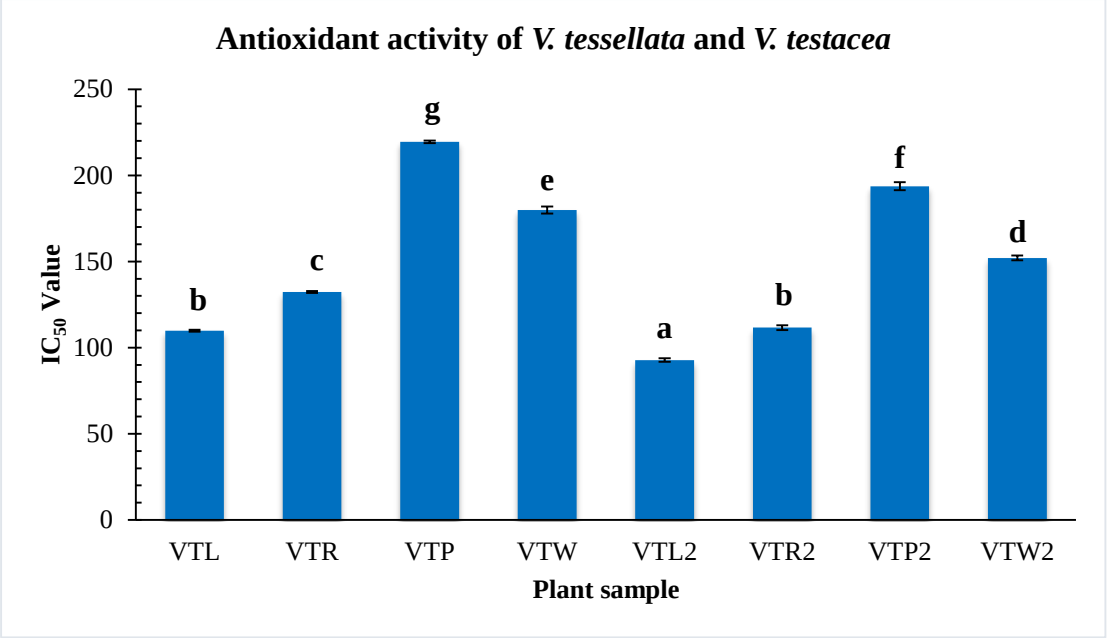


Figure 4. 16: IC_{50} values of methanolic crude extract of various samples of *V. tessellata* and *V. testacea*.

4.3.1.1 Relationship between total phenolic content, total flavonoid content and IC_{50} value of antioxidant activity

Relationship between the IC_{50} value of antioxidant activity and the total phenolic and flavonoid content of different samples of *V. tessellata* and *V. testacea* was established using Pearson's correlation method. The correlation table is presented in appendix-IV.

The $_{IC50}$ value of antioxidant activity revealed a strong negative correlation with total phenolic content ($r = -0.979$, $p<0.01$) and total flavonoid content ($r = -0.943$, $p<0.01$). This means that as the total phenolic and flavonoid content increases, the $_{IC50}$ value decreases (indicating stronger antioxidant activity). However, total phenolic content showed a strong positive correlation with total flavonoid content ($r = 0.944$, $p<0 .01$). This suggests that higher the total phenolic content, higher will be the total flavonoid content in the samples.

4.3.2 Antibacterial activity

Antibacterial activity of methanolic crude extract of different samples of *V. tessellata* (VTL, VTR, VTP, and VW) and *V. testacea* (VTL2, VTR2, VTP2, and VTW2) against different bacterial strains (*S. aureus*, *A. baumanii*, *E. coli*, *E. faecalis*, and *S. sonnei*) was screened by agar well diffusion method. The extract was tested at different

concentrations (1.25µg/mL to 10 µg/mL) and the zone of inhibition (ZOI) was measured in millimeters and the result is presented in Table 4.2.

Table 4. 2: Antibacterial activity (zone of inhibition, ZOI) of methanolic crude extract of different samples of *V. tessellata* and *V. testacea*

Plant sample		**Bacterial strains (ATCC)**				
	Concentration	***S. aureus***	***A. baumanii***	***E.coli***	***E. faecalis***	***S. sonnei***
		ZOI (in mm)	ZOI (in mm)	ZOI (in mm)	ZOI(in mm)	ZOI(in mm)
VTL	10	14.33±0.27	12.67±0.27	12.00±0.47	20.33±0.72	12.33±0.27
	5	11.67±0.27	11.33±0.27	10.33±0.27	16.33±1.52	10.00±0.47
	2.5	9.67±0.27	9.33±0.27	7.67±0.27	12.00±0.47	7.33±0.27
	1.25	7.33±0.27	7.67±0.27	7.00±0.00	9.33±0.27	0.00±0.00
VTR	10	12.67±0.27	11.33±0.27	11.33±0.27	12.67±0.27	11.33±0.27
	5	9.67±0.27	10.33±0.27	10.33±0.27	9.67±0.54	9.33±0.27
	2.5	8.67±0.27	8.33±0.27	8.33±0.27	7.33±0.27	7.33±0.27
	1.25	7.00±0.00	7.33±0.27	7.33±0.27	7.00±0.00	0.00±0.00
VTP	10	11.67±0.54	10.33±0.72	9.67±0.27	10.67±0.54	9.00±0.82
	5	8.33±0.27	7.67±0.27	7.33±0.27	7.33±0.27	7.33±0.27
	2.5	7.00±0.00	7.00±0.00	7.00±0.00	0.00±0.00	0.00±0.00
	1.25	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
VTW	10	12.33±0.27	11.00±0.47	10.33±0.72	12.00±0.47	10.67±0.27
	5	9.33±0.27	9.33±0.72	9.33±0.72	9.33±0.72	8.33±0.27
	2.5	7.67±0.27	8.33±0.72	7.67±0.27	7.33±0.27	7.00±0.00
	1.25	0.00±0.00	0.00±0.00	7.00±0.00	0.00±0.00	0.00±0.00
VTL2	10	14.67±0.27	13.33±0.27	12.67±0.27	21.67±0.98	14.67±0.27
	5	12.33±0.27	12.33±0.27	11.33±0.27	17.67±1.44	13.33±0.27
	2.5	11.00±0.47	10.67±0.54	8.33±0.27	13.33±0.72	11.67±0.27
	1.25	7.33±0.27	9.00±0.47	7.50±0.35	10.33±0.27	9.33±0.27
VTR2	10	13.67±0.27	12.33±0.27	11.67±0.27	13.67±0.27	11.67±0.54
	5	11.33±0.27	10.67±0.27	10.67±0.27	10.33±0.27	10.67±0.54

	2.5	9.33±0.27	9.00±0.47	8.67±0.27	7.67±0.27	7.67±0.27
	1.25	7.00±0.00	7.33±0.27	7.67±0.27	7.00±0.00	7.00±0.00
VTP2	10	11.00±0.47	10.33±0.27	9.00±0.47	11.33±0.27	9.67±0.27
	5	9.33±0.27	8.67±0.27	7.67±0.27	9.67±0.27	7.67±0.27
	2.5	7.33±0.27	7.00±0.00	7.00±0.00	7.33±0.27	7.00±0.00
	1.25	0.00±0.00	0.00±0.00	0.00±0.00	7.00±0.00	0.00±0.00
VTW2	10	12.00±0.47	10.67±0.27	10.67±0.27	11.67±0.27	10.67±0.27
	5	10.67±0.27	8.33±0.27	8.33±0.27	9.67±0.27	7.67±0.27
	2.5	9.00±0.47	7.00±0.00	7.00±0.00	7.33±0.27	7.00±0.00
	1.25	7.00±0.00	0.00±0.00	0.00±0.00	7.00±0.00	0.00±0.00

The highest zone of inhibition was observed for VTL2 against *S. aureus*, with a value of 14.67±0.27mm at concentration 10 µg/mL followed by VTL with ZOI 14.33±0.27 mm at concentration 10 µg/mL. While, VTP2 and VTP showed least activity against *S. aureus*, with a ZOI of 11.00±0.47mm and 11.67±0.54mm at concentration 10 µg/mL respectively. Against *A. baumanii*, VTL2 and VTL showed the highest activity with ZOI values of 13.33±0.27mm and 12.67±0.27mm at concentration 10 µg/mL, respectively. VTP and VTP2 had lower ZOI values, with the same value of 10.33±0.7mm at concentration 10 µg/mL. For *E.coli*, again VTL2 had the highest ZOI value of 12.67±0.27mm at concentration 10 µg/mL, followed by VTL with a ZOI of 12.00±0.47mm at concentration 10 µg/mL. VTP2 and VTP had lowest ZOI values of 9.00±0.47mm and 9.67±0.27mm at concentration 10 µg/mL at concentration 10 µg/mL, respectively. Against *E. faecalis*, VTL2 and VTR had the highest ZOI values of 21.67±0.98mm and VTP had the lowest ZOI value of 10.67±0.54mm at concentration 10 µg/mL. For *S. sonnei*, VTL2 had the highest ZOI value of 14.67±0.27mm, followed by VTL with a ZOI of 12.33±0.27mm and VTP had the lowest ZOI value of 9.00±0.82mm at concentration 10 µg/mL.

Overall, the results indicate that the methanolic crude extract of *V. testacea* has strong antibacterial activity against a variety of bacterial strains. *V. tessellata* sample also showed strong antibacterial activity, but it was generally lower than that of the *V. testacea* sample. The activity of the extracts against the different bacterial strains

decreases as the concentration of the extract decreases. It can also be observed that the root sample showed lower activity than leaf sample and *in vitro* raised sample of both plant had a lower activity than the wild sample.

4.3.3 Cytotoxic activity

4.3.3.1 Cytotoxic activity

4.3.3.1.1 Cytotoxic activity against HeLa cell line

The cytotoxic activity of methanolic crude extract of various samples of *V. tessellata* and *V. testacea* against HeLa cell line was measured using IC_{50} values which is presented in Figure 4.17. The results showed that the *V. testacea* leaf had the lowest IC_{50} value of 960.76±11.83 µg/mL followed by *V. tessellata* leaf with IC_{50} value of 1012.15±19.08 µg/mL. *V. tessellata* protocorm had the highest IC_{50} value among the all samples, at 3095.51±24.06 µg/mL followed by *V. testacea* protocorm with IC_{50} value of 2901.35±45.59 µg/mL.

Wild samples had comparatively better cytotoxic activity with lower IC_{50} values than *in vitro* raised samples. Also, the *V. tessellata* samples had higher IC_{50} values compared to the *V. testacea* samples, indicating that the *V. tessellata* samples had comparatively lower cytotoxic activity against HeLa cells. Overall, all the samples had very high IC_{50} values indicating inactive or very low cytotoxic activity against HeLa cells.

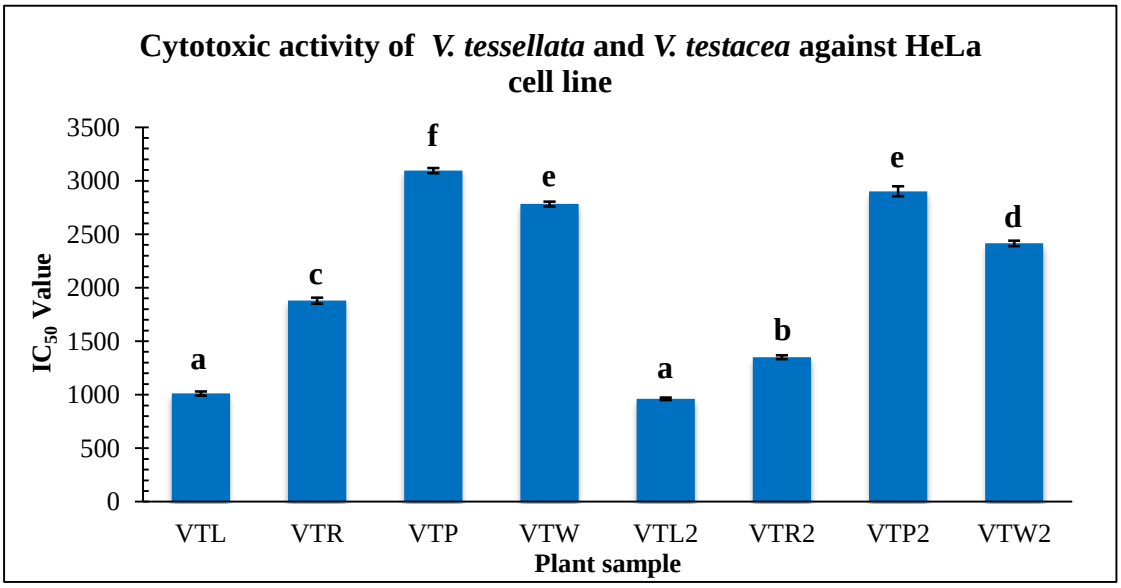


Figure 4. 17: IC_{50} values of cytotoxic activity of methanolic crude extract of various samples of *V. tessellata* and *V. testacea* against HeLa cell line.

4.3.3.1.2 Cytotoxic activity against MCF7 cell line

The cytotoxicity of methanolic crude extracts from different parts of *V. tessellata* and *V. testacea* against the MCF7 cell line was determined using IC_{50} values, as presented in Figure 4.18. The results indicated that the leaf extract of *V. testacea* had the most potent cytotoxicity, with an IC_{50} value of 1103.72±21.61µg/mL, followed by the leaf extract of *V. tessellata* with an IC_{50} value of 1383.43±44.13µg/mL. The protocorm extract of *V. tessellata* had the least cytotoxicity, with an IC_{50} value of 3269.69±35.26µg/mL, followed by the protocorm extract of *V. testacea* with an IC_{50} value of 2953.6±55.42µg/mL.

The wild samples had better cytotoxicity compared to the *in vitro* raised samples, with lower IC_{50} values. Additionally, the *V. testacea* samples had lower IC_{50} values compared to the *V. tessellata* samples, indicating higher cytotoxicity against MCF7 cells. However, all the samples had relatively high IC_{50} values, suggesting inactivity or low cytotoxicity against MCF7 cells.

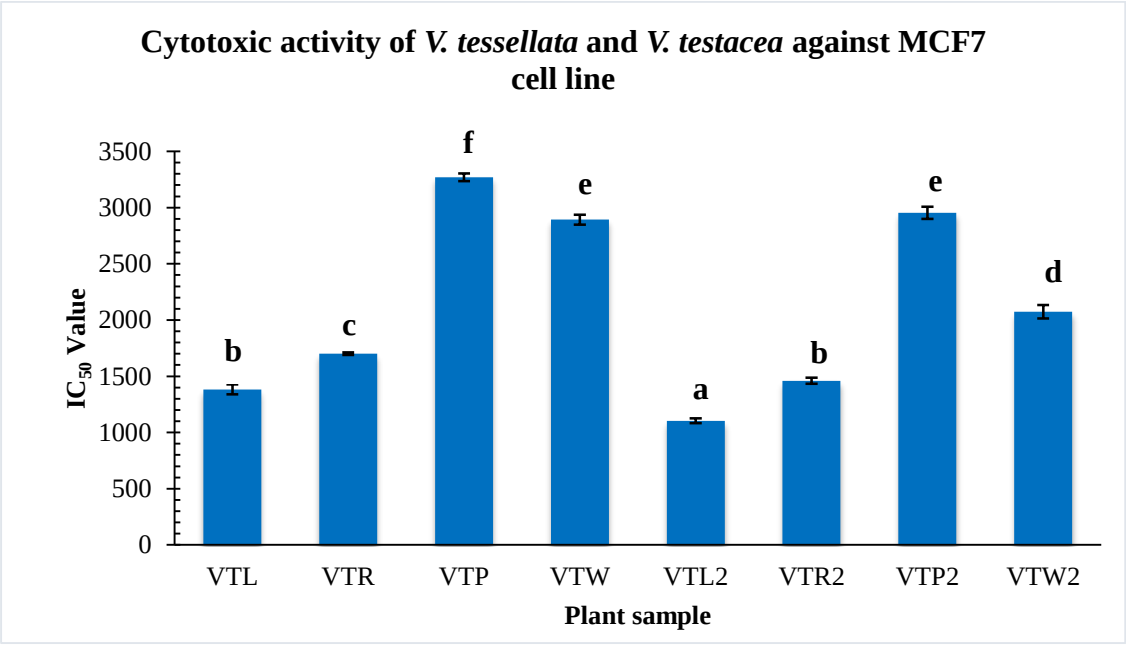


Figure 4. 18: IC_{50} values of cytotoxic activity of methanolic crude extract of various samples of *V. tessellata* and *V. testacea* against MCF7 cell line.

4.3.3.2 Cytotoxic activity on normal cell line

No cytotoxic effect was observed in all tested samples on normal cell. Instead of cell growth inhibition, normal cells were proliferated by all the tested samples. Therefore, cell proliferation was measured using AC_{50} values, as presented in Figure 4.18. The leaf extract of *V. testacea* was found to be the most potent, with an AC_{50} value of 484.94±5.31 µg/mL, followed by the leaf extract of *V. tessellata* with AC_{50} value of 592.80±8.310 µg/mL. The least cell proliferative activity was measured in the protocorm extract of *V. tessellata*, with an AC_{50} value of 1405.12±28.71 µg/mL, and the protocorm extract of *V. testacea* with an AC_{50} value of 1306.10±22.76µg/mL.

Overall, the wild samples had comparatively higher cell proliferative potential than the *in vitro* raised samples and *V. testacea* samples had greater cell proliferative potential than *V. tessellata* samples.

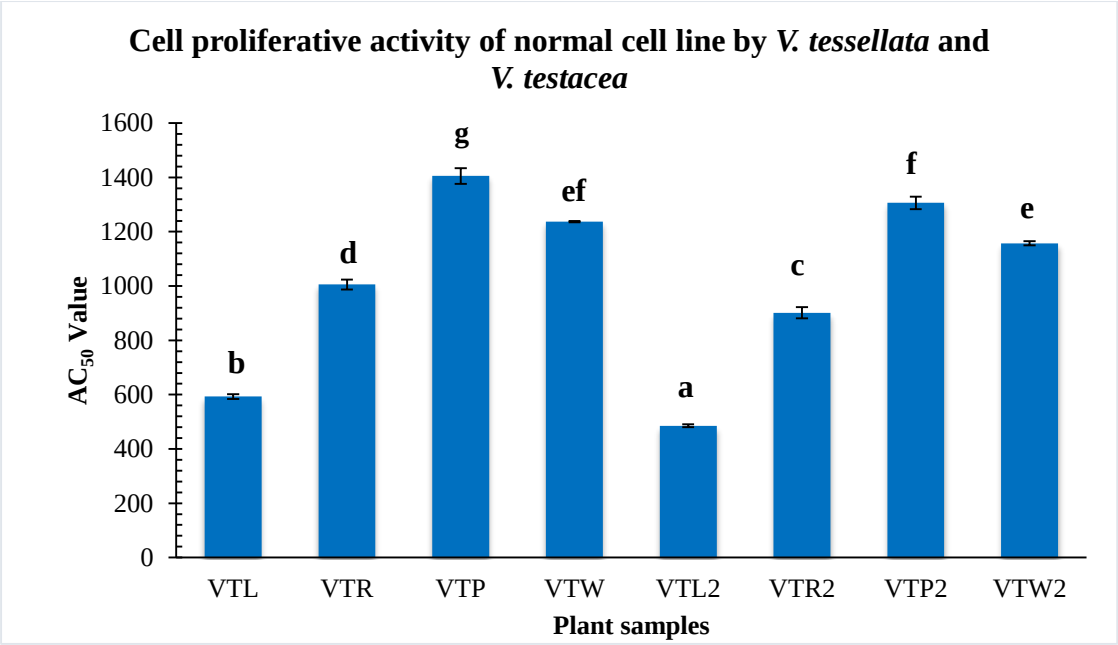


Figure 4. 19: AC_{50}values of normal cell line by methanolic crude extract of various samples of *V. tessellata* and *V. testacea*.

CHAPTER FIVE: DISCUSSION

5.1 Plant tissue culture

This study employs a modern biotechnological method of plant tissue culture to efficiently produce large quantities of raw materials for the extraction of phytochemicals from two medicinal orchids: *V. tessellata* and *V. tastacea*. The method of using plant tissue culture for phytochemical production is more efficient, rapid, sustainable, and environmentally friendly, and can also minimize pressure on natural populations of medicinal orchids. The process involves germinating asymbiotic seeds and proliferating them to generate a large number of protocorms and plantlets.

5.1.1 *In vitro* seed germination

Orchids, a unique group of plants, have evolved to heavily rely on symbiotic relationships with fungi for their survival and reproduction. However, even with this symbiotic relationship, the germination rate of orchid seeds is quite low with only 2-3% of seeds germinating in nature (Prutsch *et al.,* 2000). Tissue culture techniques can bypass this dependency and allow orchid seed to germinate and grow *in vitro*, providing a reliable way to propagate orchids that would otherwise be difficult or impossible to germinate.

In this study, both *V. tessellata* and *V. testacea* seeds were inoculated on different strengths of MS basal media, 10 % coconut water supplemented media, and plant growth regulator (BAP and NAA in combination) at different concentrations fortified with or without coconut water media. Seeds swelling and turning into the green (initiation of germination/ spherule formation), protocorm formation, shoot initiation, and germination percentage of both the species was observed.

In *V. tessellata*, seed started to germinate after 3 weeks, while protocorm development started 5 weeks after inoculation in full strength MS (FMS) medium supplemented with 10% coconut water. The highest germination percentage was also observed in FMS medium with 10% coconut water. Previous research had shown that the addition of coconut water to the medium promoted orchid seed germination (Madhavi and Shankar 2019; Obsuwan and Thepsithar, 2014). The improvement of orchid seed germination may be due to a variety of nutritional components found in coconut water such as sugars

(sucrose, glucose, and fructose), inorganic ions (Ca^{2+}, Fe^{3+}, Mg^{2+}, PO_4^{3-}, K^+, Mn^{2+}, Cu^{2+}, Na^+, SO_4^{2-}, and NO_3^-), vitamins (thiamin (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), myo-inositol, ascorbic acid (C)) organic acids(malic acid, citric acid, and succinic acid), phytohormones (IAA, trans-ZR, and ABA, zeatin-oglucoside, dihydrozeatin-O-glucoside, kinetin, gibberellins (GA_1 and GA_3), and abscisic acid (Prades *et al.*, 2012; Huh *et al.*, 2016). FMS media was better for all the tested parameters such as initiation of germination, protocorm formation, shoot initiation, and germination percentage than HMS and QMS for the seed germination of *V. tessellata*. Similar result was found by Bhattacharjee and Islam (2014) for the seed germination of *V. tessellata*. Hrahsel and Thangjam (2015) also found FMS medium optimum for seed germination of *V. coerulea* followed by HMS medium. *V. tessellata* seed may requires high levels of nutrients for seed germination. FMS media contains all necessary nutrients in high concentrations, while HMS and QMS media may not provide enough, leading to slower germination.

In *V. testacea*, seed started to germinate after 3 weeks, while protocorm development started 5 weeks after inoculation in QMS supplemented with 10% coconut water. All the germination parameters except shoot initiation was observed best in QMS with 10% coconut water. Best shoot initiation was observed in FMS fortified with 10% coconut water indicating that less nutrient requirement for seed germination and high nutrient for further growth and development i.e. shoot and root induction and proliferation. QMS media was superior to HMS and FMS for seed germination of *V. testacea*. In this case, osmotic pressure may play a role, with QMS media having a lower osmotic pressure than seed osmotic pressure in comparison to the HMS and QMS, which can prevent water loss from seed and help with easier absorption, seed swelling, and breakdown of dormancy, leading to faster germination. This result was supported by Huh *et al.*, (2016) for the seed germination of *Cypripedium macranthos* seed germination. Wu *et al*., (2014) also found low salt concentration (1/4) better for seed germination of *Renanthera imschootiana*.

The nutrient requirements for orchids may vary from species to species depending on genotype, phenotype, and the ecological niche they occupy (Wu *et al.*, 2014). In this study, two species of orchids, *V. testacea* and *V. tessellata*, were analyzed and it was found that *V. testacea* required less nutrients for seed germination. This can be

attributed to its small size and the fact that it grows in low nutrient soils, allowing it to have evolved to have low nutritional requirements. On the other hand, *V. tessellata* is known to grow in more nutrient-rich habitats and has higher nutritional requirements. This difference in nutrient requirements is due to the size and habitat of the species.

Similarly, basal media showed better seed germination in both species than hormone supplemented media. Shin *et al.*, (2011) also found strong inhibitory effect of NAA (more than 0.5 mg/L), and BAP (more than 1 mg/L) for the seed germination of *Calanthe* hybrids. This might be due to high endogenous plant growth regulator levels or potential negative effects on enzymes and metabolic processes or lack of hormone receptors in early germination stages or potential toxicity or antagonist effects of auxin to cytokinin or vice-versa.

5.1.2 Shoot elongation and proliferation

The basic information gathered from *in vitro* seed germination of both orchids such as use of high strength media for further growth, and development, use of coconut water, next experiment was designed. Therefore, the primary emphasis was placed on FMS, the utilization of organic supplements (coconut water), and use of cytokinin. However, some other strength media, basal media, and the use of gibberellins and kinetin alone or in combination with auxin were also tested to validate previously gathered information.

Protocorms of both the species were sub-cultured on FMS, HMS, and QMS media fortified with or without 10% coconut water and/or supplemented with various concentrations of BAP, Kn, GA_3 with or without combination with NAA. Both the species showed better response on FMS media supplemented with 10% coconut water. Early and repeated subculture of protocorms of both species was found to very efficient for rapid proliferation. Sinha and Roy (2004) used coconut water for rapid proliferation of new and elongation of old protocorms of *V. teres*.

For *V. tessellata*, the most effective media for shoot proliferation was found to be FMS fortified with 10% coconut water, 1.5mg/L BAP and 0.5mg/L NAA. For shoot elongation, the best media was FMS fortified with 10% coconut water, 1mg/L BAP, and 0.5mg/L NAA. Similar result was found by Bhattacharjee and Islam (2014) and Bhattacharjee and Islam (2017) for the multiple shoot induction of *V. tessellata*. Sinha

and Roy (2004) also reported synergistic effect of BAP, NAA, and coconut water for better shoot elongation. Manokari *et al.*, (2021) found 0.5 mg/L BAP and IAA most efficient medium for protocorm and shoot proliferation of *V. tessellata*.

In *V. testacea*, the media with the highest shoot proliferation was FMS fortified with 10% coconut water and 0.5mg/L BAP. Rineksane *et al.*, (2020) also used 0.5mg/L BAP for the protocorm and shoot proliferation of *V. tricolor*. The best media for shoot elongation of *V. testacea* was FMS fortified with just 10% coconut water. Kaur (2021) also found 20% coconut water for overall growth and development of *V. testacea*.

The efficiency of HMS and QMS media was found to be inferior compared to FMS and the use of Kn, and GA_3 was also not as effective as BAP, thus these combinations were relegated to the annex section only.

5.2 Phytochemical screening and Biological activity

5.2.1 Phytochemical screening

Plants produce phytochemicals through complex biochemical pathways for defense against predators, pathogens, and environmental stress. These chemicals serve as repellants, signaling molecules, and aid in various biological activities including pollination, host-pathogen communication, and animal/insect attraction (Das and Gezici, 2018). Phytochemicals not only play a crucial role in plant defense, but they also provide various health benefits to humans, as they are rich in antioxidants, anti-inflammatory agents, anticancer agents, anti-diabetic agents, antimicrobial agents and so many other bioactivities. Therefore, to understand the bioactive potential of a plant, it is essential to conduct a phytochemical screening.

The present study revealed that all the samples of *V. tessellta* and *V. testacea* showed the presence of secondary metabolites including alkaloids, flavonoids, phenols, tannins, saponins, steroids, terpenoids and phlobatannins whereas all the samples lacks anthocyanin. The anthocyanin is produced in plants in response to exposure with sunlight (Zhu *et al*., 2016). However, both the sample plants collected in the wild were from the canopy of a trees lacking the direct exposure to scorching sun. The presence of different secondary metabolites might be the reason that these two plants may possess varieties of medicinal properties while absence of anthocyanin might be due to lack of sunlight.

This result aligns with previous research on various species of *Vanda* that reported the presence of alkaloids, phenols, flavonoids, terpenoids, steroids, saponins, tannins, phalbotanin, anthocyanins, glycosides, cardiac glycosides, cyanogenic glycosides, reducing sugar, anthraquinones, and coumarins, (Maridass *et al.,* 2008; Arya *et al.*, 2012; Chowdhury *et al.,* 2014; Bhattacharjee *et al.,* 2015; Chand *et al.,* 2016; Islam *et al*., 2016; Biswas and Sinha 2020).

Chowdhury *et al.,* (2014) and Bhattacharjee *et al.,* (2015) reported the absence of saponins in different extracts of *V. tessellata* but it was present in the present study. Biswas and Sinha (2020) also found the presence of saponins in *V. tessellata* but they also report the presence of anthocyanins which is not found in this study.

Phenolic compounds are the largest and most diverse group of secondary metabolites found in plants (Vabkova and Neugebauerova, 2012). Total phenolic compounds includes simple phenols, flavonoids, tannins, lignins, phenolic acids, stilbenes, and coumarins, and anthocyanins (Blainski *et al.,* 2013; Das and Gezici, 2018). These compounds can have a wide range of functions within the plant, including providing protection against environmental stress, such as UV radiation and pathogens, attracting pollinators, and acting as allelopathic agents to inhibit the growth of nearby plants and are also known for their beneficial properties such as antioxidant, anti-inflammatory, antimicrobial, and cardio-protective effects (Zhang *et al.,* 2006; Das and Gezici, 2018).

Total flavonoids are low molecular weight one of the derivative of total phenol that play various roles in plant survival, including UV light protection, coloration, attraction of pollinators, defense against herbivores and pathogens, and stabilization and preservation of plant cell membranes and are also known for anti-inflammatory, anti-tumor, anti-viral, and anti-oxidant properties, as well as improved cardiovascular health, brain function, and reduced risk of some chronic diseases (Treutter, 2005; Cushnie and Lamb, 2011). Flavonoids are classified into subclasses such as Flavans, Flavones, Flavonols, flavanols, flavanonols, Flavanones, isoflavones, Isoflavonesflavones, chalcones dihydrochalcones, dihydroflavonols, and anthocyanidins, (Chang *et al.,* 2002; Treutter, 2005; Ravishankar *et al.,* 2013).

In this study all the samples of *V. tessellta* and *V. testacea* showed significant amount of total phenolic and total flavonoid contents ranging from 223.69±0.33 mg GAE/gm

to 108.12±0.29 mg GAE/gm and from 87.93±0.43 mg QE/gm to 34.89±0.43 mg QE/gm respectively. Similar result of TPC and TFC was reported by Prakash *et al.*, (2018) in ethanolic extract of *V. tessellata*. Nag and Kumaria (2018) also quantified the amount of TPC and TFC in *in vitro* raised samples of *V. coerulea* and reported quite similar amount of TFC from methanolic extract but they reported very less amount of TPC. Chand *et al.,* (2016) also reported similar amount of TFC in *V. cristata* but they reported less amount of TPC than current study whereas Uddin *et al.,* (2015a) reported different amount of TPC and TFC from *V. oxburghii*. This might be due to differences in extraction techniques, solvents used and nature of chemical compounds present in these plants (Kalt *et al*., 2001).

Wild plant samples tend to have higher amounts of total phenolic and total flavonoid content compared to *in vitro* raised plant samples due to several reasons such as extreme temperatures, herbivory, disease, plant age, genetic variability, nutrient availability, etc. which stimulate the synthesis of phenolic and flavonoid compounds as a defense for survival, while *in vitro* conditions do not have to struggle these stress factors. In present study TPC and TFC was also found in higher amount in wild samples than *in vitro* raised samples. Parsaeimehr *et al.,* (2010), and Khorasani *et al.,* (2015) also report similar results.

5.2.2 Biological activity

Phenolic compounds and flavonoids are known to have strong antioxidant properties due to their ability to scavenge free radicals and inhibit oxidative reactions. When these compounds come into contact with DPPH, they act as electron donors and can donate electrons to the DPPH radical, (Ratty *et al.,* 1988). The DPPH radical shows deep violet color in unpaired electron (paramagnetic) state but fades after paring (diamagnetic) with electron or hydrogen radical and the rate of decolorization in respect to number of electron accepted by DPPH (Blois, 1958). Higher the electron/hydrogen donating group to free radicals, higher will be the antioxidant potential. In addition to phenolic and flavonoid compounds other phytochemicals such as alkaloids, glycosides, terpenoids, and tannins are also responsible for enhancing the antioxidant potential of plant extract (Grabmann, 2005; Pirzadah *et al.,* 2017).

V. tessellata and *V. testacea* contains promising amount of polyphenolic and flavonoid content along with variety of other phytochemical, all samples were assessed for DPPH

free radical scavenging activity for the determination of antioxidant potential of methanolic crude extract. All the samples show significant radical scavenging activity even in crude form. Wild sample shows comparatively more effective results than *in vitro* raised samples. This might be due to presence of lower amount of total phenolic and total flavonoid content than wild samples. Subin and Williams (2018) reported similar result in ethanol extract of *V. tessellata.* Near 80% free radical scavenging activity of crude extract of *V. tessellata* at concentration 200 µg/mL was reported by (Soni *et al*., 2018). Chand *et al.,* (2016) also reported similar result from *V. cristata.* Whereas, Vijaykumar, (2013) reported less DPPH free radical scavenging activity and Uddin *et al.,* (2015a) reported more potential result from *Vanda tessellata* than the current study.

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. Vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds as an alternative that can potentially be effective in the treatment of these problematic bacterial infections (Iwu *et al*., 1999). The emergence and spread of multidrug resistant pathogens have substantially threatened the current antibacterial therapy. This has necessitated a search for a new source of antimicrobial substances such as plants as they produce a variety of bioactive compounds of known therapeutic properties.

This study has been conducted to evaluate the antimicrobial activity of *V. tessellata* and *V. testacea* of wild and *in vitro* extracts against human pathogenic bacteria. Present study reported crude methanolic extract of all samples show significant antibacterial activity in all the tested bacterial strains. This might be due to presence significant amount of polyphenols which are known to be anti to microbes (Bhattacharjee *et al*., 2015). Various phytochemicals such as tannins, alkaloids, flavonoids, terpenoids, glycosides, and steroids, (Arya *et al.,* 2012; Chowdhury *et al.,* 2014) which have been proven to have antibacterial capabilities, with flavonoids being particularly efficient against a wide spectrum of microbes and presence of these phytochemicals were already screened in this study. Present study was supported by various authors such as Gupta and Katewa, (2012), Behera *et al.,* (2013), Chaitanya *et al.,* (2013), Gupta and Katewa, (2014), Bhattacharjee *et al.,* (2015) Patra *et al.,* (2015) and Ramana, (2021) where they found potential activity in similar bacterial strains by *V. tessellata*. Kaushik,

(2019) and Ramana *et al.*, (2020b) also reported various degree of antibacterial activity of *V. testacea* against various bacterial strains.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a commonly used method for measuring cellular viability and cytotoxicity. The MTT assay measures the number of viable cells by measuring mitochondrial activity. Mitochondrial NADPH-dependent oxidoreductase enzyme reduces the MTT yellow color dye into purple colored insoluble formazan crystals, which can be dissolved in DMSO to produce a purple solution. The intensity of purple color of the solution is related to the number of viable cells (Adan *et al.*, 2016).

The MTT assay was utilized in this study to assess the cytotoxicity of various crude extracts from *V. tessellata* and *V. testacea*. The extracts were tested against the HeLa and MCF7 cell lines. Results showed that as the concentration of the plant extracts increased, the absorbance of the treated cells decreased compared to non-treated cells. The calculation of the cancer cell inhibition percentage revealed that all of the wild samples had increase in inhibition percentage from negative to positive, while most of the *in vitro* samples showed a decrease in negative inhibition percentage with increasing concentration. This suggests that the crude extracts do exhibit some level of cytotoxicity, although to a limited extent. This might be as a result of using crude extracts from plants that may have extremely low concentrations of cytotoxic compounds. The higher concentrations of the *in vitro* samples showed similar results to the wild samples at lower concentrations. Finally, the IC_{50} values for all samples were found to be very high, indicating a low level of cytotoxicity even at a concentration of 400 µg/ml.

This result was supported by various previous findings. Soot of *V. tessellata* was reported inactive against Human hepatoma (Hep G2) cell line by Ramana *et al.*, (2020a). Ramana *et al.*, (2020b) also reported inactive cytotoxic result from leaf of *V. testacea*. Similarly, Chowdhury *et al.*, (2014) and Uddin *et al.*, (2015b) also reported very low cytotoxicity against brine shrimp (*Artemia salina*) in *V. tessellata*. Nayak *et al.*, (2021) also found very low cytotoxicity of methanol extract of *V. tessellata* against brine shrimp but they also reported very strong activity of ethanol and acetone extract. Joshi *et al.*, (2020) and Rani and Nandagopalan, (2022) also reported significant

cytotoxic activity of *V. cristata* against HeLa and U251 cell line and *V. spathulata* against MCF7 cell line respectively.

To measure the cytotoxic effect of tested plant sample, all the extracts were treated against normal cell and no cytotoxic effect was observed. All the tested samples proliferated the normal cell in varied degree. Normal cell proliferation is essential for maintaining healthy tissues and organs. Therefore, any substance that can stimulate normal cell proliferation without causing harm can be considered as a potential therapeutic agent for treating various diseases. As both the plant are non-cytotoxic to normal cell may be very potential for other biological activity as well because of their safety concern. The presence of bioactive components such as total phenolic content, total flavonoid content, and other phytochemicals in different concentrations in different tested extracts might have contributed to this variation in cell proliferation. Both the plants are also reported for their significant antioxidant and antibacterial activities that might also help to provide a healthy environment for cells to grow and proliferate and these plant may possess cell tonic compounds too.

This result was supported by some previous findings. Cauchard *et al.,* (2010) found *V.coerulea* enhances the proliferation of aged normal human fibroblasts (NHF), similar to that of young cells. Simmler *et al.,* (2011) reported non cytotoxic effect of *V.teres* bioactive compounds on human immortalized keratinocyte cell line (HaCaT). Simmler *et al.,* (2010) also found non cytotoxic effect of *V.coerulea* on human normal epidermal keratinocytes (HaCaT).

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATION

6.1 Conclusion

Use of coconut water as an organic additives promotes the rapid seed germination and protocorm proliferation and addition with BAP to it enhances the shoot proliferation and elongation of both *Vanda* spp. The best results for seed germination and protocorm formation of *V. tessellata* and *V. testacea* were observed in MSCW and QMSCW medium respectively. The highest number of shoots (10.5±0.65) of *V. tessellata* was observed in FMSCW supplemented with 1.5 mg/L BAP and 0.5 mg/L NAA and longest shoot length (2.98±0.18cm) was recorded in FMSCW fortified with 1 mg/L BAP and 0.5 mg/L NAA. In *V. testacea,* highest number of shoots (10.75±0.48) was found in FMSCW and 0.5 mg/L BAP and the longest shoot length (3.1±0.24 cm) was obtained in FMSCW.

Phytochemical screening of *V. tessellata* and *V. testacea* in both wild and *in vitro* raised sampl revealed the presence of similar phytochemicals such as alkaloids, phenols, flavonoids, saponins, tannins, phliobatannins, terpenoids, etc. Quantitative analysis of total phenolic and total flavonoid content of wild and *in vitro* raised sample was found in significant amount however the concentration of the phytochemical constituents were found higher on leaf sample followed by root and tissue cultured samples. The highest amount of TPC (223.69±0.33 mg GAE/gm) and TFC (87.93±0.43 mg QE/gm) was recorded in leaf sample of *V. testacea*.

Biological activity such as antioxidant and antimicrobial activity by both *in vitro* raised and *in vivo* sample was found most effective among all tested bioactivity. The highest antioxidant and antibacterial activity was found *V. testacea* leaf with an IC_{50} value of 92.78±0.97 µg/mL and zone of inhibition against *S. aureus*, with a value of 14.67±0.27mm at concentration 10 µg/mL respectively. No significant cytotoxic activity was observed against all tested cancer cell lines as well as in normal cell line All samples showed very high IC_{50} values against HeLa and MCF7 (960.76±11.83 µg/mL to 3269.69±35.26µg/mL). In case of normal cell, instead of inhibition cell were proliferated. The wild leaf extract of *V. testacea* showed lower AC_{50} value (484.94±5.31 µg/mL) for normal cell line followed by root and *in vitro* raised samples. Non-cytotoxic effects of both plants on normal cell lines are significant in terms of

safety concerns, and they offer several benefits, including reduced side effects, potential for new drug development, supportive care, and an overall safer alternative to the available conventional medicines.

Presence of similar phytochemical constituents and somewhat similar bioactive potential of tissue cultured samples as well as in wild samples opens the alternative way for the commercial production of the biologically active compounds. Utilizing plant tissue culture could be a suitable alternative tool to reduce the pressure on the natural population of medicinal orchids and promote their sustainable utilization and conservation.

6.2 Recommendation

This study is only a preliminary one which compares *in vitro* raised samples with their respective wild species in terms of their phytochemical constituents, and biological activities. Following recommendations are recommended on the basis of present findings.

- ✓ Only *in vitro* plants should be utilized for medicinal and other purpose.
- ✓ A complete study from *in vitro* propagation to reintroduction into the wild should be conducted.
- ✓ Bioassay-guided fractionation and separation of the crude extracts is recommended.
- ✓ Further research should include isolation and identification of bioactive compounds responsible for various biological activities.

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APPENDIX

Appendix-I: Shoot elongation and proliferation of *V. tessellata*

Media composition	Shoot number	Shoot length	Root number	Root length
MS	6.00±0.41	1.23±0.13	2.00±0.41	0.93±0.09
MS+10%CW	8.75±0.48	1.50±0.27	3.25±0.48	1.15±0.23
MS+10%CW+0.5BAP	5.00±0.41	0.73±0.09	0.00±0.00	0.00±0.00
MS+10%CW+1BAP	7.75±0.85	1.08±0.17	0.75±0.25	0.35±0.13
MS+10%CW+1.5BAP	9.50±0.65	1.15±0.16	1.00±0.00	0.58±0.09
MS+10%CW+2BAP	9.25±0.48	0.85±0.13	0.50±0.29	0.23±0.14
MS+10%CW+3BAP	4.50±0.65	0.48±0.09	0.25±0.25	0.10±0.10
MS+10%CW+0.5BAP+0.5NAA	8.75±0.75	1.30±0.14	1.25±0.25	0.90±0.19
MS+10%CW+1BAP+0.5NAA	9.00±0.41	2.98±0.18	1.75±0.48	1.55±0.28
MS+10%CW+1.5BAP+0.5NAA	10.50±0.65	2.83±0.09	1.50±0.29	1.43±0.21
MS+10%CW+2BAP+0.5NAA	9.75±0.48	2.55±0.21	1.25±0.25	1.18±0.13
MS+10%CW+3BAP+0.5NAA	6.75±0.63	1.18±0.13	1.25±0.25	0.75±0.10
MSCW+0.5KN	3.00±0.18	0.60±0.02	0.00±0.00	0.00±0.00
MSCW+1KN	4.75±0.19	0.95±0.01	0.25±0.06	0.10±0.03
MSCW+1.5KN	6.25±0.12	1.03±0.04	0.75±0.06	0.48±0.05
MSCW+2KN	5.25±0.16	0.88±0.03	0.50±0.07	0.28±0.04
MSCW+3KN	4.25±0.12	0.78±0.02	0.50±0.07	0.30±0.04
MSCW+0.5KN+0.5NAA	3.75±0.12	0.88±0.01	0.50±0.07	0.33±0.05
MSCW+1KN+0.5NAA	4.75±0.12	1.15±0.04	0.75±0.12	0.48±0.07
MSCW+1.5KN+0.5NAA	5.25±0.12	1.18±0.02	1.25±0.06	0.88±0.02
MSCW+2KN+0.5NAA	5.00±0.10	1.03±0.04	0.75±0.12	0.48±0.07
MSCW+3KN+0.5NAA	3.75±0.06	0.80±0.04	0.50±0.07	0.33±0.05
MSCW+0.5GA3	10.25±0.12	1.15±0.03	0.50±0.07	0.13±0.02
MSCW+1GA3	11.50±0.24	1.48±0.07	1.25±0.06	0.93±0.07
MSCW+1.5GA3	11.25±0.28	1.23±0.04	1.25±0.06	0.95±0.02

MSCW+2GA3	9.75±0.12	0.98±0.02	1.00±0.10	0.25±0.03
MSCW+3GA3	8.75±0.16	0.93±0.03	0.50±0.07	0.43±0.06
MSCW+0.5GA3+0.5NAA	6.00±0.10	1.08±0.02	1.25±0.06	0.63±0.02
MSCW+1GA3+0.5NAA	6.25±0.24	1.60±0.04	1.50±0.07	0.70±0.04
MSCW+1.5GA3+0.5NAA	9.75±0.12	1.95±0.05	2.00±0.10	0.93±0.02
MSCW+2GA3+0.5NAA	8.75±0.16	1.65±0.03	1.50±0.07	0.80±0.04
MSCW+3GA3+0.5NAA	7.50±0.22	1.05±0.04	1.25±0.06	0.85±0.03
HMS	5.25±0.12	0.65±0.02	1.00±0.10	0.28±0.03
HMSCW	6.00±0.10	0.88±0.03	1.00±0.10	0.48±0.04
HMSCW+0.5BAP+0.5NAA	6.75±0.12	0.60±0.02	1.00±0.10	0.20±0.02
HMSCW+1BAP+0.5NAA	8.50±0.16	1.05±0.04	1.25±0.12	0.25±0.03
HMSCW+1.5BAP+0.5NAA	9.00±0.10	1.23±0.03	1.25±0.12	0.60±0.06
HMSCW+2BAP+0.5NAA	6.75±0.12	0.83±0.03	0.75±0.06	0.53±0.04
HMSCW+3BAP+0.5NAA	4.50±0.16	0.48±0.01	0.50±0.07	0.18±0.03
QMS	4.25±0.12	0.55±0.02	0.50±0.07	0.13±0.02
QMSCW	5.00±0.10	0.50±0.02	1.00±0.10	0.20±0.02
QMSCW+0.5BAP+0.5NAA	6.75±0.26	0.53±0.02	0.75±0.12	0.18±0.03
QMSCW+1BAP+0.5NAA	7.75±0.16	0.60±0.01	0.75±0.06	0.20±0.02
QMSCW+1.5BAP+0.5NAA	8.75±0.06	0.73±0.02	0.75±0.06	0.33±0.03
QMSCW+2BAP+0.5NAA	6.50±0.16	0.55±0.01	0.75±0.06	0.20±0.02
QMSCW+3BAP+0.5NAA	4.50±0.07	0.45±0.02	0.50±0.07	0.13±0.02

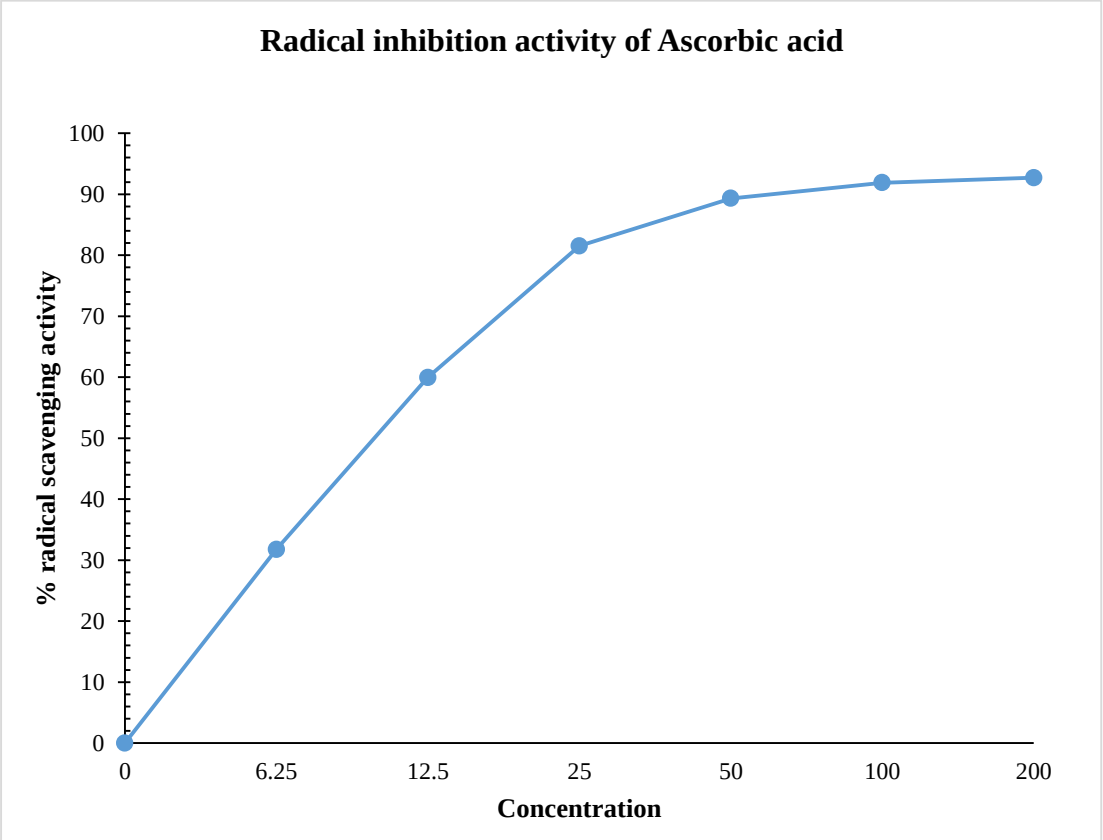
Appendix-II: Shoot elongation and proliferation of *V. testacea*

Media composition	**Shoot number**	**Shoot length**	**Root number**	**Root length**

MS	6.75±0.48	0.68±0.09	0.75±0.25	0.35±0.14
MS+10%CW	9.00±0.41	3.10±0.24	2.25±0.48	1.03±0.13
MS+10%CW+0.5BAP	10.75±0.48	1.10±0.18	0.50±0.29	0.20±0.12
MS+10%CW+1BAP	9.25±0.85	2.43±0.14	1.50±0.29	1.08±0.17
MS+10%CW+1.5BAP	8.25±0.63	1.45±0.10	1.00±0.41	0.35±0.13
MS+10%CW+2BAP	5.75±0.48	1.23±0.17	0.50±0.29	0.30±0.18
MS+10%CW+3BAP	4.50±0.29	0.48±0.05	0.00±0.00	0.00±0.00
MS+10%CW+0.5BAP+0.5NAA	9.75±0.63	0.93±0.13	0.25±0.25	0.18±0.18
MS+10%CW+1BAP+0.5NAA	8.25±0.85	1.70±0.12	2.00±0.41	1.15±0.16
MS+10%CW+1.5BAP+0.5NAA	6.50±0.65	1.28±0.14	1.00±0.00	0.93±0.15
MS+10%CW+2BAP+0.5NAA	5.50±0.29	1.05±0.16	0.75±0.48	0.48±0.29
MS+10%CW+3BAP+0.5NAA	4.75±0.48	0.63±0.08	0.25±0.25	0.10±0.10
MSCW+0.5KN	8.25±0.10	0.98±0.03	0.50±0.06	0.18±0.02
MSCW+1KN	8.00±0.15	0.95±0.04	0.25±0.05	0.13±0.03
MSCW+1.5KN	6.75±0.14	0.63±0.02	0.00±0.00	0.00±0.00
MSCW+2KN	5.75±0.14	0.60±0.02	0.00±0.00	0.00±0.00
MSCW+3KN	3.25±0.14	0.58±0.02	0.00±0.00	0.00±0.00
MSCW+0.5KN+0.5NAA	7.25±0.10	0.90±0.04	0.25±0.05	0.13±0.03
MSCW+1KN+0.5NAA	6.75±0.16	0.88±0.04	0.50±0.06	0.20±0.03
MSCW+1.5KN+0.5NAA	6.25±0.14	0.63±0.01	0.00±0.00	0.00±0.00
MSCW+2KN+0.5NAA	4.50±0.06	0.58±0.01	0.00±0.00	0.00±0.00
MSCW+3KN+0.5NAA	3.25±0.05	0.53±0.01	0.00±0.00	0.00±0.00
MSCW+0.5GA3	8.25±0.10	0.33±0.01	0.00±0.00	0.00±0.00
MSCW+1GA3	8.75±0.05	1.03±0.02	0.75±0.10	0.25±0.04
MSCW+1.5GA3	7.75±0.16	0.68±0.03	0.25±0.05	0.10±0.02
MSCW+2GA3	6.75±0.14	0.60±0.02	0.25±0.05	0.08±0.02
MSCW+3GA3	4.25±0.10	0.58±0.01	0.25±0.05	0.05±0.01
MSCW+0.5GA3+0.5NAA	7.50±0.21	0.63±0.01	0.25±0.05	0.10±0.02
MSCW+1GA3+0.5NAA	8.25±0.10	1.10±0.03	0.75±0.05	0.50±0.04

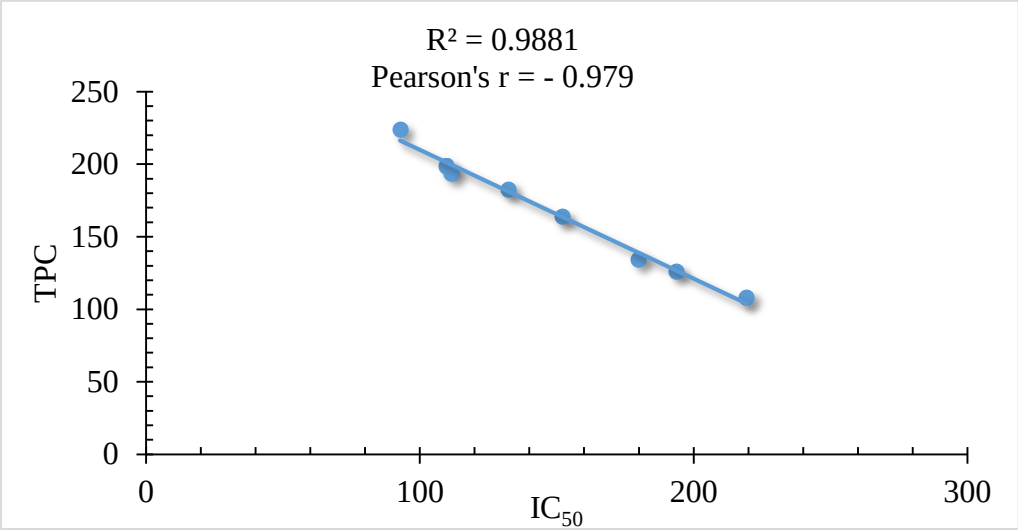
MSCW+1.5GA3+0.5NAA	7.75±0.10	0.95±0.02	0.75±0.10	0.23±0.03
MSCW+2GA3+0.5NAA	7.00±0.15	0.85±0.03	0.50±0.06	0.20±0.03
MSCW+3GA3+0.5NAA	4.25±0.05	0.63±0.02	0.25±0.05	0.08±0.02
HMS	4.50±0.06	0.63±0.02	0.00±0.00	0.00±0.00
HMSCW	7.25±0.05	0.73±0.01	0.25±0.05	0.10±0.02
HMSCW+0.5GA3+0.5NAA	7.00±0.15	0.68±0.01	0.25±0.05	0.10±0.02
HMSCW+1GA3+0.5NAA	8.75±0.10	0.70±0.01	0.50±0.06	0.13±0.02
HMSCW+2GA3+0.5NAA	6.25±0.10	0.53±0.01	0.25±0.05	0.08±0.02
QMS	3.75±0.10	0.38±0.01	0.00±0.00	0.00±0.00
QMSCW	6.75±0.10	0.63±0.01	0.00±0.00	0.00±0.00
QMSCW+0.5GA3+0.5NAA	6.25±0.10	0.58±0.01	0.00±0.00	0.00±0.00
QMSCW+1GA3+0.5NAA	7.75±0.14	0.60±0.01	0.00±0.00	0.00±0.00
QMSCW+2GA3+0.5NAA	6.00±0.15	0.50±0.01	0.00±0.00	0.00±0.00

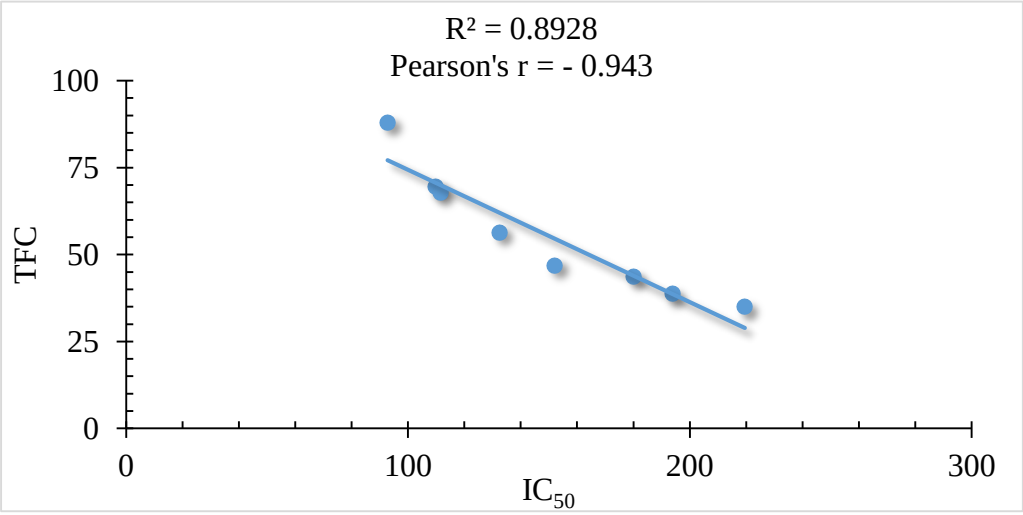
Appendix-III: Percentage DPPH radical scavenging activity of Ascorbic acid.



Appendix-IV: Correlation between Total phenolic content, total flavonoid content and IC_{50} value of antioxidant activity of different samples of *V. tessellata* and *V. testacea.*

Correlation			
	IC_{50} of Antioxidant activity	Total Phenolic Content	Total Flavonoid Content
IC_{50} of Antioxidant activity	1	-0.979**	-0.943**
Total Phenolic Content	-0.979**	1	0.944**
Total Flavonoid Content	-0.943**	0.944**	1
** Correlation is significant at the 0.01 level (2-tailed).			





Appendix-V: Photo plates

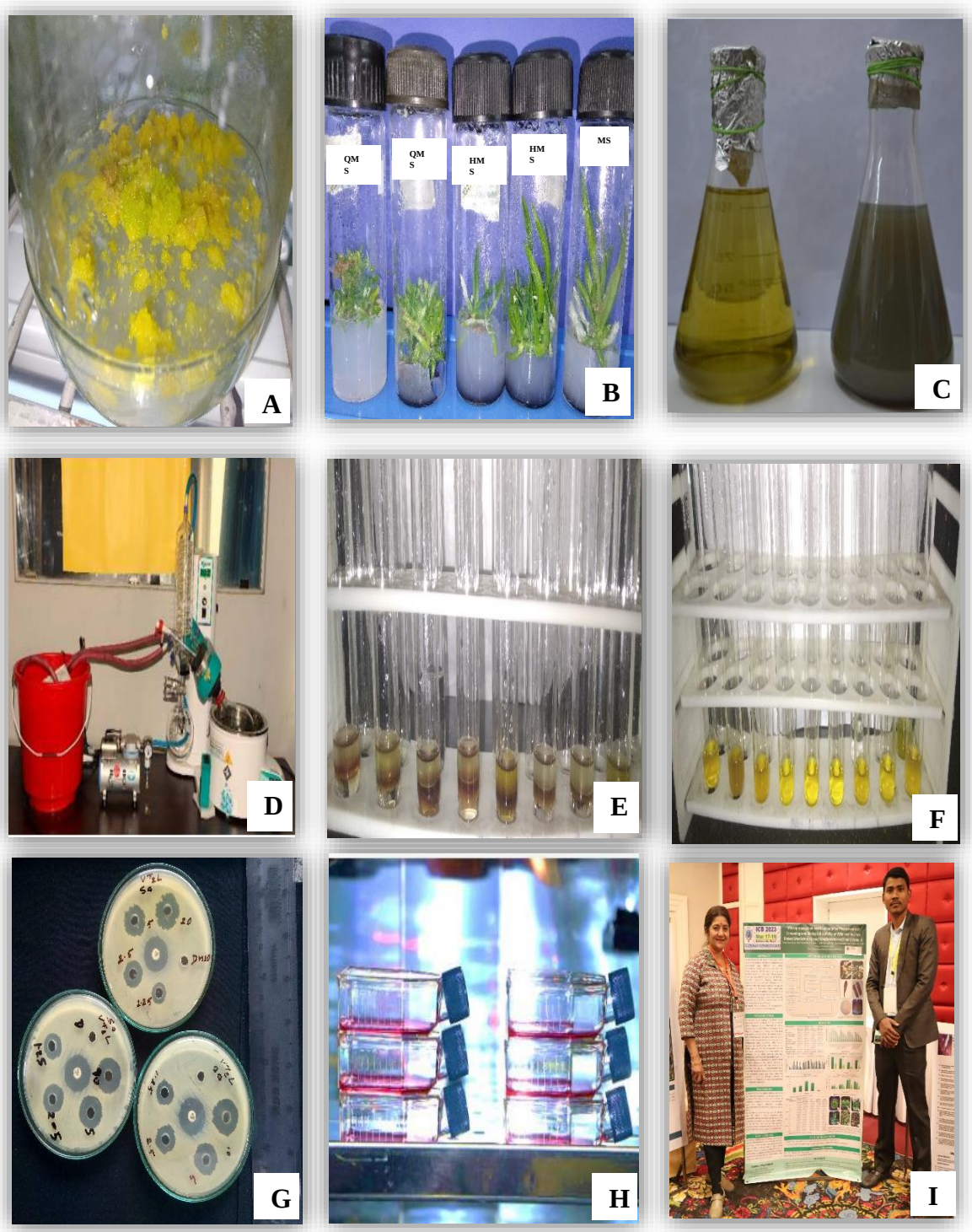


Plate 1. (A) Initiation of germination; (B) Shoot proliferation on different strength of MS media; (C) Extraction of phytochemicals in 90% methanol; (D) Evaporationg solvent with the help of rotatory evaporator; (E) Steroid test; (F) Flavonoid test; (G)Incubating T- flask containing cancer cell in CO2 incubator ; (H) Antimicrobial test ; & (I) Presenting poster in 4th international conference on biotechnology.