ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF *IN VITRO* RAISED *DENDROBIUM AMOENUM* WALL. EX LINDL.

A Dissertation Submitted for the Partial Fulfilment of the Requirement for the Master of Science in Botany

Submitted by

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DECLARATION

I declare that the thesis entitled **"Antioxidant and Cytotoxic Activity of *in vitro* raised *Dendrobium amoenum* Wall. ex Lindl."** submitted by me for the Master's degree in **Central Department of Botany, Tribhuvan University, Nepal.** This is research that I accomplished under the supervision of **Asst. Prof. Dr. Mukti Ram Paudel,** Central Department of Botany, Tribhuvan University, Nepal.

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RECOMMENDATION

This is to recommend that the dissertation entitled **"Antioxidant and Cytotoxic Activity of *in vitro* raised *Dendrobium amoenum* Wall. ex Lindl."** has been carried out by **Ms. Sujata Sharma** for the partial fulfilment of an M.Sc. degree in Botany. This original work was completed under my supervision. This dissertation work has not been submitted for any other degree, to the best of my knowledge.

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LETTER OF APPROVAL

The dissertation paper entitled **"Antioxidant and Cytotoxic Activity of *in vitro* raised *Dendrobium amoenum* Wall. ex Lindl."** submitted by Mrs. **Sujata Sharma** has been accepted for the partial fulfillment of requirements for the Master of Science in Botany.

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Sujata Sharma

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ACRONYMS AND ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxy acetic acid
BAP	6- Benzyl aminopurine
CITES	Convention on International Commerce in Endangered Species of Wild Fauna and Flora
CW	Coconut water
DADGF	*Dendrobium amoenum* dark green fraction
DAGF	*Dendrobium amoenum* green fraction
DALGF	*Dendrobium amoenum* light green fraction
DAYF	*Dendrobium amoenum* yellow fraction
DMSO	Dimethyl sulfoxide
DPPH	2.2-Diphenyl-1 -picrylhydrazyl
EMEM	Minimum Essential Medium Eagle
FMS	Full strength MS medium
GA_3	Gibberellic acid
HeLa	Human cervical cancer cell
HMS	Half strength MS medium
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IC_{50}	Half maximal (50 %) inhibition
IUCN	International Union for Conservation of Nature and Natural Resources
KC	Knudson C
KN	Kinetin
LMS	Liquid strength MS medium
MS	Murashige and Skoog
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	1 -Naphthalene acetic acid
NN	Nitsch and Nitsch
PGRs	Plant growth regulators
PLBs	Protocorm like bodies
QMS	Quarter strength MS medium
TCM	Traditional Chinese medicine
TFC	Total flavonoid content
TPC	Total phenolic content
U-2OS	Human bone osteosarcoma epithelial cell
VC	Vacin and Went

ABSTRACT

Dendrobium amoenum Wall. ex Lindl., the most prominent epiphytic orchid, is recognized for its attractive blossoms and has been used in traditional medicine in various South Asian countries. *D. amoenum* yielded a variety of compounds, including amoenylin, iso-amoenylin, moscatilin, bibenzyl derivatives, phenols, phenanthrenes, and sesquiterpenoids. The study's objectives were to assess the phenol, flavonoid, antioxidant, and cytotoxic activities of *D. amoenum* methanolic fractions grown *in vitro*. FMS medium enriched with 0.25 mg/l NAA and 10% CW produced the healthiest shoots (19.33±2.40) and shoot length (4.17±0.58cm). The highest root number (8.67±5.17) and root length (2.13±0.55cm), were found in IAA (0.25mg/l). The yellow fraction was found to have the highest phenolic (206.38 g GAE/mg extract) and flavonoid content (101.88 g QE/mg extract). The yellow, green, light green and dark green fractions of the methanolic extract inhibited DPPH free radicals (IC_{50}) at values of 63.73±0.38 µg/ml, 179.76±1.47 µg/ml, 105.79±0.57 µg/ml, and 204.27±2.75 µg/ml respectively. At 200 µg/ml concentration, the green fraction inhibited the growth of HeLa (human cervical carcinoma) cells by 82.36±5.79%, the dark green fraction inhibited the growth of U2OS (osteosarcoma) cells by 64.75±3.41%, and the light green fraction inhibited the growth of Normal cells by 13.51±4.43%. Except for Normal cells, which are non-toxic to humans, the fractions of methanolic extract suppress cell growth by 50% against HeLa and U2OS cells. The antioxidant potential of the methanolic fraction of *D. amoenum* extracts is related to the presence of antioxidant-rich chemicals such as polyphenol derivatives, which have a high potential to inhibit the proliferation of cancer cells, elucidating the orchid's potential in alternative medicine toward medication development.

CHAPTER 1: INTRODUCTION

1.1 Background

Nature has always served as a shining example of the remarkable phenomenon of symbiosis with the requirements of existence (food, clothes, and shelter) and has offered a full range of treatments to treat all human ills. Since the beginning of human evolution, natural goods have been essential to human health, and they still are today, in the most cutting-edge therapeutic period. Today, with the development of science and technology, the field of medicine has made significant advancements. Medicinal plants have been utilized as a source of medicine for millions of years, according to some written records. The foundation of traditional medicine is made up largely of medicinal herbs. The most popular and well-known type of medication in contemporary human culture is plant-derived medicine, which is utilized all around the world. Indigenous populations in both developed (Tomlinson & Akerele, 2015) and emerging countries have long used plant resources as medicine. It is a part of their traditional legacy (Chaudhary, 1998; Luitel *et al*., 2014; Rokaya *et al*., 2010). Numerous pharmacologically active substances found in plants can be exploited as natural remedies or as key building blocks for the creation of future medications for a variety of diseases (Malla *et al*., 2015). The emphasis has since shifted away from natural products due to developments in synthetic medicinal chemistry and rational drug design (David *et al*., 2015).

Today, cancer is a significant public health issue and the world's leading killer. According to World Health Organization (WHO) estimates for 2019, cancer is the third or fourth leading cause of death before the age of 70 in 23 countries and the first or second main cause in 112 of 183. In 2020, it is projected that there will be approximately 18.1 million new cases of cancer worldwide, excluding nonmelanoma skin cancer, and about 9.9 million deaths from cancer, excluding nonmelanoma skin cancer. Overall, the total number of new cancer cases worldwide, including nonmelanoma skin cancer, is expected to be around 19.3 million. According to projections, there would be 11.5 million cancer deaths by 2030, up from 7 million in 2002. With an annual occurrence rate of more than 2.6 million cases by 2015, cancer morbidity is on the rise (Siegel *et al*., 2015). The primary cause is the failure of genes that code for growth factors, growth factor receptors, anti-apoptotic proteins,

transcription factors, and tumour suppressors (Millimouno *et al*., 2014). While certain common anti-cancer medications are known to inhibit the bone marrow, leaving patients more susceptible to infections and other disorders, others are known to produce chemotherapy-induced peripheral neuropathy (CIPN), in which both big and small primary afferent sensory neurons are harmed. The rising cancer morbidity trend is a sign of the negative effects of current cancer treatments. Therefore, there is a continuing need to create brand-new, cost-efficient anticancer medications from natural sources. In truth, the idea of chemotherapy using substances derived from plants is receiving more consideration, but current cancer treatments have had only modest therapeutic success (Darwiche *et al*., 2007; Gali-Muhtasib *et al*., 2015). There are numerous plant species with a massive reservoir of bioactive compounds, but only a tiny part of them have been investigated and continue to be a key source of anticancer medications (Patel, 2016). According to the World Health Organization (WHO), medicinal plants are the primary source of pharmaceuticals for the prevention and treatment of numerous diseases. A quarter of all prescriptions have one or more active compounds that are derived from plants (Fabricant & Farnsworth, 2001; Hossain, 2011). Phytochemicals derived from plants have shown promising effects in the treatment of several cancers. As a result, they might serve as an endless supply of compounds for the development of novel medications. Although the plant's separated chemicals may not be used to make a medicine directly, they do lead to the creation of potentially innovative compounds (Chin *et al*., 2006; Fabricant & Farnsworth, 2001). A vast and diversified category of plant-derived substances used to treat cancer includes phenols, flavonoids, alkaloids, and terpenoids (Millimouno *et al*., 2014).

1.1.1 Orchids

Orchids are a highly diverse and extensive group of flowering plants, with over 28,000 species distributed across 763 genera (Christenhusz & Byng, 2016). It includes one-tenth of all blooming plants in the world, which are found everywhere from the tropics to high altitudes. These plants feature interesting flower structures, varied habits, and variations in size, shape, texture, and colour that enhance the beauty of the natural world. Plants enhance nature with charming mimics that come in a variety of forms, including birds, insects, canines, and butterflies with lovely

scents. The most well-known plant group in the global horticultural and cut flower industries is the orchid (De *et al.*, 2015). They are collected, cultivated, and marketed for a variety of reasons, including ornamentals, medicinal commodities, and food, because of their long-lasting blossoms and the presence of important components (Hinsley *et al*., 2017).

In Nepal, orchids are found in a diverse range of habitats, from tropical to alpine regions, and there are a total of 506 orchid species, including 18 endemic species (Rokaya *et al*., 2013). They are popularly known as “sunakhari”, “sungava”, “chandigava,” and “jivanti”. They are the most valuable ornamental plants, and research shows that they are also very important for human welfare. Most of the species of orchid are used in traditional medicine to cure a variety of diseases. In Nepal, more than 100 species have been employed for medicinal purposes (Rajbhandari, 2014). Because of the great demand for orchids in the horticulture business, trade in these plants has been growing daily. Illegal harvesting and trade have grown at the local, regional, and global levels (Hinsley *et al*., 2017). Despite their rich diversity and ecological value, orchids are under threat from degradation and fragmentation of habitat, indiscriminate harvesting, and illegal commerce, all of which lead to population decline and, eventually, extinction. Orchids are currently prominently featured in the International Union for Conservation of Nature and Natural Resources' Red Data Book (IUCN). Many Orchidaceae species are included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which carefully regulates and monitors international commerce.

Dendrobium represents one of the most significant orchid genera, both aesthetically and medicinally. *Dendrobium* is a large genus of orchids, containing over 1500 species, which makes it the second-largest orchid genus. It is widely distributed around the world, from tropical and warm areas in Asia to Australia and the Pacific. It is known for its impressive species diversity, with approximately 1,800 different species that vary in size, shape, structure, colour and fragrance (WCSP, 2020). There are 26 different *Dendrobium* species in Nepal (Rajbhandari & Rai, 2017). The epiphytic plant genus *Dendrobium* gets its name from the Greek words "dendros" for tree and "bios" for life. *Dendrobium* belongs to the sympodial group of orchids and

has pseudobulbs, sometimes known as "canes", which are modified orchid stems that are vulnerable to food and water reserves. The vegetative (pseudobulbs or canes, including leaves, as well as their shape and size) and reproductive parts of the genus *Dendrobium* exhibit morphological variation (flower shape, size, colours, and fragrance). Pollinators are drawn to flowers when they open with a variety of appealing colours or combinations, including white, pink, yellow, orange, green, purple, and cream.

In India and China, *Dendrobium* species have been cherished folk medicines and superior herbs for over a thousand years (Lam *et al*., 2015). This genus comprises tonic, astringent, analgesic, antipyretic, and anti-inflammatory chemicals that are used in traditional Chinese medicine (TCM) to alleviate various illnesses by, among other things, nourishing the stomach and increasing body fluid production. This genus has a wide range of pharmacological actions, including anti-inflammatory properties (Lin *et al*., 2013), antiplatelet aggregation (Hu *et al*., 2008), hepatoprotective (Tian *et al*., 2015), anti-fibrotic (Pan *et al*., 2012), anti-viral (Sukphan *et al*., 2014), anti-fungal (Sattayasai *et al*., 2009), antimicrobial (Xing *et al*., 2011), antioxidant (Luo & Fan, 2011; Luo *et al*., 2011; Lo *et al*., 2004), anti-diabetic (Pan *et al*., 2014), neuroprotective (Li *et al*., 2011), immunomodulatory and anticancer (Sun *et al*., 2016). These orchids are among the most valuable cut-flower and potted orchids, and they are produced utilizing tissue culture, *in vitro* flowering, and genetic alteration procedures (Teixeira da Silva *et al*., 2014a, b, c; 2015a, b, c; Teixeira da Silva & Winarto, 2016).

1.1.2 *Dendrobium amoenum*

Dendrobium amoenum is one of the most prominent epiphytic orchids recorded from the Central and Eastern Region of Nepal at an altitude range of 1100-2900 m asl (Rokaya *et al*., 2013). The gorgeous blossoms and medicinal properties of *D. amoenum* are highly recognized. The stems of this species are used as a tonic (Pant & Raskoti, 2013). This species has yielded compounds such as amoenylin, isoamoenylin, moscatilin, bibenzyl derivatives, phenols, phenanthrenes, and sesquiterpenoids (Majumder *et al*., 1999; Majumder & Bandoupadhyay, 2010; Venkateswarlu *et al*., 2002). These have high phenolic and flavonoid content, which showed high antioxidant properties, and have been extracted using various solvents (Paudel *et al*., 2015).

Different *Dendrobium* species have antioxidant-rich compounds that have demonstrated a cytotoxic effect against cancer cells. According to reports, the antioxidant ingredient in *D. amoenum* crude extract exerts cytotoxic effects on human cervical cancer and glioblastoma cells (Paudel & Pant, 2017). *D. amoenum* is one of the most often gathered species in the country for traditional medicine and gardening. This has resulted in a decline in population in its native environment, making the species rare. Taking this into perspective, plant tissue culture is a viable option for large production to suit the needs of conservation, commercialization, and therapeutic use.

1.2 Statement of the problem

Dendrobium amoenum is a highly prized medicinal plant. This plant has the potential to produce relatively pure compounds for chemical and biological study as well as commercial production. According to reports, *D. amoenum* extract containing antioxidant properties exerts cytotoxic effects on human cervical cancer and glioblastoma cells (Paudel & Pant, 2017). As a result, the chemical derivatives of this species may induce apoptosis in cervical cancer and glioblastoma cells by controlling the essential protein. However, the chemical mechanism through which molecule derivatives of *D. amoenum* operate as anti-oxidant and anticancer agents remains unexplained. A high deforestation rate, climate variability, and illegal trade are all contributing to the decline of these species in their natural environments. Utilization and long-term conservation will be desirable and achievable through *in situ* conservation techniques. There is no well-established standard protocol for this species *in vitro* growth. As a result, tissue culture techniques ought to be employed, which provide an ideal potential for effective conservation by mass-growing orchids in a short amount of time, and reliable phytochemical profiling methods should be used for plant genotype authentication and drug development.

1.3 Hypothesis

- *In vitro* developed seedlings of *Dendrobium amoenum* extract's column fractions are a source of potent antioxidant components and can inhibit the growth of cancer cells.

1.4 Research objectives

- To develop mass production of *in vitro* seedlings from *in vitro* grown protocorms of *Dendrobium amoenum* to generate bioactive compounds.
- To determine the total phenolic and total flavonoid contents of *in vitro* developed seedlings of *D. amoenum.*
- To evaluate the antioxidant activity of fractions of extract from *in vitro* developed seedlings *D. amoenum* by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.
- To evaluate the cytotoxic activity of fractions of extract from *in vitro* developed seedlings of *D. amoenum* by MTT assay.

CHAPTER 2: LITERATURE REVIEW

2.1 Orchid seed culture

Orchids are the most highly evolved family of flowering plants and the largest family group among angiosperms. They are admired for their stunning, long-lasting flowers, which come in a wide variety of sizes, shapes, and colours. Growing orchids is a global industry that accounts for 8% of the global floriculture market today and has the power to change a nation's economic climate. Many various cultures and tribes grow orchids mostly as ornamentals, while some use the tubers of *Cynorchis* and *Eulophia* as food and herbal remedies (Arditti, 1992). The Chinese were the first to use orchids as a herbal remedy, and their usage as medicine has a very lengthy history (Bulpitt, 2005). It is most likely essential to determine the origin of orchids (Orchidaceae) around 120 million years ago. Orchid seeds are the smallest seeds in the world. One orchid capsule contains around 3 million seeds (without endosperm or nourishment) and measures 0.05-6 mm in length, 0.01-0.93 mm in breadth, and 0.3-14 g in weight (Arditti, 1967; Arditti & Ghana, 2000). The embryo is round or spherical and made up of relatively undifferentiated isodiametric cells with dense granulated cytoplasm and prominent nuclei (Arditti, 1967).

Orchid seeds are distinct and poorly developed at maturity. Orchids are difficult to grow since the seeds do not germinate because the fruits are shed before they are fully ripe, there is no mycorrhizal association, the plants do not receive adequate nutrients, and so on. In nature, they require a combination of several variables for reproduction. They have distinct germination physiology. In nature, they require the presence of a suitable fungus for germination. The germination process of an orchid seed begins only after the fungus penetrates the seed testa and invades the embryo. During germination and early development, the orchid forms a mycorrhizal association with suitable fungi (Rasmussen *et al*., 1990). Fungi are thought to improve sugar, auxin, and vitamin transport in orchids (Mohanraj *et al*., 2009). By co-culturing fungi with orchid seeds, Bernard (1899) and Burgeff (1909) were the first to detect the role of fungus in orchid seed germination, i.e., symbiotic seed germination. The majority of an orchid's fungal companions have been categorized as Rhizoctonia and Rhizoctonia-like fungi.

Although orchids produce a large number of seeds, only 0.2-0.3 percent of them germinate in nature (Singh, 1992; Murthy & Pyati, 2001). The number of orchids in their natural habitat is decreasing due to an inappropriate physiological environment, habitat destruction, and human activities. Ex-situ conservation of orchid populations can thus be performed via tissue culture procedures that employ various growth regulators in an artificial nutrient medium under aseptic circumstances. The development of *in vitro* germination and micropropagation has substantially contributed to their scarcity. Explant *in vitro* propagation generates a high number of clones in an artificial nutritive medium under aseptic conditions in a short period, regardless of physiological or environmental constraints. A large number of plantlets can be grown in a short period from a tiny section of the plant (Roy *et al*., 2011). Exogenous growth regulators used at appropriate concentrations in artificial nutritional media promote zygotic embryos to commence protocorms that develop into plantlets. The introduction of artificial nutritional additions can boost the frequency of symbiotic germination, flowering, and profitable yields (Shin *et al*., 2011).

2.2 Micropropagation of *Dendrobium* species

The shoot tips of *Dendrobium primulinum* were cultured on solidified MS basal medium, either alone or supplemented with a mixture of plant hormones, namely naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP), which resulted in the production of shoots and multiple shoots. The combination of BAP (1.5 mg/l) and NAA (0.5 mg/l) in MS medium proved to be the most effective for shoot multiplication among the different combinations tested. The MS medium supplemented with varying amounts of rooting hormones, namely NAA, IAA, and IBA, demonstrated good root development, except for NAA (0.5 mg/l). Rooting was observed after three weeks of shoot tip culture. Unlike NAA, different amounts of IAA and IBA were found to be effective rooting hormones for *D. primulinum*. The best rooting response was observed on MS medium with exogenous input of IAA (0.5 mg/l) (Pant & Thapa, 2012). Similarly, shoot tips of *Dendrobium amoenum* were cultured on agar-solidified MS media supplemented with different doses of BAP, Kinetin, and NAA. The media supplemented with BAP (1 mg/l) and NAA (0.01-0.1 mg/l) and media supplemented with BAP (1 mg/l) and kinetin (1.5 mg/l) were found to be effective for culture development, resulting in regenerated micro shoots and PLBs. The medium supplemented with 1 mg/l BAP, 1.5 mg/l Kinetin, and

10% CW, as well as 1 mg/l BAP, 1 mg/l NAA, and 10% CW, showed the most effective results for PLB proliferation and healthy micro shoot regeneration (Rajkarnikar, 2014). The stem nodal segments of *Dendrobium amoenum* were transplanted as explants on agar solidified M and MS media, both alone and in various combinations of plant hormones, namely IAA, IBA, 2,4-D, NAA, and KN. Among these hormones, IBA was only effective at 2mg/l concentration, producing PLB in 24.25±0.95% of the explants and promoting PLB proliferation. When IBA and KN were combined at 1 mg/l, they acted synergistically to accelerate regeneration in 64.25±0.95% of the plantlets (Arora *et al*., 2016).

D. chrysotoxum seed culture in Mitra (M) medium containing 0.4% activated charcoal (AC), 2 mg/l BAP, and 2 mg/l IAA generated the maximum seed germination percentage after 2 weeks of culture. The addition of kinetin (KN) or BAP to the medium, together with low auxin, resulted in substantial shoot and leaf development. Leaf development was inhibited when cytokinins were found in the medium alone, showing a synergistic function of auxin and cytokinin in leaf development. High amounts of indole-3-butyric acid (IBA) or 1-naphthalene acetic acid (NAA) in media with low cytokinin concentrations resulted in increased *in vitro* rooting, with IBA being more effective than NAA in rooting induction (Nongdam & Tikendra, 2014). The protocorms of *Dendrobium aqueum* with shoot initials were cultivated on half macro-strength MS medium (1/2 MS) treated with different concentrations of cytokinins (BA, 2iP, KIN, and TDZ) at 1, 3, 5, 7, and 10 mg/l, natural supplements (BP and CW) at 1%, 3%, 5%, 7%, and 10%, and auxins (IBA, NAA, 2,4-D). The highest number of shoots per explant (9.4) was obtained on 3 mg/l NAA, followed by 3% BP (7.0 shoots). Shoot elongation was achieved (1.52 cm) on 1/2 MS medium supplemented with 7 mg/l NAA and 7 mg/l TDZ, compared to 1.37 cm on the control medium. Moreover, shoots grown on 1/2 MS medium with IBA 5 mg/l generated an average of 8.75 roots per shoot, while NAA 7 mg/l produced the longest roots (Parthibhan *et al*., 2015). Similarly, the protocorms of *D. chryseum* were cultured in different combinations of Kinetin, BAP, and GA3 in varying concentrations (0.5 mg/l, 1.0 mg/l, and 2.0 mg/l) with and without CW for seedling growth. The best results were obtained from 1/2 MS medium supplemented with 2.0 mg/l Kn and 10% CW for producing the most shoots, while 1/2 MS medium supplemented with 1.0 mg/l GA3 and 10% CW resulted in the longest shoots. For rooting, three separate hormones, IAA, IBA, and NAA, were used in various

concentrations, as well as each hormone at 1.0 mg/l supplemented with 10% CW. The most effective medium for producing the longest roots was found to be 1/2 MS medium with 1.5 mg/l IAA (Maharjan *et al*., 2020). *D. palpebrae* Lindl. was able to generate multiple shoot buds through organogenesis from both upper and lower regions of pseudobulb segments when cultured on MS media supplemented with auxins (IAA, IBA, NAA, Picloram) and cytokinins (BAP, Kinetin). The best average results for producing shoot buds were obtained with MS medium containing 1.0 mg/l NAA and 2.0 mg/l BAP, followed by MS medium containing 1.0 mg/l Picloram and 2.0 mg/l BAP. Agar-solidified media were more effective than liquid media for elongating shoot buds, and MS medium was found to be superior to PM. The shoot-bud-derived seedlings exhibited the best growth response in terms of length and number of roots developed when cultured on agar-solidified MS with 0.5 mg/l NAA, followed by MS with 0.5 mg/l IBA-supplemented medium (Bhowmik & Rahman, 2020).

D. crumenatum Sw. protocorm-like bodies that were grown on MS medium with 0.5 mg/l TDZ for 60 days were subjected to culture on six different media. After four months of culture, it was observed that the MS medium with 15% CW produced the highest percentage of shooting and number of shoots per explant at 96.0 and 9.5, respectively, and was significantly different from the other media. Additionally, the addition of 0.2% (w/v) AC significantly increased the number of leaves and roots. Furthermore, the MS and VW media supplemented with 15% (v/v) CW and 0.2% (w/v) AC, respectively, resulted in the highest number of roots per plantlet and root length, with 5.3 roots and 34.9 mm, respectively (Klaocheed *et al*., 2020).

The successful regeneration of shoots from stem-node segments of *D. chrysotoxum was* reported. The stem nodes of *in vitro* plantlets showed a potential for morphogenesis when cultured in an MS medium containing different growth regulators such as BA, KIN, and NAA, both separately and in combination, either in semi-solid or liquid form. The type of growth regulator and the physical state of the medium had a positive impact on the regeneration process. The plantlets were grown in MS medium enriched with 50 gdm-3 banana homogenates. The explants produced either shoot buds on cytokinin-supplemented medium or protocorm-like bodies (PLBs) on NAA-supplemented medium, and more neo-formations were observed on liquid medium, particularly in

nodal and inter-nodal regions containing NAA. The secondary buds emerged on the surface of the parent PLBs (Kaur, 2017).

2.3 Phytochemicals of *Dendrobium* species

Medicinal plants have been utilized as traditional treatments, or Ayurveda, in Nepal and other areas of the world for millennia. Different plant parts are used in the final formulation of herbal medications. Certain phytochemicals identified in these medicinal plants are thought to have therapeutic capabilities and positive biological effects. Orchid components contained a range of phytochemicals, including alkaloids, flavonoids, bibenzyl derivatives, and phenanthrenes, implying antibacterial, anticancer, anti-inflammatory and antiviral properties. *Dendrobium* species have been acknowledged as traditional medicine in the Chinese Pharmacopeia due to their phytochemicals such as phenolics, anthocyanins, and polysaccharides (Hossain, 2011; Ng *et al*., 2012). A total of 100 compounds from *Dendrobium* species were reviewed, comprising 32 alkaloids, 6 coumarins, 15 bibenzyls, 4 fluorenones, 22 phenanthrenes, and 7 sesquiterpenoids (Lam *et al*., 2015). *Dendrobium* phenols, which include bibenzyl, phenanthrene and fluorenone, are active ingredients with over 60 known structures (Bi *et al*., 2002; Bi *et al*., 2004; Yang *et al*., 2004; Zhang *et al*., 2005). The most characteristic chemical markers for the genus *Dendrobium* are bibenzyl and phenanthrene. *Dendrobium* has produced around 40 compounds of this type (Yang *et al*., 2006). Similarly, *Dendrobium* erianin and moscatilin showed anti-tumour, anti-angiogenic, anti-platelet aggregation, anti-inflammation, and immunoregulatory characteristics (Fan *et al*., 2001; Gong *et al*., 2004; Zhao *et al*., 2001). Traditional medicine systems have used various alkaloids, such as terpenoids (including dendrobine, nobiline, dendroxine and dendrowardine), and indolizidine (such as crepidamine and dendroprimine), quinolizidine (such as crepidine), as well as some other types, to treat conditions such as fevers and hypertension (Schmidt, 2017).

2.3.1 Description of *Dendrobium amoenum*

Dendrobium amoenum Wall. ex Lindl. is a prominent epiphytic orchid known for its beautiful blossoms (Figure 1) and medicinal properties. It is also known as the lovely *Dendrobium*. It is a cool- to warm-growing, medium- to large-sized epiphytic orchid with pendulous, slender stems and slightly enlarged nodes that hold numerous pale

green deciduous leaves with acute apexes that range in shape from linear to oblong-lanceolate. The inflorescence is axillary, emerging from the nodes of leafless canes. Each inflorescence bloom is white with a violet hue, and the lip is flushed green with a purple mark. The sepals are oblong to lanceolate and obtuse, but the petals are ovate and obtuse. The labellum is obovate, clawed at the base, 3-lobed with large, rounded side lobes and an orbicular mid lobe with undulating margins, and the column is broad at the apex. The labellum is amethyst in colour with a white edge and a yellow splotch on the front, while the neck is hairy and yellowish green. The gorgeous *Dendrobium* blooms in the late spring of May and is widely spread in India, Nepal, and Bhutan in tropical valleys and oak woods at elevations ranging from 1,100 to 2,900 meters (Rajbhandari & Rai, 2017).

Figure 1: Flowers of *Dendrobium amoenum*

D. amoenum has biologically active phytoconstituents that were already valued as tonics in traditional Chinese or folk medicine. The paste made from fresh pseudobulb is used topically to treat burned skin and dislocated bones (Subedi *et al*., 2013). The leaf paste of this species is used to treat skin ailments (Barua *et al*., 2006). The dried stems of this species are used as a tonic (Pant & Raskoti, 2013). The crushed stem is employed in treating bone fractures in both humans and animals (Pande *et al*., 2007; Gurung *et al*., 2008; Rana *et al*., 2015). *Hedychium wardii* rhizomes and *D. amoenum* leaves are blended to create a paste that is employed to heal wounds and various skin problems (Maikhuri & Ramakrishanan, 1992). Several significant chemicals have been identified in *D. amoenum*, including amoenylin, isoamoenylin, moscatilin, bibenzyl derivatives, phenanthrenes, and sesquiterpenoids (Majumder *et al*., 1999; Majumder & Bandyopadhyay, 2010; Venkateswarlu *et al*., 2002). Isoamoenylin 6, a dihydrostilbene isolated from *D. amoenum* roots, demonstrated moderate antioxidant and weak antibacterial activity (Venkateswarlu *et al*., 2002). Acetone and chloroform extracts of

naturally growing *D. amoenum* contained high levels of phenolic and flavonoid content as well as high antioxidant activity.

2.3.2 Antioxidant activity of *Dendrobium amoenum*

Free radicals and reactive oxygen species are produced as a result of cell metabolism in all living organisms, and they are responsible for causing oxidative damage to human cells. This damage is a critical aspect of cellular functioning. Some form of defence mechanism is required to mitigate this damage. To control oxidative stress, a variety of natural and synthetic antioxidants are used. Natural antioxidants and plant-derived substances offer effective protection against free-radical-induced oxidative stress (Salman & Ashraf, 2013; Roy *et al*., 2011). Several compounds from *Dendrobium* species were discovered to have potent antioxidative properties. The natural crude extracted with polar solvents from the stem, leaves, roots and other components of *Dendrobium* was found to have considerable antioxidant effects. *Dendrobium* species have yielded a variety of bioactive chemicals, including bibenzyl derivatives (Cheng *et al*., 2020), sesquiterpenes (Ling *et al*., 2021), phenanthrene, glucosides (Zhao *et al*., 2001), and alkaloids (Liu & Zhao, 2003). The antioxidant ability of bibenzyl derivatives obtained from *D. moniliforme* was tested using the DPPH free-radical scavenging assay (Li *et al*., 2009; 2014). The ethanolic extract of *D. nobile* has possess antioxidative properties that are comparable to or even greater than those of ascorbic acid. The antioxidant properties of *D. monoliforme* and *D. tosaense* are attributed to the presence of alkyl ferulates and quercetin, while *D. densiflorum* has the highest ABTS scavenging activity (64.4 µg/ml) and the highest total flavonoid content (35.54 mg quercetin equivalent per 100g). Among the orchid species studied, *D. moschatum* have the highest quercetin content (4518.3 mg/kg), while catechin was most prevalent in *D. densiflorum* (1211.1 mg/kg) (Natta *et al*., 2022). A DPPH experiment conducted on an ethanolic extract of *D. nobile* stems revealed notable antioxidant activity. This finding led to the isolation of bibenzyl derivatives from the plant, which exhibited antioxidant activity equivalent to or higher than that of vitamin C (Zhang *et al*., 2007).

D. moniliforme includes water-soluble polysaccharides, phenanthrenes, bibenzyl derivatives and polyphenolic chemicals. The hexane extract of *D. moniliforme* (DMH) displayed the highest DPPH radical scavenging activity, with a percentage of 94.48% at a concentration of 800 µg/ml. Following closely behind were the ethanol extract

(DME) with 94.45%, DMA with 93.71%, and DMC with 94.35%. However, the antioxidant capabilities of DMC, DMA, DMH and DME were considerably lower (42.39 µg/ml, 49.56 µg/ml, 52.68 µg/ml, and 58.77µg/ml, respectively) when compared to the IC_{50} of the methanol extract (DMM) of *D. moniliforme*, which was 223.15 µg/ml (Paudel *et al*., 2018). *D. longicornu* is composed of bibenzyl and phenanthrene, with trace levels of monoaromatics, steroids, and flavonoid derivatives that have antioxidant properties. The methanolic extract of *D. longicornu* protocorms was found to scavenge 94.31% of DPPH radicals at a concentration of 1000 µg/ml. The concentration required to scavenge 50% of the DPPH radicals was determined to be 117.56 µg/ml for this extract (Paudel *et al*., 2020). *D. crepidatum* extracts in ethanol and acetone scavenged DPPH free radicals at rates of 94.69±0.10% and 93.41±0.86%, respectively. At dosages of 73.90 µg/ml and 99.44 µg/ml, they reduced DPPH free radicals by 50% (Paudel *et al*., 2019).

The polysaccharides extracted from *D. nobile, D. huoshanense, D. chrysotoxum,* and *D. fimbriatum* possess antioxidant properties and are capable of scavenging free radicals (Lo *et al*., 2004). *D. nobile* polysaccharide exhibited the greatest ability to scavenge free radicals such as hydroxyl, ABTS, and DPPH (Zhang *et al*., 2007; Luo *et al*., 2009). The polysaccharide extracted from *D. fimbriatum* has a robust ability to scavenge ABTS free radicals but only a moderate capacity to scavenge DPPH free radicals. Conversely, the polysaccharide from *D. denneanum* exhibits potent DPPH free radical scavenging activity but lacks any significant effect on ABTS scavenging (Fan *et al*., 2009). Polysaccharides extracted from *D. huoshanense* and *D. chrysotoxum* were both found to have limited ability to scavenge ABTS free radicals. However, they do possess significant hydroxyl radical scavenging activity. Generally, polysaccharides from different *Dendrobium* species exhibit potent scavenging effects on hydroxyl and DPPH free radicals, but their ability to inhibit ABTS free radicals is comparatively weak (Lam *et al*., 2015). Many substances, including dihydrostilbene and isoamoenylin from *D. amoenum* roots and Cis-Cismelilotoside, dihydromelilotoside, and trans-melilotoside) from *D. aurantiacum* stems demonstrated antioxidative effects. The antioxidant activity of *D. amoenum* stem extract was investigated using the DPPH radical scavenging test (Paudel *et al*., 2015). Chloroform, acetone, and hexane extracts showed the greatest percentage of DPPH scavenging capabilities, whereas acetone (53.19µg/ml) and chloroform (36.48µg/ml) extracts showed the lowest IC_{50}.

2.3.3 Cytotoxic activity of *Dendrobium amoenum*

Phytochemicals have shown promising results in cancer chemoprevention against a range of malignancies (Shukla *et al*., 2014). Secondary metabolites produced by plants have the potential to be a never-ending source of molecules for the development of new drugs. The plant chemical may not be immediately useful as a treatment, but it may lead to the discovery of novel drugs (Dias *et al*., 2012). Plant-derived compounds have been examined for their effects on cell cycle regulation and apoptotic pathways (Darwiche *et al*., 2007; Lee *et al*., 2013), but less is known about their impact on non-apoptotic processes such as autophagy, mitotic catastrophe, senescence, and planned necrosis, or "necroptosis (Chan *et al*., 2015). Plant-derived cancer-fighting substances include polyphenols, flavonoids, alkaloids, terpenoids, and sulfhydryl compounds. *Dendrobium* plants have been employed as stomachic, pectoral, analgesic, and antipyretic therapies in traditional medicine (Kong *et al*., 2003). Current pharmacological research based on ethnic and folk information gathered from local practitioners found that various *Dendrobium* species components and extractives exhibited anti-tumour (Lee *et al*., 1995), anti-platelet aggregation (Chen *et al*., 1995), anti-mutagenic (Miyazawa *et al*., 1997), anti-angiogenic, and immuno-regulatory activities (Zhao *et al*., 2001). Moscatilin, produced from the stem of *D. loddigessi*, exhibits strong cytotoxicity against cancer cell lines obtained from various tissues (Ho & Chen, 2003; Tsai *et al*., 2010). Erianin, a compound extracted from the stem of *D. chrysanthum*, is a potent inhibitor of the proliferation of HL-60 cells. The inhibition may be attributed to erianin's ability to induce apoptosis and cause changes in the expression of the bcl-2 and bax genes in HL-60 cells (Li *et al*., 2001; Zhang *et al*., 2007). Moscatilin, which was derived from an ethanol extract of *D. chrysanthum*, was found to be cytotoxic to T-cell lymphoma and the FaDu cell line (Nam *et al*., 2019; Prasad & Koch, 2016). In hepatoma BEL7402 and melanoma A375, eriein from *D. chrysanthum* causes severe tumour necrosis, growth delay, and fast vascular shutdown (Gong *et al*., 2004). TNF-alpha, IL8, IL10, and iNOS mRNA levels in mouse peritoneal macrophages were reported to be suppressed by dendrochrysanene extracted from *D. chrysanthum* stems (Yang *et al*., 2006).

Similarly, the extract of *D. chrysotoxum* stem and its components, erianin, chrysotoxine and confusarin have been observed to possess properties that may help in the treatment of cancer (Gong *et al*., 2006; Wang *et al*., 1997; Chen *et al*., 2008). Antitumoral

phenanthrenes were found in the aerial parts of *D. nobile* (Lee *et al*., 1995). The compounds denbinobin and 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene isolated from *D. nobile* exhibited cytotoxic effects against various cancer cell lines, including human lung cancer, ovarian adenocarcinoma and promyelocytic leukemia (Lee *et al*., 1995). Denbinobin, derived from *D. nobile*, has demonstrated cytotoxicity against cell lines A549, SK-OV-3, and HL-60 (Gutierrez, 2010). The strong cytotoxic effects of *D. thyrsiflorum* are attributed to the presence of bicyclic and tricyclic chemicals (Zhang *et al*., 2005). *D. candidum* has been found to inhibit the growth of breast cancer cells (MCF-7) by causing cell cycle arrest at the G2/M phase and regulating certain proteins in MCF-7 cells (Sun *et al*., 2016).

The cytotoxic effect of *D. moniliforme* aqueous extract against human embryonic kidney cells (HEK 293) has been reported (Lee *et al*., 2016). Similarly, methanol extracts of *D. moniliforme* and *D. amoenum* showed cytotoxic action against the HeLa and U251 cell lines, respectively (Paudel *et al*., 2018; Paudel & Pant, 2017). Several phenolic compounds of *D. longicornu* have antioxidant and cytotoxic actions (Paudel *et al*., 2017). This plant's bibenzyl compounds and lignin glycosides exhibit anti-platelet aggregation action (Hu *et al*., 2008). Crepidatuols from *D. crepidatum* have been demonstrated to improve nerve growth factor-mediated neurite outgrowth in PC12 cells (Li *et al*., 2013). Moscatilin extracted from *D. brymerianum* was found to be cytotoxic to the H-460 cell line (Klongkumnuankarn *et al*., 2015). *D. chryseum* methanol extract was cytotoxic to Hela and U-251 cell lines (Pant *et al*., 2021). The extract of *D. amoenum* has a cytotoxic effect against human cervical cancer and glioblastoma cells (Paudel & Pant, 2017). The mechanism of apoptosis has been demonstrated by activating caspase-8 and cytochrome C in receptor-mediated and mitochondrial routes, respectively (Elmore, 2007; Evan & Vousden, 2001). The renewed interest in natural products as potential chemopreventive and chemotherapeutic anti-cancer medications is due to the possibility that these products could play a significant role in the critical pathways involved in the growth and spread of cancer (Gali-Muhtasib *et al*., 2015; Lombardi *et al*., 2017). The present study will contribute to the examination of the pharmacological importance of this orchid in the development of anti-cancer medicine. This research will serve as a foundation for future research into the pharmaceutical applications of this orchid.

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant materials

The *in vitro*-grown protocorms/shoots of *Dendrobium amoenum*, cultured by my senior in the plant biotechnology laboratory of the Central Department of Botany, Tribhuvan University, were taken as plant materials for the micropropagation in terms of the number and length of shoots and roots.

3.2 Methods of tissue culture

An explant's growth, development, and morphogenic response in culture is determined by its genetic make-up, surrounding environment, and culture medium composition. The success of a plant tissue culture experiment is heavily dependent on the choice of culture medium. Murashige and Skoog (MS) medium were utilized as basal media for the tissue culture. MS media was prepared by using different stock solutions, viz. macronutrients (stock A), micronutrients (stock B), Iron-EDTA (stock C), vitamins (stock D), KI, sucrose, agar, and myo-inositol. The method for culture of the *in vitro* capsule of *Dendrobium amoenum* is described below.

3.2.1 Preparation of Culture media

The Murashige and Skoog medium (1962) was utilized as the basal medium for this experiment, coupled with various hormone concentrations. To begin the preparation of the MS medium, various stock solutions for macronutrients (stock A), micronutrients (stock B), iron-EDTA (stock C), and vitamins (stock D) were prepared. As shown in Table 1, each stock solution was made by precisely weighing and dissolving the ingredients in double-distilled water. The solution was agitated using a magnetic stirrer to help the ingredients dissolve more easily. The final volume was 500 ml for stocks A and C and 200 ml for stocks B and D. Due to their sensitivity to light, all stock solutions were maintained in sterile brown bottles and refrigerated.

To prepare one litre of MS medium, a 1-litre sterilized conical flask was used first. A mixture was prepared in a sterilized conical flask by sequentially adding 100 ml of stock A, 1 ml of stock B, 10 ml of stock C, and 1 ml of stock D to 400 ml of distilled water. Sucrose (30g) was weighed and dissolved in stock solution.

Table 1: Preparation of MS medium from its stock solutions and compositions.

S.N.	Stock solutions/Compositions	Concentration	Quantity
1	Macronutrients (stock A solution)	10X(g/l)	100ml
2	Micronutrients (stock B solution)	100X(mg/100ml)	1 ml
3	Iron-EDTA (stock C solution)	10X(mg/100ml)	10 ml
4	Vitamins (stock D solution)	100X(mg/100ml)	1 ml
5	Sucrose		30g
6	Myo-inositol		0.1g
7	Agar		0.8g

A total of 1000 ml of distilled water was added. The solution was swirled with a magnetic stirrer to ensure that the stock and sugar solution were mixed evenly. Hormone stock was mixed in ten separate beakers to generate 100 ml media for the preparation of hormonal medium, according to the media requirement. It was heated to boiling with a heater to melt the agar. When the solution completely dissolved, approximately 16 ml was poured into each of the six sterilized culture tubes. Afterward, aluminum foil caps were used to seal each of the tubes. The medium-containing tubes were sterilized in an autoclave for 20 minutes at 121^0C and 15lb/sq. inch pressure. After the autoclave had cooled, the tubes were removed and placed in a slanting orientation in an air-conditioned culture chamber.

3.2.2 Hormones and their preparation

a. Auxins

IAA, IBA, NAA, and 2,4-D are widely used auxins. Among these, NAA and 2,4-D are stable and can be preserved at a temperature of 4°C for multiple months. IAA stock solution can be preserved for no longer than a week. It is better to prepare the IAA solution fresh. For the preparation of hormones, 5 mg of NAA was dissolved first in 2.5 ml of 95%ethyl alcohol. After that, the final volume was made to 50 ml by the addition of distilled water. Adjust pH 5.8. NAA can be dissolved in 2.5 ml of 1N KOH or NaOH, which can be used to dissolve IAA or 2,4-D.

b. Cytokinin

Cytokinins are stable and can be stored at -20^0C. Cytokinins are difficult to dissolve. For the preparation of hormones, 5 mg of BAP was dissolved in 0.25 ml of 0.5N NaOH

and heated gently. After that, the distilled water was added to make a final volume of 50 ml. Adjust the pH to 5.8.

3.2.3 Sterilization of glassware and metal instruments

The essential glassware was steam sterilized before use during the experiment. Petri plates, culture tubes, pipettes, beakers, and conical flasks made of glass were immersed in a solution of detergent for 24 hours. They were subsequently washed with tap water and distilled water. The glassware was then sterilized in an autoclave at 121ºC for 15 minutes at 15 lb/sq. Metal instruments such as forceps, scalpels, and surgical blade holders were wrapped in aluminium foil before being sterilized in an autoclave.

3.2.4 Sterilization, shoot tip culture and seedling growth

The surgical instruments and glassware used for the tissue culture were autoclaved at 121ºC for 45 minutes and dried in an oven at 150ºC for 1 hour for sterilization. Before the transfer of the medium, the laminar air flow cabinet was cleaned with 70% (v/v) ethanol. The culture tubes and jars containing the medium, glassware, and sterile instruments were autoclaved and placed inside the laminar air flow under UV (ultraviolet) radiation for 45 minutes.

All the surgical instruments, such as forceps, blades, and scalpels, were sterilized using a red hood in Bunsen flame. The blower was kept running during the process of inoculation. The initial shoot tips of *D. amoenum* were removed from the jars and inoculated on various MS media supplied either alone or in combinations with various hormone concentrations using sterile forceps.

All the inoculation processes were done in the laminar air flow cabinet with a flame to avoid fungal and bacterial contamination. The cultured tubes were transferred to an incubation room at 25±2ºC temperature, 78±5% humidity, and a 12–16-hour photoperiod. The growth of seedlings was observed every week for five months. 40 different media combinations were used for seedlings development. The media composition for seedling growth and development of *D. amoenum* is listed below in Table 2.

Table 2: Media composition for seedling growth and development of *Dendrobium amoenum.*

✓ FMS	✓ HMS+0.5BAP	✓ FMS+0.25IBA+10%cw
✓ HMS	✓ HMS+1BAP	✓ FMS+0.5IBA+10%cw
✓ FMS+10%cw	✓ FMS+0.25IBA	✓ FMS+1IBA+10%cw
✓ HMS+10%cw	✓ FMS+0.5IBA	✓ FMS+0.25IAA+10%cw
✓ FMS+0.25NAA	✓ FMS+1IBA	✓ FMS+0.5IAA+10%cw
✓ FMS+0.5NAA	✓ FMS+0.25IAA	✓ FMS+1IAA+10%cw
✓ FMS+1NAA	✓ FMS+0.5IAA	✓ HMS+0.25NAA+10%cw
✓ HMS+0.25NAA	✓ FMS+1IAA	✓ HMS+0.5NAA+10%cw
✓ HMS+0.5NAA	✓ FMS+0.25NAA+10%cw	✓ HMS+1NAA+10%cw
✓ HMS+1NAA	✓ FMS+0.5NAA+10%cw	✓ HMS+0.25BAP+10%cw
✓ FMS+0.25BAP	✓ FMS+1NAA+10%cw	✓ HMS+0.5BAP+10%cw
✓ FMS+0.5BAP	✓ FMS+0.25BAP+10%cw	✓ HMS+1BAP+10%cw
✓ FMS+1BAP	✓ FMS+0.5BAP+10%cw	
✓ HMS+0.25BAP	✓ FMS+1BAP+10%cw	

3.3 Phytochemical extraction

3.3.1 Extraction from *in vitro* raised seedlings of *Dendrobium amoenum*

About 200 gm of *in vitro*-grown *Dendrobium amoenum* plantlets were shade dried at room temperature for 30 days. The dried plantlets were powdered using an electric grinder. 5 gm of the fine powder thus obtained was mixed with a 9:1 mixture of methanol and distilled water and stored at 4ºC for 72 hrs of maceration. The mixture was mixed with a stirrer for 15 to 20 minutes and filtered using Whatman No.1 filter paper (Whatman, UK). Again, a 9:1 mixture of methanol and distilled water was poured into the residue and stored at 4ºC for 72 hours. The mixture was filtered and added to the previous. The filtrate thus obtained was partially concentrated using a rotatory evaporator (Hahnvapor HS-2005V-N). The extract was dried at room temperature.

3.3.2 Sephadex Separation

Sephadex separation is among the most effective ways for separating and purifying chemical compounds based on their migration across a stationary bed of a porous semi-liquid material (mainly silica gel). It is also referred to as size exclusion chromatography. It separates substances through the differential adsorption of

compounds to the absorbent as the compounds pass along the column at various rates, allowing them to be fractionated. The different fractions are gathered separately as they depart the bottom of the column by shifting receivers. Colourless chemicals are commonly separated by collecting numerous small fractions from the column in succession, testing each fraction chemically or otherwise, and merging all fractions having a single component.

The crude methanolic extract of *D. amoenum* was separated by Sephadex separation using methanol (Figure 2). Initially, the column was poured with methanol to purify and maintain the level of wax. The methanol was poured repeatedly into the column to maintain the level. At first, the crude dried extract was dissolved in 5 ml of methanol. Then, the solution of methanol extract was poured into a column. The column was kept in an iron stand for 30 minutes and the methanol was poured into the column. The different fraction bands were seen on the column. All the fractions were collected according to their colour bands in separate vessels along with the solvent. The colour band fractions were then concentrated using the rotary evaporator under reduced pressure below 40ºC. The dried form thus obtained was weighed and stored at 4 ºC.

3.4 Total phenolic content

To determine the total phenolic content in the methanolic fractions of *D. amoenum* extract, the Folin-Ciocalteau phenol reagent colourimetric method was used, following the protocol by Zhang *et al*. (2006) with slight modifications. In a 96-well plate, 75 µl of distilled water was added to each well, followed by 25 µl of either the sample (1mg/ml) or standard (25-200 mg/ml) and 25 µl of F–C reagent (diluted 1:1 with distilled water at first). The mixture was pipetted repeatedly to mix and left to incubate for 6 minutes. Then, 100 µl of 1M Na_2CO_3 was added to each well. The solutions were mixed again, covered, and left in the dark at room temperature for 90 minutes. The absorbance at 765 nm was measured with a microplate reader (Azure Biosystems Microplate Spectrophotometer) after an incubation of 90 minutes. A control was prepared by replacing the same volume of plant extract with absolute methanol.

Figure 2: Extraction of phytochemicals in 90% Methanol (A); Evaporating solvent with the help of rotary evaporator (B); Sephadex separation (C); Fractionated extracts collected based on the colour band in different vessels (D).

The total phenolic content was determined using a calibration curve obtained from a linear regression equation with gallic acid as a standard. The equation was $y=0.0061x+0.0224$, with an R^2 value of 0.9975, where y represents absorbance and x represents gallic acid concentration (Figure 3). Gallic acid was used at concentrations of 25, 50, 100, and 200 µg/ml to generate the calibration curve. The total phenolic content was expressed in milligrams of gallic acid equivalent per gram of plant extract (mg GAE/gm). The absorbance was measured in triplicate at each concentration.

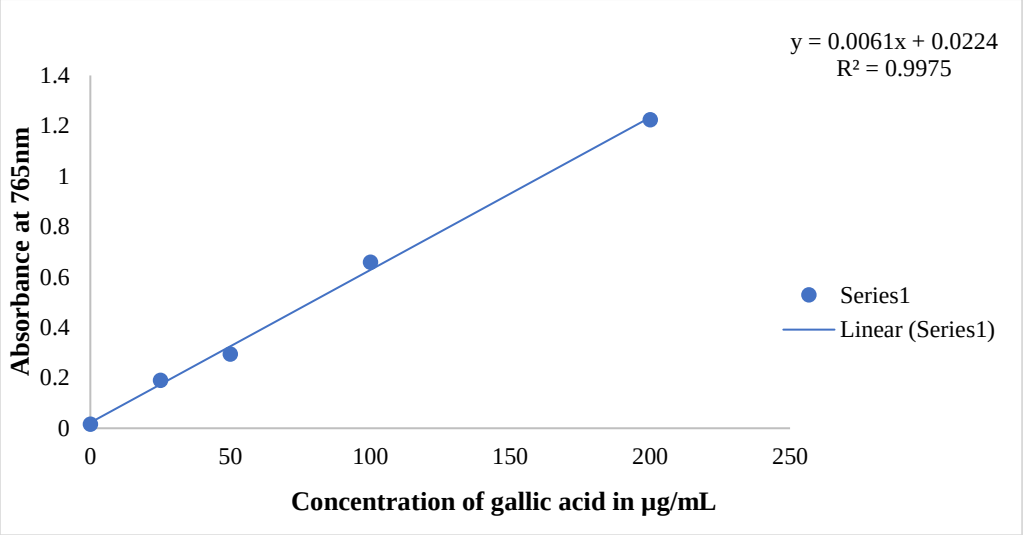

Figure 3: Calibration curve of gallic acid

3.5 Total flavonoid content

The total flavonoid content of *D. amoenum* methanolic extracts was determined using the Aluminum Chloride ($AlCl_3$) colourimetric method based on the protocol of Chang *et al*. (2002) with some modifications. Firstly, a 1 mg/ml plant extract solution was prepared, and 250 µl of this solution was mixed with 750 µl of 10% AlCl3 and 50 µl (1M) of potassium acetate in a test tube. The mixture was diluted by adding 1.4 ml of distilled water and left to incubate at room temperature for 30 minutes. The blank was prepared with absolute methanol. Then, 200 µl of the reaction mixture was transferred into triplicate wells of a 96-well plate. The absorbance at 415 nm was measured with a microplate reader (Azure Biosystems Microplate Spectrophotometer).

The total flavonoid content was calculated using the quercetin equation (y= 0.0031x-0.0195, R^2=0.9978) obtained through linear regression analysis (Figure 4). The x-axis represents the concentration of quercetin, while the y-axis shows the absorbance in triplicate at each concentration. A calibration curve was generated using quercetin as a standard at concentrations of 25, 50, 100, and 200 µg/ml. The total flavonoid content was expressed in milligrams of Quercetin equivalent (mg QE/gm) per gram of plant extract.

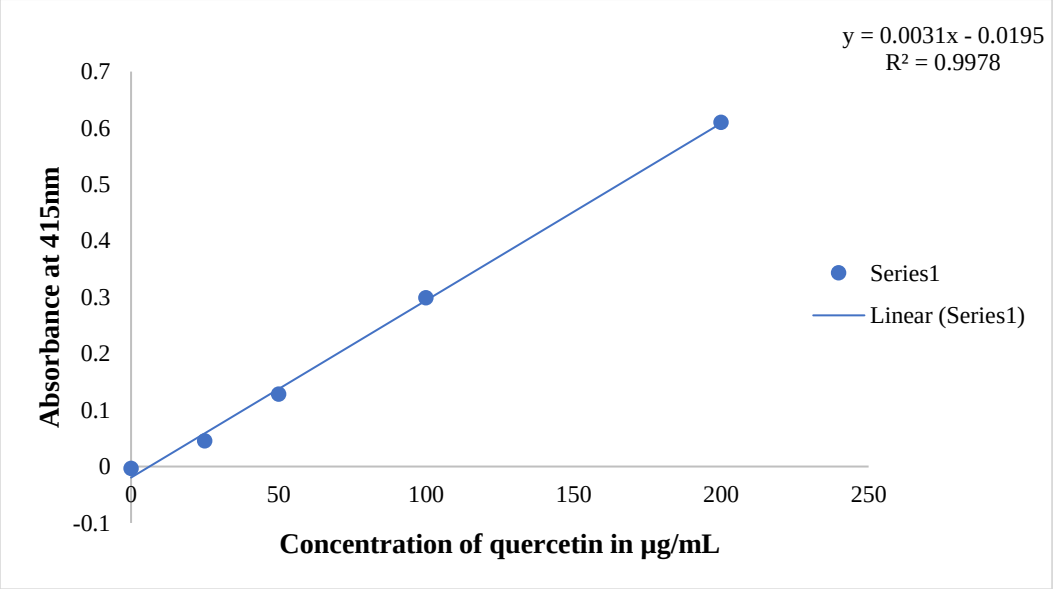

Figure 4: Calibration curve of quercetin

3.6 Antioxidant activity

Free radicals are harmful molecules that can cause various degenerative diseases like cancer, cardiovascular problems, and ageing (Singh & Singh, 2008). They can be generated within the body or come from external sources. Antioxidants are substances that can counteract the harmful effects of free radicals by interrupting their oxidative process (Cui *et al*., 2004). The DPPH method is commonly used to evaluate the antioxidant activity of substances by measuring their ability to neutralize free radicals or donate hydrogen. This method is affordable, simple, and widely used in complex biological systems. The DPPH molecule is considered a stable free radical because of the electron delocalization that prevents dimerization.

The antioxidant activity of fractions of *Dendrobium amoenum* extract was evaluated using the free radical 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), following the protocol by Ben Mansour *et al*. (2016). Stock solutions of all fractionated extracts of *D. amoenum* and ascorbic acid (positive control) were prepared using methanol. The solutions were then serially diluted to obtain concentrations of 200, 100, 50, and 25 µg/ml. A fresh DPPH solution of 0.2 mM was prepared by dissolving 7.88 mg of DPPH powder in 100 ml of methanol and kept away from direct light. Next, 50 µl of each sample (ranging from 25 to 200 µg/ml) was mixed with 150 µl of DPPH in a 96-well plate in triplicate. A control sample was also prepared using absolute methanol. The reaction mixture was incubated in the dark at room temperature for 30 minutes.

The absorbance of the reaction mixture was measured at 517 nm using a microplate reader (Azure Biosystems Micro-plate Spectrophotometer) after incubation at room temperature for 30 minutes. The measurement was carried out in triplicate, and the sample with the lowest absorbance value was considered to have the highest activity. The free radical scavenging activity of the plant samples was calculated as a percentage using the following equation:

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

Using the standard curve, a graph was created with concentration plotted on the X-axis and the percentage of radical scavenging activity on the Y-axis. The antioxidant potential of the plant extract was determined by calculating the concentration required to suppress 50% of the DPPH radicals, which is expressed as IC_{50} (in µg/ml of extract). The IC_{50} value was calculated using the following linear equation:

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

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

3.7 Cytotoxic activity

3.7.1 Cell line

A cell line refers to a group of cells that can multiply independently, typically in a laboratory outside of an organism. There are two types of cell lines, namely immortal and finite. Immortal cell lines can replicate continuously, while finite cell lines have a limit to their replication. Cancer cell lines, which are immortal, have been extremely useful for cancer research. Normally, human cells have finite lifespans due to internal controls that regulate their replication. However, cancer cells are immortal and can divide endlessly, making them valuable for scientific studies. This property of immortal cell lines, such as the HeLa cell line, makes them resilient and abundant for research purposes.

3.7.1.1 HeLa cell line

The HeLa (human cervical cancer) cell line was the first immortal cell line and is still the most widely used human cell line. HeLa cells have been employed by scientists to

make a wide range of scientific discoveries. HeLa cell lines were isolated from Henrietta Lacks in 1951 by researchers George Otto Gey, Margaret Gey, and Mary Kucibek at the Johns Hopkins Hospital in the city of Baltimore, Maryland. HeLa cells, which were first isolated from the tumour of a woman suffering from cervical cancer in the 1950s, have been grown indefinitely for scientific purposes. They have been used for a variety of reasons, including the development of a polio vaccine, the search for a treatment for diseases like leukaemia and cancer, and the investigation of the cellular effects of medications and radiation.

3.7.1.2 U-2OS cell line

U2OS is a type of cell line derived from an osteosarcoma tumour found in a 15-year-old female (Ponten & Saksela, 1967). Osteosarcoma is a type of bone cancer that develops from mesenchymal cells transforming into osteoblasts. It is a common type of cancer in pediatric patients, accounting for 2.4% of all cancers in this age group, and its causes are still not fully understood (Jaffe *et al*., 2010). U2OS is a widely used cell line in osteosarcoma research and has been utilized in 35% of research publications related to osteosarcoma listed in the PubMed database.

3.7.1.3 Normal cell line

A human normal cell line is a group of cells generated from normal human tissue and can continuously divide and multiply in laboratory culture conditions. These cell lines are extensively utilized in scientific research and medical applications as control or reference cells because they serve as a baseline of normal cellular behaviour and are free of genetic or functional defects that are present in diseased or malignant cells. Fibroblasts, epithelial cells and blood cells are some of the most common types of human normal cell lines.

3.7.2 MTT assay

MTT is a tetrazolium salt that is converted to a colored formazan by enzymes found only in cells that are metabolically active. Mosmann (1983) described the MTT test as a fast colorimetric enzyme-based assay. The MTT assay involves the use of a water-soluble yellow dye called MTT, which is converted to an insoluble purple formazan through the action of mitochondrial reductase. This assay is commonly used to measure

viable cells in a high-output format (such as 96-well plates) without the need for complicated cell counting. The underlying principle of the assay is that mitochondrial activity is relatively constant in most viable cells, so changes in viable cell number are proportional to mitochondrial activity. The MTT assay measures mitochondrial activity by detecting the conversion of MTT into formazan crystals, which can be solubilized and measured for homogeneous analysis. Optical density (OD) readings at 540 and 720 nm are used to measure the concentration of formazan, which reflects the mitochondrial activity of the cells. In drug sensitivity studies, the OD values of cells treated with drugs are compared to the OD values of cells that were not treated with drugs.

To assess the cytotoxic effects of methanolic fractions of *Dendrobium amoenum* extract, a modified MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) colourimetric assay was carried out in 96-well flat-bottomed microtiter plates (Corning). The study involved three cell lines, including two cancer cell lines (HeLa and U2OS) and a normal human cell line. These cell lines were cultured in Minimum Essential Medium Eagle (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine and maintained at 37°C in a 5% CO_2 incubator.

The cells were cultured in 96-well plates and treated with different concentrations (25, 50, 100, 200 µg/ml) of plant extracts for 48 hours. After the incubation period, MTT reagent was added, and a purple formazan product was formed. The formazan crystals were dissolved in DMSO, and the plate was read at 595 nm using an Elisa reader. The percentage of dead cells was calculated as a percentage of the control, which allowed for the assessment of cell death caused by the plant extract fractions. The IC_{50} value, representing the concentration of extract that suppresses cell growth by 50%, was calculated using a dose-response curve. The percentage of cytotoxic activity was determined using a formula.

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

Whereas, Abs1 indicates the absorbance of the cell containing all the components except for the plant extracts, Abs2 indicates the absorbance of the cell containing all the components, including the plant extracts.

3.8 Statistical Analysis

The complete seedling growth was recorded based on the number and length of shoot and root development at different concentrations in the culture medium. The mean values of the weekly measurements were analyzed using the Duncan test at $p \leq 0.05$ significance.

A conventional linear equation was used to estimate the total polyphenol and flavonoid contents. The Duncan multiple-range test was employed at $p \leq 0.05$ to determine the significance of differences in total polyphenol and flavonoid content, mean IC_{50} of antioxidant activity, and mean IC_{50} of HeLa, U2OS, and normal cell growth across fractions of methanolic extract. Standard equations for gallic acid and quercetin were obtained using a linear regression model. A regression equation ($y = mx + c$) in MS Excel was used to establish the relationships between antioxidant activity and total flavonoid and total polyphenol levels. The IC_{50} values of the extracts' antioxidant and cytotoxic activities were estimated using an appropriate linear regression equation derived from the mean percentage activity of each fraction of methanolic extract at various concentrations. All measurements were conducted in triplicate, and the data were collected in MS-Excel 2019 and analysed using SPSS software.

CHAPTER 4: RESULT

4.1 Growth and development of seedlings

An explant's growth, development, and morphogenic response in culture is influenced by its genetic make-up, surrounding environment, and culture medium composition. The fresh medium of different combinations with supplementation of different concentrations of PGRs and CW was used for the development of seedlings. The shoot tips were cultured in small groups into MS medium. Twenty-eight combinations of different strengths of MS medium were used for the growth of seedlings.

In a basal medium of different strengths, the growth of shoot number and the length of the shoot in FMS medium (3.33±0.33) and (1.5±0.17 cm) respectively were found to be higher than in HMS medium (2.67±0.88) and (1.03±0.09 cm) respectively. Among the basal medium of different concentrations, FMS, FMS media supplemented with BAP (0.25 mg/l), FMS supplemented with NAA (1 mg/l) and HMS supplemented with NAA (1 mg/l) showed equal growth in terms of shoot number, while the length of the shoot in FMS supplemented with NAA (1 mg/l) and HMS medium supplemented with NAA (1 mg/l) was found to be lower as compared to FMS and FMS medium supplemented with BAP (0.25 mg/l) as shown in Table 3 and Figure 5. Similarly, the basal medium of full and half strengths supplemented with different concentrations of CW, NAA, and BAP showed a significantly higher shoot number and growth of shoot length. The addition of CW to the medium showed a significantly greater shoot number as well as in the growth of the length of shoot than the basal medium. The FMS medium fortified with 10% CW performed better in the growth of shoot number 13.67±1.86 with shoot length 3.00±0.51 cm than the HMS medium fortified with 10% CW with shoot number 7.33±0.88 with shoot length 2.57±0.23 cm.

FMS medium supplemented with NAA (0.25 mg/l) and the addition of 10% CW achieved the highest growth in shoot length 4.17±0.58 cm with an increase in shoot number 19.33±2.40 which was followed by FMS medium supplemented with NAA (0.5 mg/l) and 10% CW of shoot number 18.67±3.92 and shoot length 2.0±0.23cm respectively. HMS medium supplemented with NAA (0.25 mg/l) and 10% CW yielded the lowest growth in terms of shoot number (5.67±1.76) but recorded healthy growth in shoot length (3.13±0.18 cm). The lowest growth in shoot number (1.67±0.33) was

achieved in HMS fortified with NAA (0.25 mg/l). However, the increase in concentrations of NAA in both FMS and HMS medium performed lesser growth in shoot length with a lesser shoot number. With the addition of different concentrations of BAP in both FMS and HMS medium, an equal increase in shoot number was found in BAP (1 mg/l) in both FMS (11.67±2.03) and HMS medium (11.66±0.88) with supplementation of 10% CW. The highest growth in the shoot was achieved in both full and half MS medium supplemented with BAP of 0.5 mg/l and 1mg/l along with 10% CW as shown in Table 3. However, the increase in the concentration of BAP in both FMS and HMS medium led to higher growth in shoot length as well as shoot number.

Table 3: Growth and development of *Dendrobium amoenum* shoot measured in terms of number and length in basal MS medium supplemented with different hormone compositions.

Medium	**Shoot number (Mean±SE)**	**Shoot length (cm) (Mean±SE)**
FMS	3.33 ± 0.33^{c}	1.50 ± 0.17^{b}
HMS	2.67 ± 0.88^{b}	1.03 ± 0.09^{a}
FMS+10%CW	13.67 ± 1.86^{g}	3.00 ± 0.51^{d}
HMS+10%CW	7.33 ± 0.88^{e}	2.57 ± 0.23^{c}
FMS+0.25NAA	4.00 ± 0.58^{d}	1.87 ± 0.27^{b}
FMS+0.5NAA	6.67 ± 0.67^{e}	1.00 ± 0.12^{a}
FMS+1NAA	3.00 ± 0.58^{c}	1.17 ± 0.09^{a}
FMS+0.25NAA+10%CW	19.33 ± 2.40^{h}	4.17 ± 0.58^{e}
FMS+0.5NAA+10%CW	18.67 ± 3.92^{h}	2.00 ± 0.23^{c}
FMS+1NAA+10%CW	5.33 ± 1.20^{e}	1.37 ± 0.35^{a}
HMS+0.25NAA	1.67 ± 0.33^{a}	1.13 ± 0.18^{a}
HMS+0.5NAA	5.67 ± 0.33^{e}	1.10 ± 0.06^{a}
HMS+1NAA	3.00 ± 0.58^{c}	1.07 ± 0.07^{a}
HMS+0.25NAA+10%CW	5.67 ± 1.76^{e}	3.13 ± 0.18^{d}
HMS+0.5NAA+10%CW	4.33 ± 0.88^{d}	1.77 ± 0.15^{b}
HMS+1NAA+10%CW	4.00 ± 1.00^{d}	1.33 ± 0.24^{a}
FMS+0.25BAP	3.33 ± 0.88^{c}	1.77 ± 0.12^{b}
FMS+0.5BAP	4.67 ± 0.67^{d}	1.37 ± 0.09^{a}
FMS+1BAP	6.00 ± 0.58^{e}	2.17 ± 0.12^{c}
FMS+0.25BAP+10%CW	2.33 ± 0.67^{b}	1.47 ± 0.18^{a}
FMS+0.5BAP+10%CW	8.33 ± 1.20^{f}	2.16 ± 0.18^{c}
FMS+1BAP+10%CW	11.67 ± 2.03^{g}	2.93 ± 0.37^{c}
HMS+0.25BAP	4.33 ± 1.20^{d}	1.67 ± 0.09^{b}
HMS+0.5BAP	4.67 ± 1.20^{d}	1.20 ± 0.12^{a}
HMS+1BAP	5.33 ± 1.45^{e}	2.16 ± 0.18^{c}
HMS+0.25BAP+10%CW	4.67 ± 0.67^{d}	1.27 ± 0.13^{a}
HMS+0.5BAP+10%CW	9.33 ± 0.88^{f}	1.90 ± 0.12^{b}
HMS+1BAP+10%CW	11.67 ± 0.88^{g}	2.67 ± 0.13^{c}

Values with different alphabets differ significantly at $p\leq0.05$ (Duncan test)

Similarly, FMS mediums of different combinations with supplementation of different concentrations of rooting hormones, i.e., NAA, IBA, IAA, and CW, were used for the development of roots. The seedlings with small leaves were sub cultured in small groups into MS medium. 19 different combinations of FMS medium were used for the root proliferation of *Dendrobium amoenum* (Table 4).

Table 4: Growth and development of *Dendrobium amoenum* roots measured in terms of number and length in basal MS medium supplemented with different hormone compositions.

Medium	Root number (Mean±SE)	Root length (cm) (Mean±SE)
FMS+10%CW	4.33±0.33[a]	0.37±0.06[a]
FMS+0.25NAA	3.67±0.67[a]	0.97±0.42[a]
FMS+0.5NAA	3.00±1.00[a]	0.30±0.05[a]
FMS+1NAA	3.00±1.00[a]	0.33±0.08[a]
FMS+0.25NAA+10%CW	3.00±1.15[a]	0.33±0.08[a]
FMS+0.5NAA+10%CW	4.00±1.73[a]	0.67±0.17[a]
FMS+1NAA+10%CW	4.00±1.00[a]	1.03±0.73[b]
FMS+0.25IBA	4.67±0.88[a]	1.33±0.22[b]
FMS+0.5IBA	6.33±0.67[b]	2.33±0.52[c]
FMS+1IBA	5.33±1.33[b]	1.03±0.33[b]
FMS+0.25IBA+10%CW	4.33±1.86[a]	1.47±0.12[b]
FMS+0.5IBA+10%CW	2.67±0.33[a]	0.27±0.06[a]
FMS+1IBA+10%CW	5.67±1.76[b]	0.80±0.23[a]
FMS+0.25IAA	8.67±5.17[b]	2.13±0.55[c]
FMS+0.5IAA	5.00±0.58[b]	0.73±0.24[a]
FMS+1IAA	5.00±1.73[b]	0.47±0.18[a]
FMS+0.25IAA+10%CW	3.00±0.58[a]	0.93±0.44[a]
FMS+0.5IAA+10%CW	2.33±0.88[a]	0.53±0.24[a]
FMS+1IAA+10%CW	3.33±0.33[a]	1.00±0.30[b]

Values with different alphabets differ significantly at $p \leq 0.05$ (Duncan test)

FMS medium fortified with IAA (0.25 mg/l) and IBA (0.5 mg/l) induced the longest roots (2.13±0.55 cm) and (2.33±0.52 cm) and with the more roots of 8.67±5.17 and 6.33±0.67 and respectively. 10% CW developed healthy growth in the number of roots in the FMS medium. FMS medium supplemented with different concentrations of IAA showed the best root growth in terms of number and length per shoot. Apart from IAA, the FMS medium supplemented with IBA provided healthy root growth as shown in Table 4. The lowest growth in root was achieved in the FMS medium fortified with different concentrations of NAA. Overall, the FMS medium supplemented with 0.25 mg/l NAA and 10% CW was found to be the best medium for the successful growth and development of seedlings into healthy plants of *Dendrobium amoenum* (Figure 5).

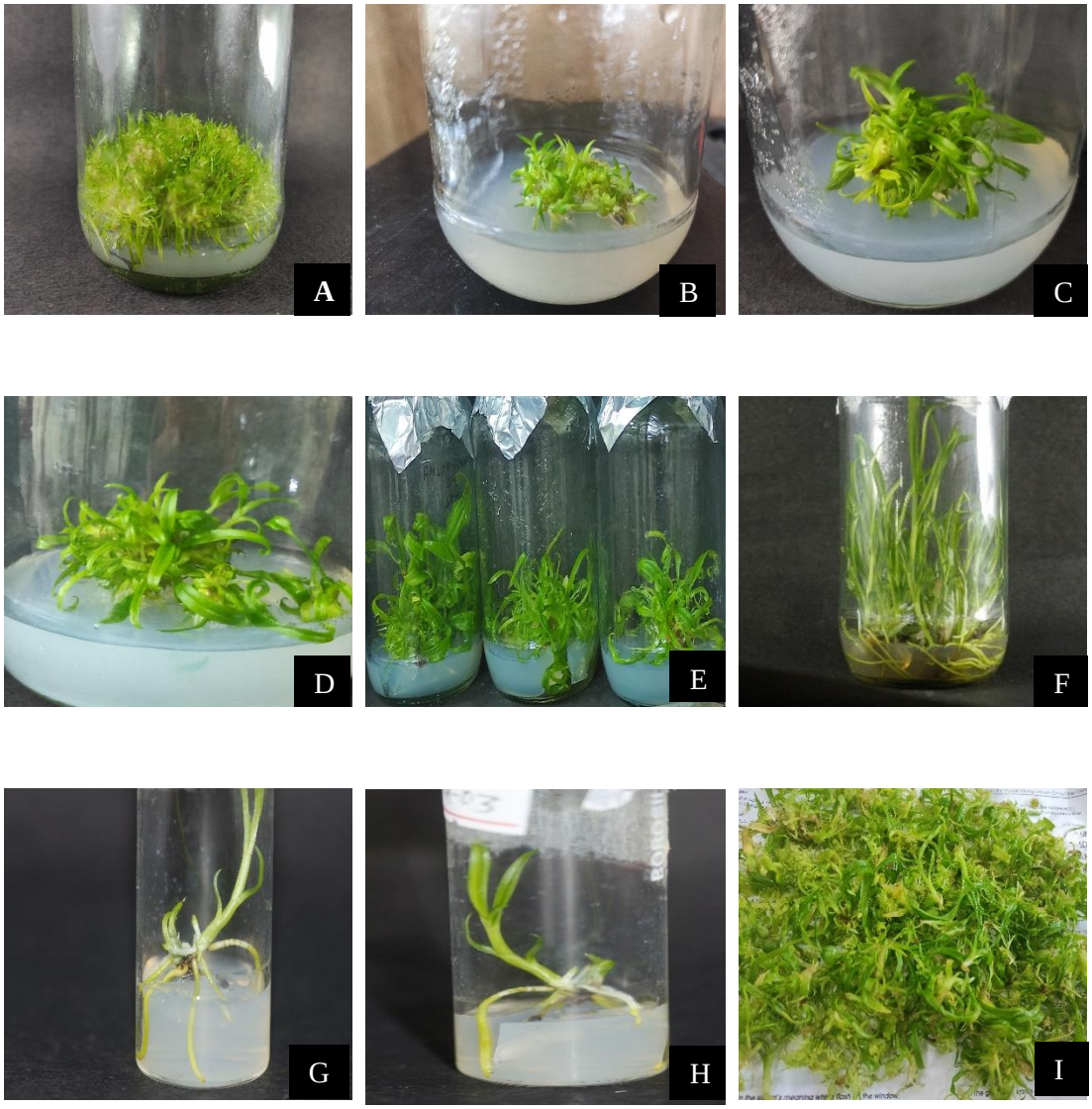

Figure 5: *In vitro* seedlings growth of *Dendrobium amoenum*.

(*In vitro* grown plant in lab (A); A month old plants in FMS with 0.25 mg/l NAA+10% CW (B); Two months old plants in FMS with 0.25 mg/l NAA+10% CW (C); Three months old plants in FMS with 0.25 mg/l NAA+10% CW (D); Four months old plants in FMS with 0.25 mg/l NAA+10% CW (E); Five months old plants in FMS with 0.25 mg/l NAA+10% CW (F); Longest root in FMS with 0.5 mg/l IBA (G); Highest root number in FMS with 0.25 mg/l IAA (H); and Green seedling taken out from jar for shade drying (I).)

4.2 Extract yield

About 1.089 gm of crude methanolic extract was obtained from 5 gm dry powder by extraction which was later fractionated by Sephadex separation.

4.3 Total Phenolic and flavonoid content

The total phenolic content was calculated in terms of gallic acid equivalent using the Folin-Ciocalteau phenol reagent colourimetric method. The equation of standard gallic acid ($y = 0.0061x+0.0224$, $R^2= 0.9975$) obtained from the linear regression model was statistically significant. The highest mean percentage of phenolic content was found in DAYF (206.38 µg GAE/mg extract), and the lowest mean percentage of phenolic content was found in DADGF (74.79 µg GAE/mg extract) as shown in Figure 6.

Similarly, the total flavonoid content was determined using the colourimetric method of Aluminium Chloride ($AlCl_3$) and expressed in terms of quercetin equivalent. The equation of standard quercetin obtained from the linear regression model ($y =0.0031x-0.0195$, $R^2 = 0.995$) was also significant. The highest mean percentage of flavonoid content was also found in DAYF (101.88 µg QE/mg extract), and the lowest mean percentage of flavonoid content was found in DADGF (35.21 µg QE/mg extract), which was shown in Figure 6.

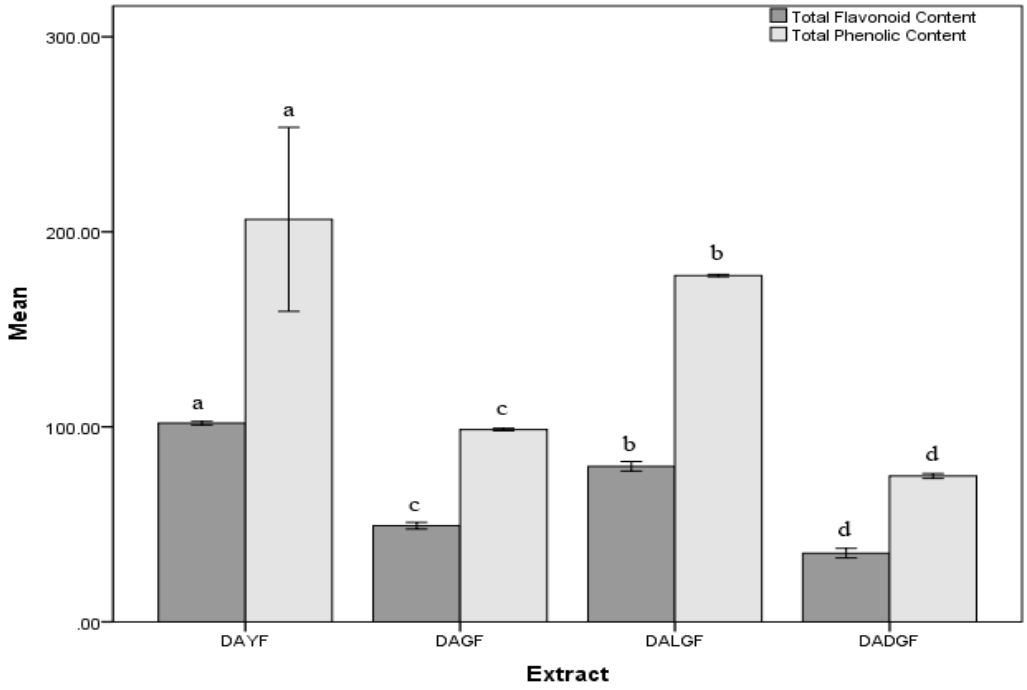

Figure 6: Mean values of total phenolic and flavonoid contents of methanolic extracts of column band fractions.

4.4 Antioxidant activity using DPPH

The DPPH free-radical scavenging test was used to evaluate the antioxidant activity of four fractions of methanolic extract. The extract fractions exhibited varying degrees of antioxidant activity, and the mean percentage of DPPH free-radical scavenging activity was determined for concentrations ranging from 25 to 200 µg/ml. The yellow fraction (DAYF) was found to have the highest percentage of DPPH free-radical scavenging activity (83.24±0.44%), followed by the light green fraction (DALGF), the green fraction (DAGF), and the dark green fraction (DADGF) at a considerably lower level at their 200 µg/ml concentrations, as indicated in Table 5. It was also observed that the mean percentage of radical scavenging activity increased as the concentration increased in all cases.

Table 5: Percentage of DPPH radical scavenging activity of *Dendrobium amoenum*.

Fractions	**Concentration of extract (µg/ml)**			
	25	**50**	**100**	**200**
DAYF	22.58±0.39	53.49±0.56	76.98±0.30	83.24±0.44
DAGF	10.01±0.39	16.93±0.44	38.97±0.25	51.66±0.45
DALGF	21.55±0.47	38.97±0.44	57.19±0.44	69.94±0.53
DADGF	10.74±0.53	19.85±0.39	35.82±0.61	46.38±0.52

The values are given in terms of a mean ± standard deviation.

The IC_{50} value of each extract was used to determine its antioxidant activity, which represents the quantity of the extract required to scavenge 50% of DPPH free radicals. It was observed that the IC_{50} value of the yellow fraction (DAYF) of *D. amoenum* was found to be the lowest (63.73±0.38µg/ml) which means the yellow fraction (DAYF) has the highest antioxidant activity. Similarly, the IC_{50} value of dark green fraction (DADGF) was found highest (204.27±2.75µg/ml) which means the dark green fraction (DADGF) has low antioxidant activity. The IC_{50} value of the yellow fraction (DAYF) was found to be significantly different from other fractions as shown in Figure 7. The antioxidant activity of fractions is ranged from yellow (DAYF)> light green (DALGF) >green (DAGF) >dark green (DADGF).

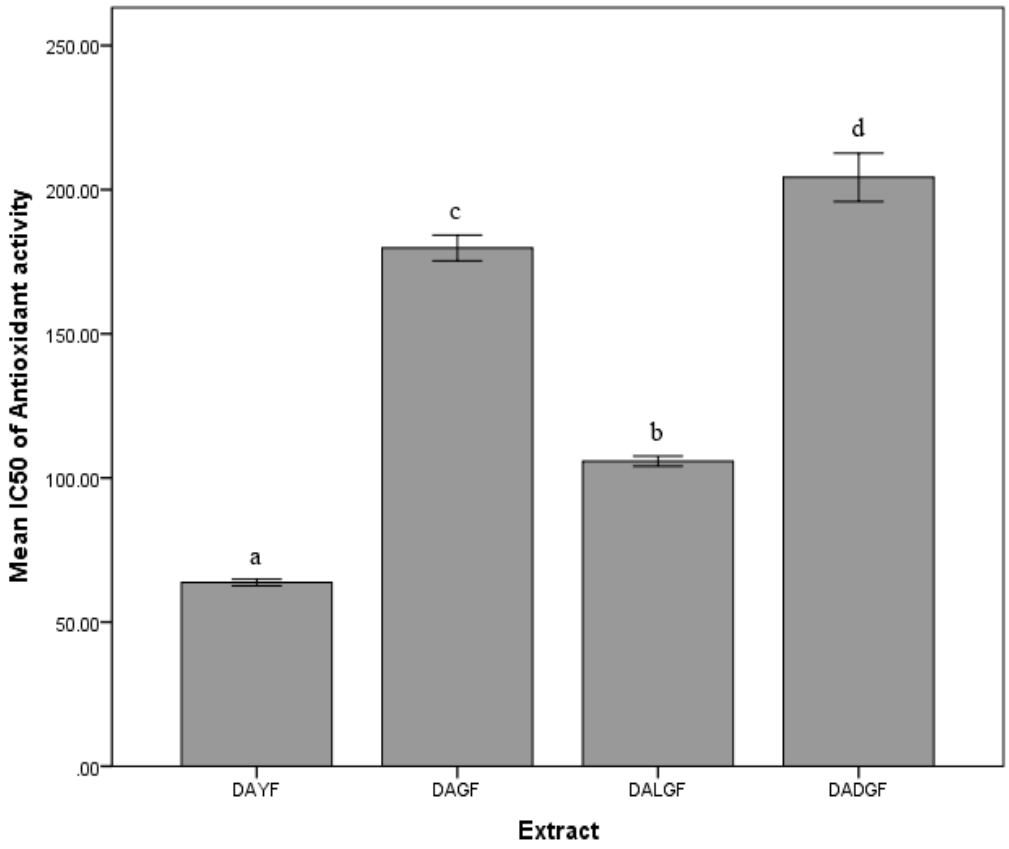

Figure 7: Mean IC_{50} of antioxidant activity of four fractions of a methanolic extract of *D. amoenum*.

4.5 Relationship between antioxidant activity and total phenolic and flavonoid contents

The IC_{50} values for the DPPH radical scavenging activity of methanolic extract fractions are strongly and negatively correlated with both total phenolic and flavonoid content implying a direct relationship between these factors. The strong negative correlation between IC_{50} values and total phenolic content, as indicated by a Pearson correlation coefficient of -0.987, implies that these two factors have a strong and direct relationship, as shown in Figure 8. The IC_{50} value decreases as the total phenolic content increases, or vice-versa, indicating that the compound becomes less efficient at inhibiting the biological process being tested. The coefficient of determination (R^2) value of 0.974 confirms this relationship, suggesting that total phenolic content can explain 97.4% of the variance in IC_{50} values. This indicates that total phenolic content is a good predictor of the IC_{50} value, and the association between the two variables is statistically significant.

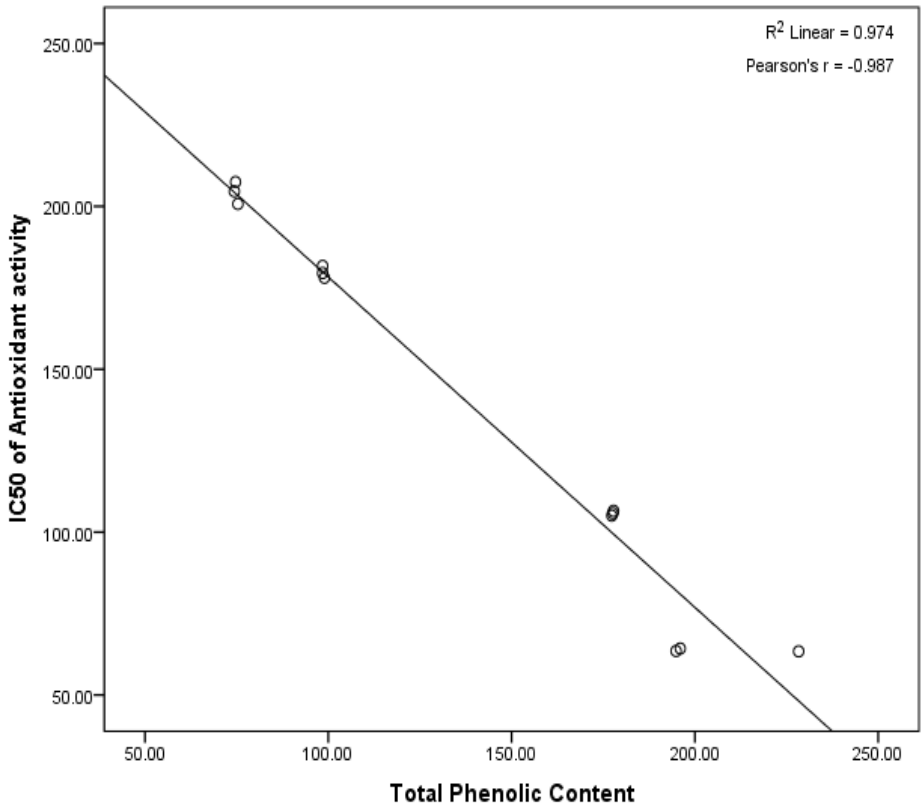

Figure 8: Regression of IC_{50} with total phenolic content.

Similarly, as in Figure 9, Pearson's correlation value (r) of -0.998 between IC_{50} and total flavonoid content indicates a significant negative relationship between the two factors. A negative correlation of -0.998 in this context indicates that as the IC_{50} value (a measure of pharmacological potency) increases, the total flavonoid content decreases, and vice-versa.

In this case, a high R-squared value of 0.995 implies that the total flavonoid content can explain 99.5% of the variation in IC_{50}, showing a significant relationship between these two factors. The results suggest that as the total polyphenol and flavonoid content of the fractions increases, the IC_{50} value decreases, indicating that the substance becomes more effective at neutralizing the DPPH radical.

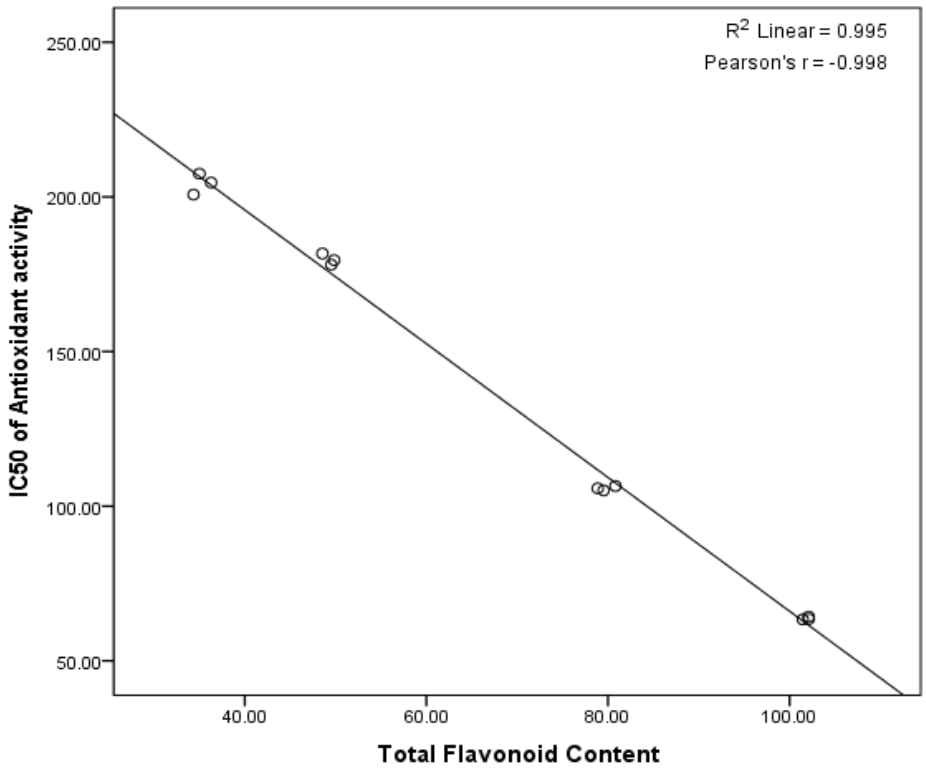

Figure 9: Regression of IC_{50} with total flavonoid content.

4.6 Cytotoxicity activity

The fractions of methanolic extract obtained through Sephadex separation were used in cytotoxicity tests according to their different colour fractions. MTT colourimetric test was used to assess the cytotoxic activity toward HeLa (human cervical cancer), U2OS (human osteosarcoma), and Normal cell lines. The present study found that the fractions of methanolic extract of *D. amoenum* were found to have the most effective cytotoxic activity toward both HeLa and U2OS cancer cells. After the addition of MTT dye, formazan crystals developed in live cells, but no crystals formed in cells killed by extracts. Table 6 shows the proportion of HeLa cell growth inhibition caused by various fractions. When the concentration of the extract increases, so does the percentage of cell growth inhibition in extract-treated cells. The growth inhibition percentage of HeLa cells was discovered to range from 7.76±5.27% for the extract of the yellow fraction (DAGF) at 25 µg/ml to 82.36±2.10% for the extract of the green fraction at 200 µg/ml. At 200 µg/ml, the green fraction (DAGF) inhibited the proliferation of HeLa cells by a significant amount (82.36±2.10%). However, the extract of yellow fraction (DAYF) inhibited the proliferation of HeLa cells much more than other extracts at 100 µg/ml (77.82±0.37%). Similarly, at 50 µg/ml and 100 µg/ml,

the green (DAGF) and dark green (DADGF) fractions inhibited HeLa cell proliferation by the same amount. However, the dark green fraction (DAGF) inhibited the least amount of HeLa cell development.

Table 6: Percentage of HeLa cell growth inhibition by the extracts at varying concentrations.

Fractions	Concentration of extract (µg/ml)			
	25	50	100	200
DAYF	7.76±5.27	25.32±7.08	77.82±0.37	79.07±0.70
DAGF	17.36±9.59	23.02±10.03	47.82±8.29	82.36±2.10
DALGF	10.13±5.32	20.78±4.54	67.5±5.01	81.84±2.27
DADGF	13.42±14.62	16.77±16.47	47.36±9.94	78.15±1.37

The values are given in terms of a mean± standard deviation.

The U2OS cell lines showed an increase in the percentage of cell growth inhibition as the concentration of the extract increased. The growth inhibition percentage varied from 7.69±2.41% for the extract of the green fraction (DAGF) at 25 µg/ml to 64.76±3.41% for the extract of the dark green fraction (DADGF) at 200 µg/ml, as shown in Table 7. The dark green fraction (DADGF) exhibited the highest inhibition percentage (64.76±3.41%) at 200 µg/ml, which was significantly higher than the other fractions. However, the yellow fraction (DAYF) had relatively low inhibition capacities compared to the other fractions.

Table 7: Percentage of U2OS cell growth inhibition by the extracts at varying concentrations.

Fractions	Concentration of extract (µg/ml)			
	25	50	100	200
DAYF	15.86±6.31	23.22±1.97	38.76±1.53	46.2±0.31
DAGF	7.69±2.41	22.16±1.92	43.5±2.16	58.38±2.2
DALGF	13±6.03	30.17±2.58	47.83±1.42	60.59±5.34
DADGF	18.23±5.15	32.05±10.07	58.63±1.53	64.76±3.41

The values are given in terms of a mean ± standard deviation.

In the case of Normal cell lines, the percentage of cell growth inhibition of different fractions against normal cell lines was shown in Table 8. The growth inhibition percentage of normal cells varied from -16.56±1.78% to 13.51±4.43%, which is significantly non-toxic to human beings. The inhibition percentage of extracts against

a normal cell line was found to be lowest on the (DAGF) green fraction (-16.56±1.78%) which was followed by (DADGF) dark green fraction (-12.67±3.43) at 25µg/ml.

Table 8: Percentage of normal cell growth inhibition by the extracts at varying concentrations.

Fractions	Concentration of extract (µg/ml)			
	25	**50**	**100**	**200**
DAYF	-7.33±10.35	1.07±6.28	5.27±2.62	9.69±2.67
DAGF	-16.56±1.78	-4.73±3.57	6.26±4.03	9.92±15.38
DALGF	2.52±4.96	7.25±6.03	9.39±1.77	13.51±4.43
DADGF	-12.67±3.43	0.08±6.29	4.27±3.9	7.86±8.98

The values are given in terms of a mean ± standard deviation.

The IC_{50} value assesses an extract's ability to inhibit cell growth. The yellow (DAYF) and light green (DALGF) fractions exhibited similar mean IC_{50} values of 67.03 µg/ml and 77.99µg/ml respectively against HeLa cells as shown in Figure 10. The green (DAGF) and dark green (DADGF) fractions, on the other hand, had higher mean IC_{50} values of 110.92µg/ml and114.87µg/ml, respectively, which were similar to each other. This indicates that the green (DAGF) and dark green (DADGF) fractions are less cytotoxic, or have a lower ability to destroy/kill HeLa cells, than the yellow (DAYF) and light green (DALGF) fractions. The difference in cytotoxic capabilities between the extracts was statistically significant, meaning that it is unlikely to be due to chance.

In the U2OS cell line, the dark green fraction (DADGF) had the lowest mean IC_{50} value of 119.54 µg/ml, suggesting it was the most cytotoxic to the cells among the four fractions. The green (DAGF) and light green (DALGF) fractions had similar IC_{50} values of 155.91 µg/ml and 142.92 µg/ml respectively (Figure 10), indicating similar efficacy in inhibiting cell growth. The yellow fraction (DAYF) had the highest mean IC_{50} value of 207.40±9.11 µg/ml, indicating that it was less toxic or less effective in inhibiting cell growth compared to the other three fractions.

The green fraction (DAGF) was found to have a low IC_{50} value of 942.75 µg/ml in the case of Normal cells, indicating that it is more powerful in inhibiting normal cell growth than the light green fraction (DALGF). This suggests that lower concentrations of the green fraction are needed to inhibit Normal cell growth by 50% as compared to the light green fraction. The yellow (DAYF) and dark green (DADGF) fractions were

found to have similar IC_{50} values against normal cells of 1316.18 µg/ml and 1227.8 µg/ml, respectively, implying that they are also more powerful in inhibiting Normal cell growth than the light green fraction. The yellow (DAYF) and dark green (DADGF) fractions were found to have similar IC_{50} values against Normal cells of 1316.18 µg/ml and 1227.8 µg/ml, respectively (Figure 10), implying that they are also more powerful in inhibiting Normal cell growth than the light green fraction. Hence, the IC_{50} value of Normal cells was found to be highest among HeLa and U2OS cells. It was revealed that the extracts of *D. amoenum* can inhibit the growth of cancer cells in the human body.

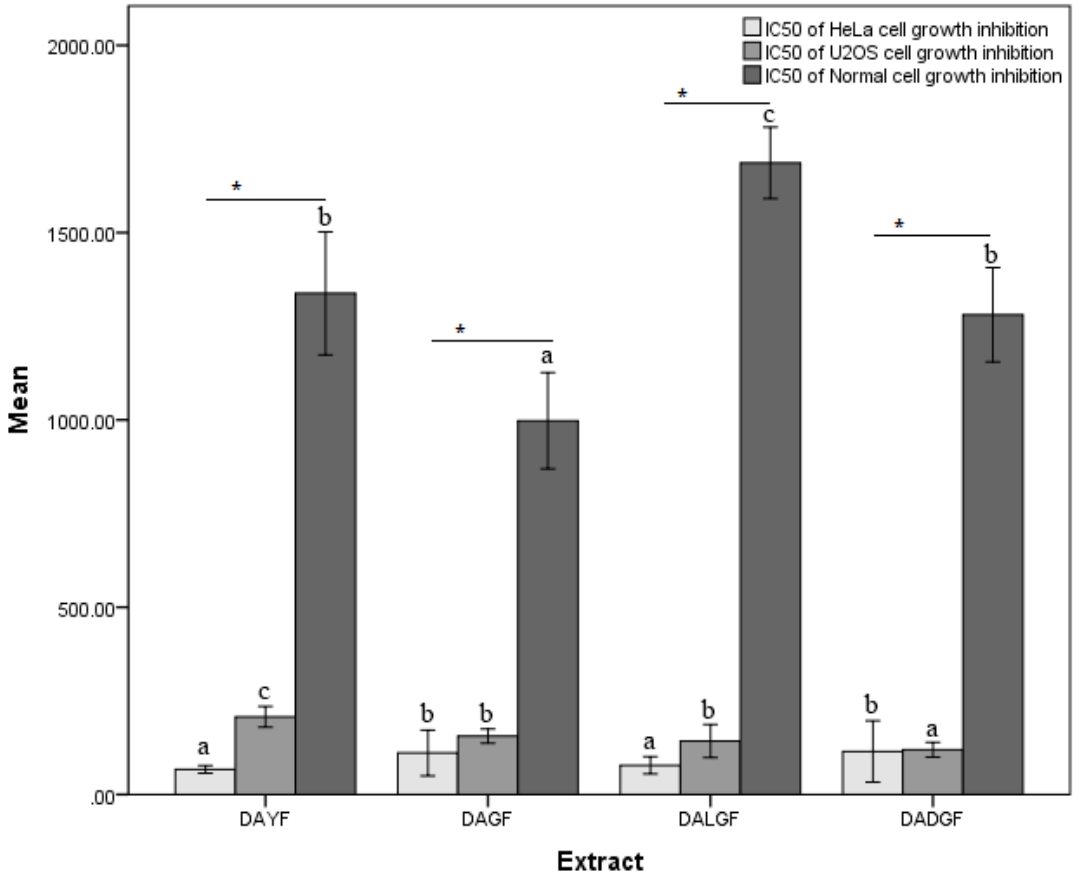

Figure 10: Mean IC_{50} of cytotoxic activity of four fractions of methanolic extract of *Dendrobium amoenum*.
Bars with different alphabets and asterisk (*) represent the significant difference at $p \leq 0.05$.

4.7 Relationship between the cytotoxic activity of cell lines and antioxidant activity

The IC_{50} value of HeLa cell growth inhibition shows a strong positive correlation (0.757 at a significance level of 0.004) between the IC_{50} values of HeLa cell growth

inhibition and antioxidant activity, meaning that as the concentration of the treatment required to inhibit HeLa cell growth increases, the concentration required to produce antioxidant activity also increases. On the other hand, the correlation coefficient of -0.524 at a significance level of 0.080 suggests a weak negative relationship between the IC_{50} values of HeLa cell growth and U2OS cell growth inhibition, meaning that as the concentration of the treatment required to inhibit HeLa cell growth increases, the concentration required to inhibit U2OS cell growth decreases. Similarly, the correlation coefficient of -0.429 at a significance of 0.164 between HeLa cell growth and Normal cell growth inhibition indicates a weak negative relationship between the two, meaning that as the concentration of the treatment required to inhibit HeLa cell growth increases, the concentration required to inhibit normal cell growth decreases. The correlation between HeLa cell growth and antioxidant activity has a very low p-value, suggesting a strong correlation, while the correlation between HeLa cell growth and U2OS cell growth inhibition and Normal cell growth inhibition has relatively high p-values, indicating weaker correlations as shown in Table 9.

In the case of U2OS cells, the IC_{50} values of U2OS cell growth inhibition have been compared to the IC_{50} values of Normal cell growth inhibition and antioxidant activity. The correlation between the IC_{50} values of U2OS cell growth inhibition and Normal cell growth inhibition is negative ($r = -0.056$), but the correlation is not statistically significant ($p = 0.862$). This means that there is no strong relationship between the two variables, and the IC_{50} values do not vary consistently in opposite directions. However, the correlation between the IC_{50} values of U2OS cell growth inhibition and antioxidant activity is negative ($r = -0.769$) and statistically significant, ($p = 0.003$), meaning that there is a strong and consistent relationship between the two variables. This suggests that substances with higher IC_{50} values for U2OS cell growth inhibition may also have higher IC_{50} values for antioxidant activity.

In Normal cell growth inhibition, there is a moderate negative correlation ($r=-0.524$) between the IC_{50} values of Normal cell growth inhibition and antioxidant activity and a very weak negative correlation ($r=-0.056$) between the IC_{50} values of Normal cell growth inhibition and U2OS cell growth inhibition. The negative correlation between the IC_{50} values implies that the substance has a stronger effect on antioxidant activity than on Normal cell growth inhibition, and as the concentration of the substance

increases, the effect on antioxidant activity becomes stronger while the effect on Normal cells becomes weaker. The IC_{50} values for U2OS cell growth inhibition are not strongly related to changes in the IC_{50} values for Normal cell growth inhibition. These correlations are not statistically significant as shown in Table 9.

Table 9: Relationship between antioxidant activity and IC_{50} of HeLa, U2OS and Normal cell growth inhibition.

		IC_{50} of HeLa cell growth inhibition	IC_{50} of U2OS cell growth inhibition	IC_{50} of Normal cell growth inhibition	IC_{50} of Antioxidant activity
IC_{50} of HeLa cell growth inhibition	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	12			
IC_{50} of U2OS cell growth inhibition	Pearson Correlation	-.524	1		
	Sig. (2-tailed)	.080			
	N	12	12		
IC_{50} of Normal cell growth inhibition	Pearson Correlation	-.429	-.056	1	
	Sig. (2-tailed)	.164	.862		
	N	12	12	12	
IC_{50} of Antioxidant activity	Pearson Correlation	.757**	-.769**	-.524	1
	Sig. (2-tailed)	.004	.003	.081	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

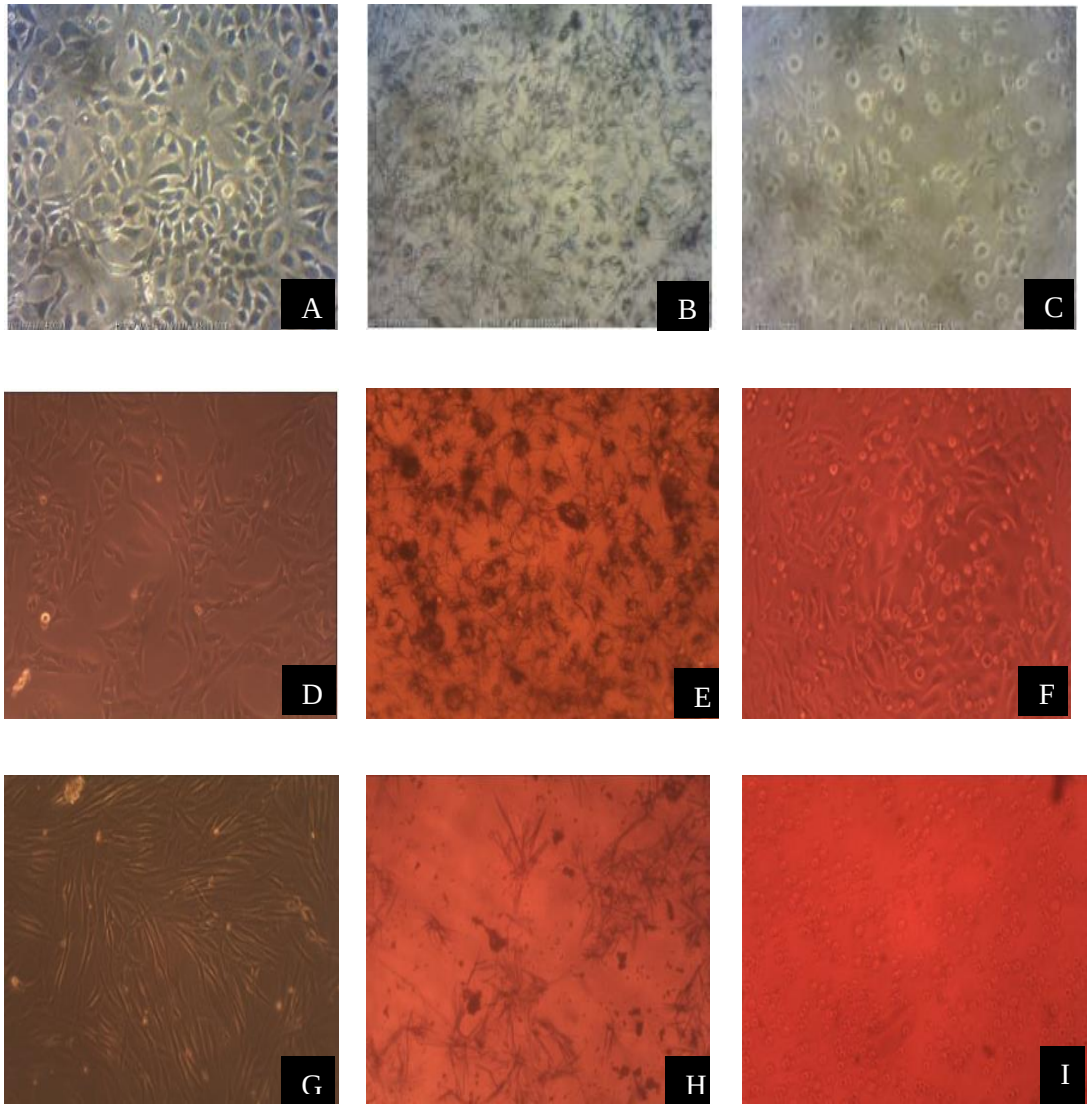

Figure 11: Apoptosis of HeLa cells after methanol extracts addition: HeLa cells before extract addition (A), formazan crystals in treated cells with the extract after MTT addition (B), dead HeLa cells (C); U2OS cell apoptosis after methanol extract addition: U2OS cells before extract addition (D), formazan crystals in treated cells with the extract after MTT addition (E), dead U2OS cells (F); Normal cell apoptosis after methanol extract addition: Normal cells before extract addition (G), formazan crystals in treated cells with the extract after MTT addition(H), dead Normal cells(I).

CHAPTER 5: DISCUSSION

5.1 Factors affecting *in vitro* seedling development

Several factors can influence *in vitro* seedling development, including the composition and concentration of the culture medium, the presence of plant hormones and growth regulators, and the physical and environmental conditions of the culture system. The growth and development of seedlings can be significantly influenced by the nutrient type and concentration present in the culture medium. In the current study, it was observed that seedlings transferred to a basal medium devoid of PGRs grew slowly. Some seedlings began to divide and form white, globose entities known as PLBs, which did not develop into plantlets. To achieve active seedling growth and development, or protocorm prolongation, coconut water and various PGRs ought to be added to the initial medium. As a result, the medium was supplemented with various PGRs and coconut water in the current study for seedling growth and development.

Following five months of culture, the FMS medium fortified with 0.25mg/ml NAA and 10% coconut water showed the greatest shoot and leaf development. Sharma *et al*. (2002) found that supplementing the medium with 0.1mg/l NAA and 15% coconut water increased protocorm proliferation and seedling growth in *Dendrobium fimbriatum*. Since coconut water contains both organic and inorganic chemicals, it functions as a complicated multifunctional growth promoter that modulates growth factors such as shoot quantity and length (Peixe *et al*., 2007; Nasib *et al*., 2008; Akhtar *et al*., 2008). Coconut water is a complex addition that comprises a variety of nutritional and/or hormonal compounds (Chugh *et al*., 2009). It is a natural growth promoter that contains additional zeatin, zeatin riboside, 1,3-diphenylurea (which has cytokinin-like activity), auxin, nitrogen-containing chemicals, inorganic compounds, organic acids, sugar and their alcohols, peptides, vitamins, amino acids, as well as numerous unidentified components (Tokuhara & Mii, 2001; Nasib *et al*., 2008). It has been used successfully in the propagation of numerous orchids, either alone or in combination with a plant growth regulator. Roslina (2010) also showed effectiveness in protocorm, and shoot regeneration using coconut milk as an adjuvant. Pyati (2002) revealed that the inclusion of coconut water in the Murashige and Skoog growth medium resulted in 78% of *Dendrobium macrostachyum* nodal plantlets producing axillary buds, which then developed into axillary shoots within one month of being cultured. Islam *et al*.

(2014) exposed plantlet growth from the protocorm of *Vanda roxburghii* to MS medium enriched with 15% coconut water. Sinha & Roy (2003) discovered shoot regeneration from *Vanda teres* protocorm in ½MS medium with coconut water. Sudeep *et al*. (1997) discovered that adding coconut milk (5, 10, or 15%) to ½ MS medium boosted the quantity of *Dendrobiun nobile* shoots.

Several orchid species have been shown to require exogenous cytokinin for protocorm regeneration, shoot buds, and plantlet development. BAP is an initial-generation synthetic cytokinin that stimulates cell division to impact plant development and growth responses, such as flowering and fruit richness. The amount of cytokinin required for shoot proliferation and plantlet development differs among orchid genera. (Pradhan *et al*., 2013; Pant *et al*., 2019). The MS medium in combination with 1mg/l BAP along with 10% coconut water had also shown a successful increment in the growth and development of seedlings in the present study. The result shows similarity with Vijayakumar *et al*. (2012) in *Dendrobium aggregatum* where the MS medium fortified with 3% sugar,1.5mg/l BAP, and 15% coconut water reported the maximum number of shoots, elongation of shoots and formation of roots.

The role of auxins is essential for root initiation in the *in vitro* cultures of various orchid species. Auxins have previously been shown to stimulate root development and multiplication in various orchid species (Bhadra & Hossain, 2004; Nongdam & Chongtham, 2012; Zhao *et al*., 2013; Julkiflee *et al*., 2014). In the current study, various concentrations of IAA, NAA, and IBA were employed for rooting, either alone or in conjunction with coconut water. Overall, the MS medium supplemented with 0.25 IAA mg/l had the largest root number as well as the longest root. The result is identical to Maharjan *et al*. (2020) in *Dendrobium chryseum* where MS media supplemented with 1.5 mg/l IAA created longer roots.

The importance of auxin and cytokinin alone or in conjunction with coconut water has been employed for *in vitro* growth and development of plantlets was reported in many orchid species. The most commonly utilized plant growth regulators for inducing shoot formation are NAA, which is an auxin, and BAP, which is a cytokinin (Tripepi, 1997; Nasiruddin *et al*., 2003). Rajkarnikar (2010) discovered that MS significantly boost with BAP (1mg/l) and NAA (1mg/l) was the optimal combination for the multiplication of PLBs and the growth of healthy seedlings of *Dendrobium amoenum*. Rajkarnikar &

Niroula (1994) employed 5.0 mg/l BAP and 1.0 mg/l NAA for shoot tip explant micropropagation of *Dendrobium fimbriatum* Hook. To be optimal for shoot bud production and multiplication of *Paphiopedilum hangianum*, a 1/2 MS medium enriched with CW and containing 1.0 mg/l BAP and 2.0 mg/l NAA was used (Zeng *et al*., 2013). Rajbahak & Rajkarnikar (2017) reported that robust and healthy elongated shoots were produced from *Dendrobium longicornu* protocorms by culturing them in MS media supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA, in combination with CW. The multiplication of shoots in *Cymbidium gradiflorum* was achieved by utilizing 5.0 mg/l BAP and 1.0 mg/l NAA, along with 10% coconut water (Shrestha & Rajbhandary,1993). However, in the present study, a full MS medium supplemented with 0.25 mg/l NAA along with 10% coconut water was found as the best medium for the growth and development of seedlings.

5.2 Total phenolic and flavonoid contents

Polyphenols, which include both flavonoid and phenolic molecules, are the most often recognized phytochemicals in plants because of their capacity to remove free radicals. Since ancient times, they have played an important part in the effective treatment of an extensive variety of ailments (Panche, 2016; Lobo, 2010). Phenolics and flavonoids exhibit noteworthy antioxidant properties by transferring hydrogen from their hydroxyl groups to free radicals, which generates stable phenoxyl radicals. Phenolic compounds were discovered to vary in various solvents due to the polarity of the solvents. The total phenolic content of four fractions of methanol extract was determined using the Folin-Ciocalteau technique and gallic acid as the chemical standard in this experiment. The results demonstrated that the yellow fraction (DAYF) performed with the highest total phenolic content and highest flavonoid content at 206.38 µgGAE/gm extract and 101.88 µg GAE/gm respectively than other fraction extracts. The methanolic extract of stem and leaves from the Chinese medicinal orchid, *D. nobile*, exhibited considerably more polyphenols and flavonoids than chloroform and acetone extracts (Bhattacharya *et al*., 2014). Among the plants derived from ISO, *D. thysiflorum* exhibited the highest phenolic content (75.66±0.2 GAE/g DW) and the greatest flavonoid content (8.23± 0.1 QE/g DW). On the other hand, the root extract of the mother plant obtained using chloroform displayed the lowest flavonoid content. Among the ISO-cultivated *D. thysiflorum* plants, the ones with the highest phenolic content (75.66±0.2 GAE/g DW)

and flavonoid content (8.23± 0.1 QE/g DW) were identified. In contrast, the chloroform root extract from the original plant had the lowest flavonoid content (Bhattacharya *et al*., 2015). The methanol extracts of *Dendrobium herbaceum* Lindl. stems have a significant total phenolic content utilizing tannic acid and gallic acid as standards, as well as high antioxidant activity (Vattakandya & Chaudhary, 2013). The methanolic extracts from *Dendrobium denudans* D. Don stem also showed high phenolic contents and exhibited high antioxidant activities (Singh *et al*., 2015).

In a previous report, the chloroform and acetone extracts of *D. amoenum* stem demonstrated higher phenolic and flavonoid content than hexane, ethanol, and methanol extracts (Paudel *et al*., 2015). In this report, the yellow fraction (DAYF) of methanolic extracts had the highest total phenolic content and the highest flavonoid content at 206.38 µg GAE/mg extract and 101.88 µgGAE/mg respectively, than other fractions of extracts. The suitability of methanol as a solvent for extracting TPC was established, as supported by the findings of other researchers (Chavan *et al*., 2012; Jagtap *et al*., 2011). The current study found that the phenolic and flavonoid content of the yellow fraction (DAYF) of the methanol extracts of *D. amoenum* was substantially higher than that of the other fractions. It indicates that polyphenol concentration varies across column bands as a result of the extraction of diverse compounds with varied polarity solvent systems. The findings indicate that phenolics and flavonoids are important contributions to antioxidant and cytotoxic properties.

5.3 Antioxidant activity using DPPH assay

The antioxidant potential of fractions from methanol extracts was evaluated by monitoring the alteration in the decolourization of DPPH free radicals as they react with antioxidant-rich compounds and receive electrons. Several studies have found that the phenolic and flavonoid content of *Dendrobium* species extracts showed antioxidant potential (Paudel *et al*., 2017, Mukherjee *et al*., 2012). A part from *D. amoenum*, there have been numerous studies on the antioxidant properties of *Dendrobium* species (Fan *et al*., 2009; Luo & Fan, 2011; Luo *et al*., 2011; Mukherjee *et al*., 2012; Moretti *et al*., 2013; Xing *et al*., 2013). Only isoamoenylin extracted from *D. amoenum* has been demonstrated to possess antioxidant properties (Venkateswarlu *et al*., 2002). The DPPH technique has been successful for determining the antioxidant activities of methanolic extracts of *D. tosaense*, *D. moniliforme* & *D. linawianum* by UV spectrophotometric

assay (Lo *et al*., 2004). The stem extracts from *D. amoenum* using acetone and chloroform are rich in phenolic and flavonoid content, and they demonstrate robust antioxidant activity (Paudel *et al*., 2015). In the present study, the yellow fraction (DAYF) methanolic extract of *D. amoenum* showed the highest DPPH activity (83.24 ± 0.77%) with a 63.73 ± 0.38µg/ml IC_{50} value, which is greater than that reported by Paudel *et al*. (2015). A similar finding was found in the methanolic leaf extract of *D. nobile*, which had a higher level of scavenging activity for free radicals than the mother plant (Bhattacharya *et al*., 2014). The maximum antioxidant activity was similarly found in methanolic extracts of Pineapple peel (Hossain & Rahman, 2011) and *S. chinensis* fruit pulp (Chavan *et al*., 2012). Similar results were obtained in *Piper nigrum* (Ahmad *et al*., 2010) and *Aloe arborescens* (Amoo *et al*., 2012), both of which have a higher potential for free radical scavenging action. The antioxidant capacity of the extracts is closely related to the solvent used, which is because molecules with antioxidant activities dissolve differently in different polarity solvents (Boeing *et al*., 2014). The differences in IC_{50} values are mainly due to the extraction of different chemical compounds based on the use of different solvents for extraction. The higher effectiveness of radical scavenging activity in wild plants in comparison to *in vitro*-grown plants is due to lower environmental and reproductive stress in the *in vitro*-grown plants, resulting in the development of a lower number of secondary metabolites. Our findings demonstrate that the antioxidant capacity of *D. amoenum* four fractions methanol extract is associated with the presence of antioxidant-rich chemicals such as polyphenol derivatives, which have great potential for pharmacological applications and drug discovery.

5.4 Cytotoxic activity using MTT assay

Free radicals and reactive oxygen species (ROS) are extremely reactive molecules that contain unpaired electrons and can cause damage to biological components such as DNA, proteins, and lipids. This damage can result in the emergence of mutations, which can accumulate over time and contribute to the development of diseases like cancer, heart disease, and ageing. Almost all orchid species inhibit endophytic fungi, surface saprophytes, dormant pathogens, and mycorrhizal fungi at some point in their lives (Petrini & Fisher, 1990; Rasmussen & Rasmussen, 2009; Pant *et al*., 2016). Biologically active secondary metabolites with a variety of structural variations, such

as alkaloids, benzopyranones, chinones, flavonoids, phenolics, quinones, steroids, terpenes, tetralones, and xanthones, are found in endophytes (Tan & Zou, 2001; Shah *et al*., 2018). The utilization of plant bioactive compounds extends to various fields, which comprise agrochemicals, antibiotics, immunostimulants, anti-diabetic drugs, anti-inflammatory agents, anti-parasitics, antioxidants, and anti-cancer agents (Farzaneh & Carvalho, 2015; Gunatilaka, 2006; Kharwar *et al*., 2011; Schulz *et al*., 2002; Strobel *et al*., 2004). In the current investigation, the green (DADG) and dark green (DADGF) fractions of the *D. amoenum* methanolic extract displayed the most effective cytotoxic activity against HeLa cells, whereas the dark green fraction (DADGF) had the most effective cytotoxic activity against U2OS cells. The green (DAGF) and dark green (DADGF) fractions inhibited HeLa cells by 82.36±2.10% and 81.84±2.27%, respectively. In contrast, the dark green fraction (DADGF) inhibited 64.76±3.41% of U2OS cells. The fractions have no cytotoxic effect on the Normal cell. In a previous study, the methanol (DAM) extract of *D. amoenum* showed excellent cytotoxic action against HeLa cells (Paudel & Pant, 2017). The presence of many bioactive chemicals in the extract of *D. amoenum* accounts for its cytotoxic action. The presence of antioxidant-rich substances such as polyphenol derivatives in *D. amoenum* fractions is an essential characteristic responsible for cancer chemopreventive activities (Dai & Mumper, 2010; Paudel & Pant, 2017). The cytotoxicity of both methanol and ethanol extracts of *D. moniliforme* on HeLa and U251 cell lines and the mechanisms by which phenol derivatives can decrease cancer proliferation by stopping the cell cycle and inducing apoptosis was observed (Paudel *et al*., 2018). Both extracts contain phenol derivatives that can reduce free radicals and prevent oxidation. Several phenol compounds in *D. moniliforme* extracts prevent cancer cells from proliferating by stopping the cell cycle and triggering apoptosis through morphological changes including cell shrinkage, chromatin condensation, as well as nuclear disintegration. The result was supported similarly by Paudel *et al*. (2019) on *D. crepidatum*, in which the methanolic extracts exhibited more cytotoxic activity against both HeLa and U251 cell lines due to their numerous polyphenol derivatives, which play an essential role in the prevention of cancer cell proliferation. Previous research has demonstrated that phenolic derivatives cause the death of cancer cells (Prasad & Koch 2014; Chen *et al*., 2015; Milutinoviae *et al*., 2015). Polyphenol compounds present in plants may decrease cancer cells by modifying the metabolic activation of carcinogens via xenobiotic-metabolizing enzymes, whereas some flavonoids may change hormone production to

inhibit cancerous cells development (Chen *et al*., 2007; Gali-Muhtasib *et al*., 2015). Phenols decrease the quantity of protein expression, the mitotic index, and colony formation during cell growth (Li *et al*., 2001; Wu *et al*., 2006; Paudel *et al*., 2019). The anticancer activity of the flavonoid molecule is a result of the incorporation of a 4-carbonyl group into its structure. (Cragg & Newman, 2013). Additionally, a flavonoid molecule with a 2,3-double bond has been associated with mitochondrial dysfunction and cancerous cell apoptosis (Prasad *et al*., 2009). As a result, *D. amoenum* methanol extract fractions have the potential to deliver important antioxidants and anticancer compounds, potentially resulting in the discovery of valuable drugs.

CHAPTER 6: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Orchid *in vitro* multiplication helps to save rare species from extinction. An efficient methodology for *in vitro* seedling development of *D. amoenum* has been established in the current study. The growth and development of seedlings, along with the highest growth based on shoot length and shoot number were achieved best in the basal FMS medium supplemented with 0.25mg/l NAA+10% coconut water. Further, the synergistic effect of 1mg/l BAP with 10% CW in both half and full basal media was also successful in obtaining healthy growth in *D. amoenum*. The phenolic and flavonoid content in four fractions of a methanolic extract of *D. amoenum* were measured by using the Folin- Ciocalteau phenol reagent and the Aluminium Chloride ($AlCl_3$) colourimetric method, respectively. The highest phenolic and flavonoid content was found in the (DAYF) yellow fractions. The four fractionated extracts of *in vitro*-grown *D. amoenum* have shown antioxidant activities in the DPPH assay. The IC_{50} value of the yellow fraction (DAYF) was found to be the lowest, which was followed by the light green fraction (DALGF). The cytotoxic activity of fractionated extracts against HeLa (a type of human cervical cancer), U2OS (a type of human osteosarcoma), as well as normal cell lines was evaluated using an MTT colourimetric test. The IC_{50} value of the yellow (DAYF) and light green (DALGF) fractions was found to lower, which indicates that the yellow and light green fractions are more cytotoxic, and have a higher ability to destroy/kill HeLa cells. The dark green fraction (DADGF) had the lowest IC_{50} value, suggesting it was the most cytotoxic against U2OS cells among the four fractions. While the IC_{50} value of Normal cells was higher comparatively than that of HeLa and U2OS cells, indicating it the less toxic to humans. The high level of phenolic and flavonoid components presents in the methanolic extract of *D. amoenum* fractions implies that they could be more efficient in eliminating both DPPH radicals and inducing apoptosis in cancer cells. This approved the proposed hypothesis that the column fractions of *in vitro*-raised *D. amoenum* are a source of potent antioxidant compounds and can inhibit the growth of cancer cells. Using the protocol created from the present research, it would be feasible to produce viable, uniform, and healthy plants suitable for large-scale growth. The protocol may make it easier to save this therapeutic orchid from extinction and reintroduce it into its natural environment. In addition, it

would be possible to extract bioactive compounds from this species, which could potentially lead to the development of new and more effective anti-cancer treatments.

6.2 Recommendation

The following recommendations are made for the present research work:

1. The *in vitro* culture methodology thus established offers a foundation for crucial species regeneration. Attention must be paid to the further improvement of culture techniques and the preservation of *in vitro* cultivated plants.
2. In addition, other tissue culture procedures, including direct organogenesis employing explants, may offer an additional choice for the *in vitro* regeneration of *D. amoenum*.
3. The separation and purification of bioactive components from plants grown *in vitro* are of utmost significance.
4. To achieve the efficiency of essential compounds obtained from *in vitro*- grown plant material, the following phase involving in vivo testing should be carried out.
5. Commercialization of the tissue culture method and the active compounds is necessary to improve the economy and develop plant-based medicines for a variety of therapeutic uses.

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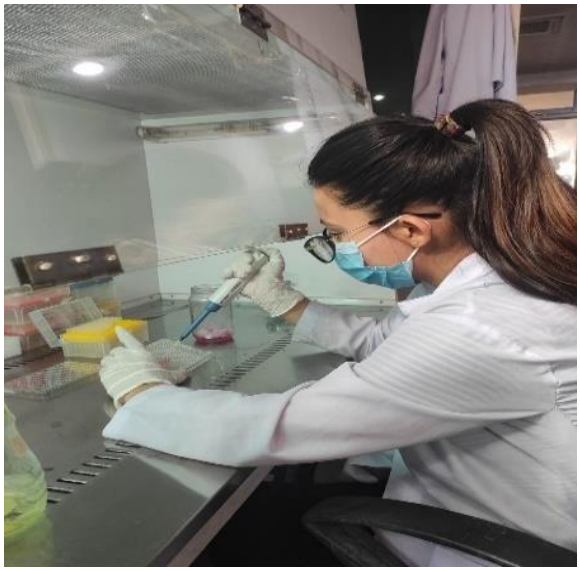
APPENDIX-I

Photoplates

Subculture of seedlings in lab



Cell lines culture in Annapurna Research Center (ARC)

CERTIFICATE
OF ACHIEVEMENT

THIS CERTIFICATE IS PRESENTED TO

Mrs. Sujata Sharma

Tribhuvan University, Nepal

as a Poster Presenter

on

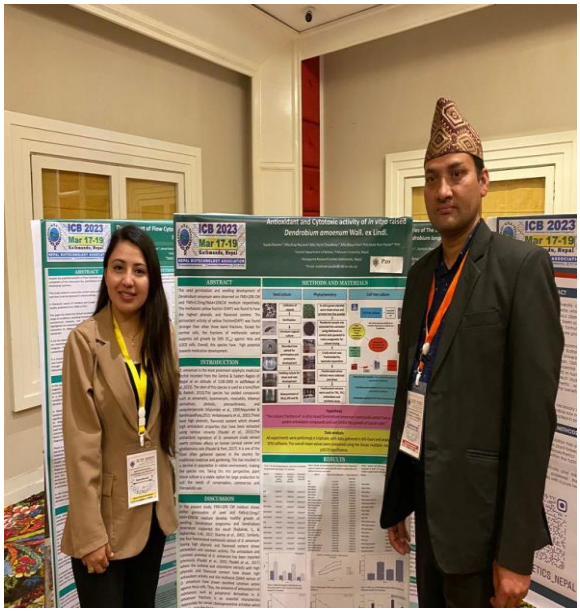
Antioxidant and cytotoxic activities of in-vitro grown Dendrobium amoenum extracts

in the 4th International Conference on Biotechnology: Academia to Industry (ICB - 2023) held on 17-19 March, 2023 at Kathmandu organized by Nepal Biotechnology Association. www.nepalbiotech.org

Prof. Dr. Bijaya Pant
Convener, ICB 2023

Prof. Dr. Tribikram Bhattarai
President, NBA

Certificate of Achievement as a Poster Presenter in 4th International Conference on Biotechnology:Academia to Industry(ICB-2023)



Photograph with my supervisor Asst.Professor Dr.Mukti Ram Paudel during 4th International Conference on Biotechnology:Academia to Industry
(ICB-2023)