Antioxidant and Anticancer Activities of Protocorms Extract of *Dendrobium longicornu* Lindl. (Orchidaceae)

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DECLARATION

The dissertation entitled "**Antioxidant and Anticancer Activities of Protocorms Extract of *Dendrobium longicornu* Lindl. (Orchidaceae)**" which is being submitted to the Central Department of Botany, Tribhuvan University for the completion of Master of Science in Botany is genuine work carried out by me under the supervision of **Dr. Mukti Ram Paudel, Assistant Professor** at Central Department of Botany. I further declare that the work reported in this research has not been previously deposited for the award of any degree, in this or any other institute or University.

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RECOMMENDATION

This is to certify that the dissertation work entitled **"Antioxidant and Anticancer Activities of Protocorms Extract of *Dendrobium longicornu* Lindl. (Orchidaceae)"** submitted by **Ms. Khageshwari Saud** was accomplished under my supervision. The candidate has carried out the original research and, to the best of my knowledge, the work has not been submitted anywhere for any academic purpose. I hereby recommend for the approval of this dissertation as a partial fulfillment of the requirements of Master's Degree in Botany at Tribhuvan University.

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LETTER OF APPROVAL

The M.Sc. Dissertation entitled **"Antioxidant and Anticancer Activities of Protocorms Extract of *Dendrobium longicornu* Lindl. (Orchidaceae)"** submitted at the Central Department of Botany, Tribhuvan University by **Ms. Khageshwari Saud** for the partial fulfilment of her Master's degree in Botany has been approved.

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ABBREVIATIONS AND ACRONYMS

°C = degree centigrade

µg = microgram

µL = microliter

µM = Micromolar

DCM = Dichloromethane

DMSO= Dimethyl sulfoxide

DPPH = 1, 1-Diphenyl-2Picrylhydrazyl

EMEM= Eagle's Modified Minimal Medium

GAE = Gallic Acid Equivalent

IC_{50} = Half-Maximal Inhibitory Concentration

mg = milligram

mL= millilitre

Mm = millimolar

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

QE = Quercetin Equivalent

ROS = Reactive Oxygen Species

rpm = revolutions per minute

RSA = Radical Scavenging Activity

SE = Standard error

TFC = Total Flavonoid Content

TPC = Total Phenolic Content

ABSTRACT

Dendrobium longicornu Lindl. (Orchidaceae) is a traditionally used medicinal orchid that has many bioactive compounds such as bibenzyl, phenanthrenes, monoaromatics, steroid and flavonoid derivatives. This study aimed to investigate the total phenolic and flavonoid contents and the antioxidant and cytotoxic activity of the different sub-fractions obtained from the *in vitro*-grown protocorms of *Dendrobium longicornu*. The protocorm's methanol extract was separated using a separatory funnel in hexane, DCM, methanol and water. Each solvent fraction was subjected to a Sephadex LH-20 silica gel column chromatography and 4 sub-fractions (M1, M2, M3 and M4) of methanol, 2 sub-fractions (A1 and A2) of aqueous, 3 sub-fractions (DY, DB, DG) of DCM and 3 sub-fractions (HBG, HY, and HYG) of hexane fraction were obtained. The total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of all the 12 sub-fractions was determined using Folin-Ciocalteu phenol reagent colourimetric method, Aluminium Chloride ($AlCl_3$) colourimetric method and DPPH free radical scavenging assay respectively. The cytotoxic activity of only 6 sub-fractions M1, M2, M3, M4, A1, and A2 was evaluated against human breast cancer cells (MCF-7) and cervical carcinoma cells (HeLa) using MTT assay. TPC and TFC were found to be highest in the M1 sub-fraction with the value of 187.58±0.23 µg GAE/mg and 84.68±0.37 µg QE/mg respectively. The highest antioxidant activity against DPPH free radical with the lowest IC_{50} value of 99.68±3.31µg/mL was shown by the M1 sub-fraction. M1 sub-fraction also showed the highest inhibitory effect on the growth of both MCF- 7 and HeLa cells with the lowest IC_{50} value of 92.43±9.44 µg/mL and 283.48±17.55 µg/mL respectively. It concluded that the M1 sub-fraction of protocorms produced by *in vitro* seed culture has bioactive secondary metabolites, therefore, protocorms can be used to isolate chemical compounds for the formulation of anticancer drugs without harming natural populations.

CHAPTER ONE: INTRODUCTION

1.1. Background of the study

Plants have been considered as one of the most important sources of medicine. Among 2,50,000 higher plant species reported worldwide, more than 80,000 species are being used as medicinal. Medicinal plants are extensively utilized worldwide as a major resource in the traditional medicine and herbal industry, providing the base for livelihood to a large segment of the world population (Jain, 1968). From the beginning of human civilization, medicinal plants have been identified and used traditionally throughout the world (Bauri *et al*., 2015). The discovery and development of drugs have been significantly aided by the use of natural products. Many plant-derived compounds have been found to possess a wide range of biological activities including antimicrobial, antiviral, cytotoxic, antioxidant, anti-hepatotoxic, and anti-inflammatory activities and the non-success of possible drug discovery techniques to deliver many lead compounds in key therapeutic areas have led to a renewed interest in natural product research, including the study of plant-derived compounds (Butler, 2004; Rathee *et al*., 2009).

Cancer is an illness defined by the unchecked proliferation of normal human cells that have undergone subtle alterations. The majority of the regulatory activities that normally govern cells have been lost by cancer cells, and they continue to divide, which is the opposite of what normally happens to cells. In both economically developed and developing nations, cancer is regarded as a terrible killer that poses a significant public health burden. After ischemic heart disease and stroke, cancer is regarded as the second most common cause of death worldwide among non-communicable diseases. Globally, cancer has a significant influence on both society and the economy. In 2018, it was projected that the US would spend $150.8 billion on cancer treatment. These expenses will undoubtedly rise in the coming years as the population ages and as more people develop cancer (Bray *et al*., 2018).

According to statistics, breast cancer accounts for about 11.7% of all cancer cases which is chased by lung cancer (11.4%), colorectal cancer (10%), prostate cancer, stomach cancer and liver cancer (Sung *et al*., 2021). Breast, lung and colorectal cancers are the three most prevalent cancers in women in the US, accounting for over half of all new diagnoses in 2020 (NIHcancer.gov/about-cancer/understanding/statistics,2020). Most

individuals with breast cancer experience recurrent, progressive disease due to an increase in the resistance phenotype to several conventional chemotherapy treatments.

Several anti-cancer medications derived from plants vinblastine, vincristine, etoposide, teniposide, docetaxel (Taxotere), paclitaxel (taxol), camptothecin, homoharringtonine and elliptinium are now used in the cure of cancer (Cragg and Newman, 2013; Oberlies and Kroll, 2004). Many of the chemicals that are discovered in orchids have been isolated. Erianin, dendrochrysanene, moscatilin, denbinobin, cirrohopetalanthrin and fimbriatone were extracted from several orchids which show strong anticancer activity (Ma *et al*., 1998; Xia *et al*., 2005; Chen *et al*., 2007; Peng *et al*., 2007; Wu *et al*., 2006). Due to the potential for cancers to acquire resistance to chemotherapy and the rising number of people dying from cancer, there is a constant need for new anti-cancer medications (Newman, 2008).

Orchidaceae, one of the largest families in the plant kingdom having 28,000 accepted species, spread over 763 genera comprising 6-11% of seed plants, has abundant scientific studies carried out on their phytochemistry and available bioactive compounds (Christenhusz and Byng, 2016). It has been mentioned that orchids have been known widely for their medicinal use in the traditional medical system of Ayurveda. Some of the compounds identified for their bioactivity are alkaloids, flavonoids, anthraquinones, cardiac glycosides, glycosides, phlorotannins, quercetin, saponins, reducing sugars, steroids and terpenoids (Vaidya, 2019). The genus *Bulbophyllum* is the largest of the family followed by *Dendrobium* genus, distributed in Asia, Europe and Australia with more than 1500 species (Cakova *et al*., 2017).

The genus *Dendrobium* Sw. is one of the largest genera in Orchidaceae with 800 to 1400 species in the world (Xiaohua *et al*., 2009). About 31 species of *Dendrobium* were recorded in Nepal and have been listed in Appendix-II of the Convention of International Trade in Endangered species (CITES) (Rokaya *et al*., 2013). The *Dendrobium* genus has often been used as a tonic, and its phenolic contents have attracted attention for their anti-tumour and anti-diabetic complications. It manifests various medicinal effects including antiangiogenic, antidiabetic, immunomodulating, antiplatelet aggregation, cataractogenesis-inhibiting, neuroprotective, hepatoprotective, anti-inflammatory, antifungal, antibacterial, antimalarial, antiherpetic, aquaporin-5 stimulating and hemagglutinating activities, and also exerts

beneficial actions on colonic health and reduce symptoms of hyperthyroidism (He *et al*., 2020; Teixeira and Ng, 2017).

D. longicornu Lindl. (Orchidaceae) is an orchid of very high medicinal value which is greatly used for curing coughs and fever in the traditional medicine system (Manandhar, 1995). *Dendrobium longicornu* is an epiphytic orchid and the stem of this orchid is tufted and covered with black hairs. The flowering period of the orchid is from September to November. It is found at an elevation range of 1300 - 2900 m in Nepal (Pant and Raskoti, 2013). There have been reports of a wide range of its chemical components, including bibenzyls, phenanthrenes, monoaromatics, steroids, lignin glycoside, and phenolic compounds from this plant (Hu *et al.*, 2008; Hu *et al.*, 2010).

Reactive nitrogen and oxygen species, such as singlet oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical and nitrous oxide are frequently produced in human beings as byproducts of biological reactions and have been recognized to play a significant part in oxidative cellular damage. Oxidative stress caused by these free radicals contributes significantly to the manifestation of many ailments, including diabetes, cancer, and cardiovascular diseases (Hu *et al.*, 2008; Chen *et al.*, 2010). Due the presence of secondary metabolites as bibenzyl, phenanthraquinone, lignin glycosides and phenolic compounds, *D. longicornu* can scavenge free radicals and show anticancer activity (Hu *et al.*, 2008; Hu *et al.*, 2010; Paudel *et al*., 2020)

Overharvesting of wild resources to supply traditional medicine has resulted in the depletion of the natural population of *D. longicornu*. Without a pre-existing association with particular mycorrhizal fungi, the seeds of this plant have trouble germinating in the wild (Pant *et al*., 2017; Shah *et al.*, 2018; Chand *et al.*, 2020). Since it is threatened, it is listed in Appendix II of the Convention on International Trade in Endangered Species (CITES). The technology of plant tissue culture offers a path to preserve its natural population (Dohling *et al*., 2012) and the creation of beneficial secondary metabolites (Ochoa-Villareal *et al*., 2016; Espinosa-Leal *et al.*, 2018). Alkaloids, polyphenols, anthocyanins and carotenoids are just a few chemical compounds with pharmacological importance that are generated and accumulate in tissues that have been developed *in vitro* (Bhattacharya *et al.*, 2015). Protocorm is a unique organ created from the seed culture of orchids which goes through growth and plant differentiation. They

are highly proliferative tissues that accumulate high amounts of secondary metabolites (Cui *et al*., 2014; Wang *et al*., 2016).

1.2. Objectives of the study

1.2.1 General objective

The general objective of the present study is to identify the anticancer activity of different sub-fractions of *in vitro*-grown protocorms of *Dendrobium longicornu*.

1.2.2 Specific Objectives

The specific objectives of the present study are:

1. To estimate the total flavonoid and phenolic contents in the samples.

2. To identify the antioxidant activity of the samples.

3. To analyze the anticancer activity of the samples on different cancer cell lines (MCF-7 and HeLa).

1.3 Rationale of the study

Orchids are regarded as a significant source of therapeutic and pharmaceutical unique molecules required in the discovery of new medications, as well as beneficial medical natural goods. Since there is a good probability that these traditional medicinal plants will yield pharmacologically effective chemicals, bioprospecting of these plants is crucial (Newman *et al*., 2000, 2003; Cragg and Newman, 2013).

Cancer has been a serious concern in public health since it causes 8.8 million deaths worldwide (Sung *et al*., 2021), and that number is predicted to climb to 13.1 million by 2030. Drugs' toxicity to healthy cells and lack of specificity for cancer cells are two of the key reasons we haven't conquered cancer. Different chemotherapeutic medications that are often employed as anti-cancer medicines have different toxicities. As a result, there has been a need for the identification of innovative medications that are more targeted and have fewer harmful side effects. Natural products have been thought to be developed as prospective cancer treatment agents due to their lower toxicity. Several anticancer medications derived from plant materials are examined in clinical studies on cells (including numerous cancer cell lines) and experimental animals after being

purified. Newly discovered natural chemicals have been found at a rapid rate in recent years (Lichota and Gwozdzinski, 2018).

Dendrobium longicornu is a widely used traditional medicinal orchid having valuable secondary metabolites such as bibenzyl, phenanthrenes, phenolics, lignin glycoside, phenanathraquinone and monoaromatic derivatives (Chen *et al*., 2010; Hu *et al*., 2008; Hu *et al*., 2010; Li *et al*., 2009). Antioxidant-riched *Dendrobium longicornu* have shown cytotoxic activities against human cervical carcinoma and glioblastoma cells, the compound derivatives of *D. longicornu* exhibit the apoptosis of cervical carcinoma and glioblastoma cells (Paudel *et al*., 2017; 2020). The biological activities of the extract's sub-fractions derived from *in vitro*-developed protocorms of this orchid have not been explored very well. So, the present study will explore the anticancer activity of the extract's sub-fractions obtained from protocorms of this orchid on different human cancer cell lines (MCF-7 and HeLa). The major target of developing protocorms is to reduce the overexploitation of wild habitats as well as to obtain effective secondary metabolites. This study will prove a baseline for the pharmacological uses of this orchid.

CHAPTER TWO: LITERATURE REVIEW

Natural resources derived from plants have been used by humans since the beginning of time. People have performed plant-based medicine based on their experience and knowledge collected through trial and error, which established information was then passed down from generation to generation, without knowing the precise chemical makeup of its extracts. Indigenous healthcare systems in underdeveloped nations were built based on medicinal plants.

Since the dawn of human civilization, medicinal plants have been employed by people for their therapeutic benefits. For thousands of years, natural resources have served as the foundation for medicinal agents, and an amazing number of new medications have been extracted from these sources. The applications of the substances in traditional medicine provided the basis for many of these isolations. With nearly about 80% of the world's population relying mostly on traditional medicines for their primary health care, the plant-derived, traditional system of medicine continues to play a crucial role in healthcare (Owolabi *et al*., 2007).

Bioactive substances produced by medicinal plants are mostly employed for therapeutic purposes. Therefore, it is crucial to identify bioactive compounds in plants, isolate, purify, and characterize the active components in crude extracts using various analytical techniques. According to Cowman (1999) and Adesokan *et al*. (2008), plants' therapeutic benefits may be based on the antioxidant, antibacterial, and antipyretic actions of their phytochemicals. In contrast, because knowledge of therapeutic plant uses has been passed down through oral tradition from one generation to the next, it has begun to deteriorate and become outmoded due to the lack of concern and awareness by younger generations as a result of a change in view point and socio-economic changes (Kala *et al*., 2006).

Drugs with a natural origin are significant because it is thought that they may be able to treat and prevent disease. This has increased interest in examining biological impacts, isolating natural substances, and figuring out their structures. Although research of this kind was initially challenging, the development of new and thorough screening techniques has greatly simplified them (Sarkar *et al*., 1996).

There is increased concern about the potential of natural products as chemopreventive and chemotherapeutic anticancer medicines since they may be important mediators for the crucial pathways involved in the development and progression of cancer (Gali-Muhtasib *et al*., 2015; Lombardi *et al*., 2017). The rising cost of prescription medications for maintaining human health and well-being as well as the bioprospecting of new plant-derived medications have stoked attentiveness to medicinal plants as a re-emerging health help (Lucy and Edgar, 1999). Numerous factors, including a growing belief in herbal medicine, contribute to the continued growth in awareness of medicinal plants.

2.1 Phytochemicals in antioxidant and cytotoxic activity

Phytochemicals are organic substances that are naturally present in plants and have been linked to several positive health effects. Although they are not regarded as essential nutrients like vitamins and minerals, they can aid in the prevention of chronic illnesses including diabetes, cancer and heart disease. There are countless varieties of phytochemicals, such as lignans, carotenoids, polyphenols and flavonoids. It is thought that phytochemicals serve as antioxidants, reduce inflammation, and hinder the formation of cancer cells, among other actions, to improve health. Free radicals are unstable chemicals that can cause oxidative stress and damage to cells. Antioxidants are substances that shield cells from this damage. It has been demonstrated that phytochemicals with antioxidant capabilities, including carotenoids, flavonoids and phenolic acids, can help shield cells from harm brought on by free radicals. According to several studies, phytochemicals with antioxidant qualities may help lower the chance of developing cancer. For the prevention or treatment of various ailments, including cancer, the use of herbal extracts or naturally occurring chemicals derived from herbs is still a popular alternative to utilizing drugs. The goal of numerous investigations on plant chemicals was to identify molecules that have specific cytotoxicity on aberrant cells (Teodor *et al*., 2020).

Flavonoids have significant impacts on cancer chemotherapy and prevention, according to compelling evidence from laboratory research, epidemiological investigations, and human therapeutic trials. A few of the several modes of action that have been identified include the inactivation of carcinogens, antiproliferation, cell cycle arrest, promotion of apoptosis and differentiation, suppression of angiogenesis, antioxidation, and

reversal of multidrug resistance (Ren *et al*., 2003). Flavonoids were also discovered to be connected to cell growth suppression and apoptosis activation in the breast cancer cell lines MCF-7 and MDA-MB-231 *in vitro* (Kosmider and Osiecka, 2004). Similarly, phenolics are potent antioxidants that can scavenge free radicals and protect cells from oxidative stress. This property makes them favourable for human health because oxidative stress and several chronic diseases are linked. Moreover, phenols have anti-inflammatory qualities. They also assist in reducing the body's inflammatory response, which can help avoid diseases like cancer and diabetes. As phenolics have been shown to have antibacterial and antiviral actions, they are useful for treating or preventing infections. Phenolic acids have much stronger in vitro antioxidant activity than well-known antioxidant vitamins (Tsao and Deng, 2004).

2.2 *Dendrobium* species, their phytoconstituents and biological activities

Different species of *Dendrobium* have been used and are still being used for a variety of purposes, including treating stomachaches, treating night sweats, fortifying the body, strengthening the kidneys, curing impotence, and acting as a tonic to help strengthen medicine and treat a wide range of illnesses (Chen *et al*., 1994).

According to the ancient Chinese medicinal book, "Shen Nong's Materia Medica," *Dendrobium* orchid has been used widely as a tonic and valuable food for thousands of years. According to earlier studies, *Dendrobium* species contain polysaccharides, sesquiterpenoids, alkaloids, and amino acids. As bioactive compounds, carbohydrates exhibit a wide range of anticancer, antiglycation, immune-stimulating, antiviral, antioxidant, and other actions. The precise forms and latent activities of polysaccharides from *Dendrobium* species are therefore heavily studied since they are the primary physiologically active component (Han *et al*., 2020).

Dendrobium species have been used in traditional or folk medication as stomachic, pectoral, analgesic and antipyretic medication (Kong *et al*., 2003). Recent pharmacological studies depending on the information obtained from local practitioners have shown that some of the components of *Dendrobium* species displayed anti-tumour properties. In a phytochemical analysis, the presence of flavonoids, reducing sugars, cyanogenic glycosides, terpenoids, and tannins was examined in 61 orchid species, including various *Dendrobium* species from south India (Maridass *et al*., 2008). Bibenzyls, phenanthrenes, sesquiterpenoids and polysaccharides isolated from various

Dendrobium species have shown potent antioxidant and anticancer activities (Paudel *et al.*, 2020). *Dendrobium longicornu* has been reported for having many important compounds, such as bibenzyl, phenolics, phenanthrenes, lignin glycoside, phenanathraquinone and monoaromatic derivatives (Chen *et al*., 2010; Hu *et al*., 2008; Hu *et al* 2010; Li *et al*., 2009).

The synthesis of numerous pharmaceutical ingredients for novel therapies has benefited greatly from the use of tissue culture technologies. Tissue culture is the way to conserve the natural population of plants. The creation of an extensive range of pharmaceuticals, including alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids, and amino acids, has been made possible by the advancement in the field of cell cultures for the manufacture of medicinal substances.

Using the MTT test, the ethanolic extracts of *Dendrobium crepidatum* and *Dendrobium chrysanthum* were evaluated for their capacity to induce apoptosis and cause cytotoxicity in T-cell lymphoma. The entire investigation's findings showed that ethanolic extracts of *D. crepidatum* and *D. chrysanthum* considerably boosted cytotoxic and apoptotic activities in T-cell lymphoma (Prasad and Koch, 2016).

The antioxidant and cytotoxic properties of *Dendrobium moniliforme* were investigated. The plant's TPC concentration was highest in the chloroform extract (DMC), while the TFC level was highest in the acetone extract of *D. moniliforme*. The DPPH radical scavenger with the highest percentage of action was *D. moniliforme* hexane extract (DMH). HeLa cells' growth was most inhibited by a methanolic extract of the plant, while U251 cells' growth was most inhibited by an ethanolic extract of the same plant at the same concentration. Many bioactive substances were found in the plant's methanolic and ethanolic extracts. The presence of several bioactive chemicals in the plant extract of *D. moniliforme* that demonstrated antioxidant and cytotoxic properties points to the potential pharmacological value of this plant (Paudel *et al*., 2018). Methanolic extract of *in vitro*-grown protocorms of *Dendrobium longicornu* scavenges DPPH free radicals and inhibits the growth of cancer cell lines namely HeLa and U251 (Paudel *et al*., 2020).

Using the MTT assay, it was determined that the ethanolic extract of *Dendrobium formosum* had anticancer properties against Dalton's lymphoma. Fluorescence microscopy was used to measure apoptosis, flow cytometry was used to examine cell

cycle progression, and Dalton's lymphoma-bearing mice were used to test *in vivo* anticancer activity. In Dalton's lymphoma cells, the ethanolic extract's IC_{50} value was found to be 350 g/mL (Prasad and Koch, 2014).

The cytotoxic activity of the stem extract of *Dendrobium crepidatum* was assessed with the identification of bioactive secondary metabolites. The growth of the HeLa cell line was most effectively inhibited by the chloroform extract, while the growth of the U251 cell lines was most effectively inhibited by the hexane extract. The cytotoxic activity of these extracts can be attributed to various compounds, including tetracosane, triacontane, stigmasterol, as well as several phenol derivatives such as 2-methoxy-4-vinyl phenol, 2-methoxy-5-(1-propenyl)-phenol, p-mesyloxyphenol, and 2,6-dimethoxy-4-(2-propenyl)-phenol (Paudel *et al*., 2019).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Plant materials

In vitro developed protocorms of *Dendrobium longicornu* (Figure 3.1) cultured by my senior Santoshi Khatri in the laboratory of Biotechnology at the Central Department of Botany were taken as starting plant materials.

Figure 3.1: *In vitro*-developed protocorms of *Dendrobium longicornu*

3.2 Preparation of plant materials

Plant materials i.e., protocorms were taken out from the jars and the fresh weight was taken using an electric balance. *In vitro*-developed protocorms were dried at room temperature and finely powdered using an electric grinder.

3.3 Extraction and fractionation of the extract

A maceration method was used for the extraction of phytochemicals. In this method, the powder was dissolved in 90% methanol 1:10 (w/v) for 48 hours and filtered using Whatman's filter paper. The filtrate was dried under reduced pressure in a rotatory evaporator by evaporating the solvent. The crude extract was collected in a clean and dry sample vial and stored in the refrigerator at 4°C.

The crude extract was then subjected to fractionation using a separatory funnel (Figure 3.2). The solvents hexane, dichloromethane (DCM), methanol and water were used in the ratio of 1:10 w/v. Fractions of hexane, Methanol, DCM and water were obtained and dried under reduced pressure in a rotary evaporator.

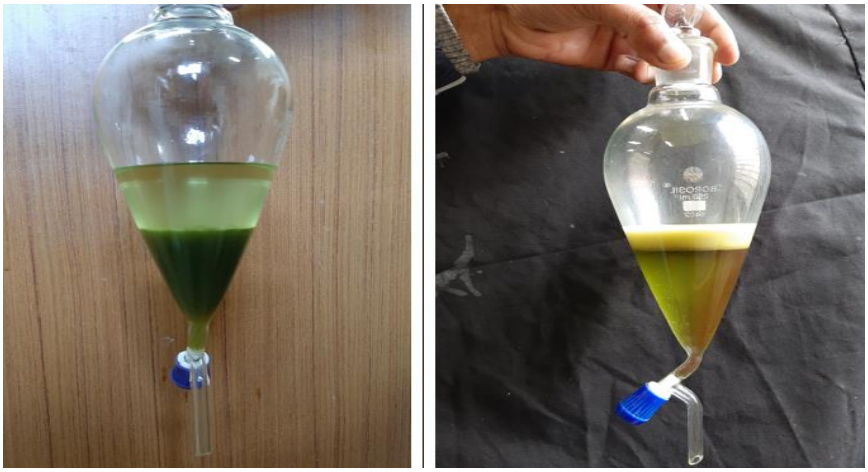

Figure 3.2: Solvent fractionation

3.4 Sub-fractionation of the extract

Sephadex LH-20 separation, also referred to as size exclusion chromatography, is a technique employed to separate chemical substances based on their size as they migrate through stationary beds consisting of a porous semi-liquid substance, primarily silica.

Each hexane, methanol, DCM and the aqueous fraction was subjected to further fractionation using methanol in the Sephadex LH-20 column chromatography (Figure 3.3). Different bands of the sub-fractions were seen in the column and were collected in different beakers based on colour appearance. Four sub-fractions (M1, M2, M3 and M4) of methanol, two sub-fractions (A1 and A2) of aqueous, three sub-fractions (DY, DB, DG) of DCM and three sub-fractions (HBG, HY, and HYG) of hexane fraction were obtained. All the sub-fractions obtained were dried under the reduced pressure of the rotary evaporator. The weight of all the collected bands was noted and stored at 4ºC for further use.

Figure 3.3: Column chromatography

Figure 3.4: Sub-fractions collected based on the colour bands

3.5 Total Phenolic Content (TPC)

Total phenolic content in all the hexane, methanol, DCM and aqueous sub-fractions was determined using the Folin-Ciocalteau phenol (FC) reagent colourimetric method of Zhang *et al.* (2006). This colourimetric FC reagent assay was done on a 96-well plate. In each well, 75 µL of distilled water was introduced followed by adding 25 µL of either sample (1 mg/mL) or gallic acid (25-200 mg/mL) and 25 µL of FC reagent (diluted 1:1 (v/v) with distilled water). The reaction mixture was delivered through a repeating pipette. It was left for about 6 minutes and then 100 µL of 1 M Na_2CO_3 was added to each well. Then, the plates were covered with aluminium foil and incubated in the dark for 30-45 minutes. The absorbance was measured at 765 nm with a micro-plate reader (Azure bio-systems Micro-plate reader). The control was made by replacing an equivalent amount of plant sample with absolute methanol. This process was performed in triplicate. The total phenolic content of the samples was quantified by expressing it in micrograms of gallic acid equivalent per milligram of the extract (µg GAE/mg) through the utilization of the gallic acid calibration curve (Figure 4.1).

3.6 Total Flavonoid Content (TFC)

Total flavonoid content in all the sub-fractions was evaluated using Aluminium Chloride ($AlCl_3$) method given by Chang *et al.* (2002) with minor modifications. First of all, 1 mg/mL of plant extracts were prepared and from these 1 mg/mL stocks, 250 µL of the plant extract was taken in a test tube. Then, 750 µL of 10% Aluminium chloride was added to the test tubes followed by 50 µL (1M) potassium acetate (CH_3COOK). Then the solutions were diluted by adding 1.4 mL of distilled water and then incubated at room temperature for 30 minutes. The control was prepared by

replacing the plant extract with the same volume of absolute methanol. Then, 200 µL reaction mixtures were transferred from the test tube to the 96-well plate in triplicate. The absorbance at 415 nm was measured with a micro-plate reader (Azure biosystems Micro-plate reader). This process was performed in triplicate. The calibration curve was obtained using Quercetin, which was used as a standard at 25, 50 100 and 200 µg/mL to obtain a calibration curve (Figure 4.2). The flavonoid content (TFC) was quantified by expressing it as milligrams of Quercetin equivalent per gram of the plant extract (µg QE/mg).

3.7 Antioxidant activity

The change in optical density of DPPH radicals was monitored to test the antioxidant capacity of the sub-fractions through a free radical scavenging assay. The antioxidant capacity of the sub-fractions was determined by following Ben Mansour *et al.* (2016). The stock solution of each sample was prepared (1 mg/mL) by using methanol. Then, a dilution was carried out serially to obtain a solution at concentrations of 200, 100, 50 and 25 µg/mL. A fresh DPPH solution of 0.02 mM was prepared by dissolving 7.88 mg of DPPH powder (molecular weight 394.32 gm/mol) in 100 mL of methanol away from the direct light. Then, a volume of 50 μL of each sample (25–200 µg/mL) was mixed with 150 μL of DPPH in a 96-well plate in triplicate. The blank or control was made by replacing the same volume of plant sample with an equal volume of absolute methanol and was incubated for 30 minutes at room temperature. After 30 minutes of incubation, the absorbance of the 96-well plate was measured at 517 nm using a micro-plate reader (Azure biosystems Micro- plate reader). A lower absorbance value indicates the higher antioxidant activity of the sample.

The percentage of DPPH free radical scavenging activity by the sub-fractions of plant samples was calculated using the formula:

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

A standard graph was represented by taking the concentrations on the X-axis and the percentage of radical scavenging activity on the Y-axis. The IC_{50} value was also calculated by using a linear equation of the curve obtained:

$$Y = mX + C;\ IC_{50} = (50 - C)/m$$

Where X=concentration, Y=50% RSA, m and C are the coefficient and constant of the linear equation respectively.

3.8 Anticancer Activity

The cytotoxicity of the sub-fractions was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. In the present study, two human cancer cell lines namely HeLa (cervical carcinoma) and MCF-7 (breast cancer) (Figure 3.5) were used. The cytotoxicity assay was performed at Annapurna Research Centre.

3.8.1 Cell Culture

The cell lines were grown separately in EMEM medium supplemented with 10 % FBS and 1% Penicillin as antibiotics and 1% L-Glutamine in a T-flask maintained at 37 ºC with 5% CO_2 in an incubator before testing anticancer activity (Mosmann, 1983).

3.8.2 Resuscitation of cells

Previously cryopreserved frozen cells were thawed and 5 mL EMEM medium was added to it. The cell suspension was centrifuged at 4000 rpm for 4 minutes. The supernatant was removed and cells were suspended in the medium. The cell suspension was then transferred into the T-flask and incubated at 37 ºC with 5% CO_2 in an incubator.

3.8.3 Subculture of cells

Once the cells get attached and reached more than 80% confluency, the cells were sub-cultured. Before sub-culture, the medium was removed and the cell layer was rinsed with PBS to remove the traces of the medium. About 2/3 mL trypsin was added to the flask and incubated for 3-4 minutes for the detachment of the cells, the process is called trypsinization. The detached cells were centrifuged at 4000 rpm for 4 minutes. The pallet formed was resuspended in the fresh medium and cell suspension was transferred to other T-flasks. Then, the T-flasks were kept for incubation at 37 ºC with 5% CO_2 in an incubator. The medium was changed when necessary in 2 to 3 days.

3.8.4 Cell harvesting

The culture medium was removed from the T-flask and the cells were cleaned with PBS. The cells were trypsinized with 2/3 mL Trypsin-EDTA solution for detachment.

The cell suspension was placed in a falcon tube and centrifuged, the supernatant was removed and the cell suspension was used for further assay.

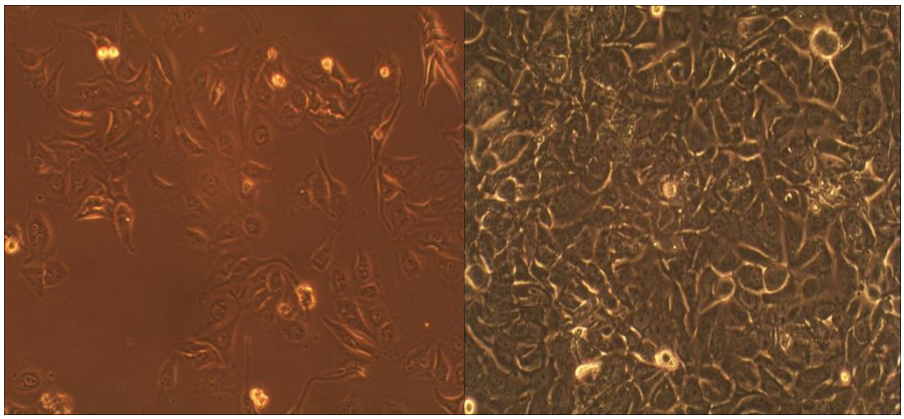

Figure 3.5: HeLa cells (left)**,** MCF-7 cells (right)

3.8.5 Cell counting

Cells were counted using a haemacytometer. Cells were harvested as described earlier, and about 20 μL of the cell suspension was transferred in a haemacytometer and observed under an electron microscope. The cells of each of the four corners and central square were counted and the cells per microlitre were calculated using the formula below.

Cells/ μL medium= (Total no. of counted cells in 5 squares x 4) /5 × 10^4 cells/mL

3.8.6 MTT assay

From the cell culture suspension, about 10000 cells with 100 µL of the medium were distributed into each well of a 96-well microtiter cell culture plate and incubated at 5% CO_2 incubator at 37 ºC for 48 hours. After 48 hours, following the attachment, the old medium was removed and the cells were treated with 100 μL of different concentrations (50, 100, 200, 400 µg/mL) of the sub-fractions and 100 μL of medium and incubated for 24 hours. The test was carried out in triplicates. After 24 hours of incubation, the supernatant was discarded and 100 µL of medium with 20 µL of MTT was added to each well and incubated for 4 hours. Following 4 hours of incubation, purple formazan crystals were formed in living cells. To dissolve formazan crystals, DMSO (100 µL, 2.5%) was used followed by incubation for another 15 minutes at room temperature. The absorbance was measured at 595 nm with an ELISA microplate reader. The number of dead cells per well was determined by calculating the percentage relative to the

control, thereby obtaining an average measurement of cell death following exposure to the sub-fractions.

The percentage of cytotoxicity was determined by using the following formula.

$$\% \text{ cytotoxic activity} = \frac{control\ absorbance(\ Abs1) - sample\ absorbance\ (Abs2)}{control\ absorbance} \text{ X } 100\ \%$$

Where, Abs1 indicates the absorbance of the cell with all the components except plant extracts and Abs2 indicates the absorbance of the cell with all components with plant extract.

3.9 Data analysis

Data analysis was done by using SPSS version 25 software. The values were presented in the form of Mean±SE (standard error of means) and the data were put through an analysis of variance (ANOVA) using Duncan multiple range test.

CHAPTER FOUR: RESULTS

Based on the colour of the bands in a column chromatography using Sephadex LH-20, a total of 4 sub-fractions of methanol extract, 2 sub-fractions of aqueous extract, 3 sub-fractions of hexane extract and 3 sub-fractions of DCM extract obtained from *in vitro* grown protocorms of *Dendrobium longicornu* were used for quantitative analysis of their antioxidant activity, total phenolic content (TFC) and total flavonoid content (TFC). And only the sub-fractions of methanol and aqueous fractions (based on their antioxidant capacity) were further processed for their cytotoxicity assay on human breast cancer cells (MCF-7) and human cervical cancer cells (HeLa).

4.1 Sub-fractions of the fractions

The hexane, methanol, DCM and aqueous fractions were subjected to Sephadex LH-20 column chromatography. Altogether, 3 sub-fractions of hexane, 4 sub-fractions of methanol, 3 sub-fractions of DCM and 2 sub-fractions of aqueous extracts were collected (Table 4.1).

Table 4.1: Sub-fractions of protocorms of *Dendrobium longicornu.*

S.N.	Extract	Sub-fractions	Colour of the sub-fraction	Weight (mg)
1.	Methanol extract	M1	Light brown	41
		M2	Light yellow	83
		M3	Blackish	38
		M4	Dark brown	29
2.	Aqueous extract	A1	Brown	123
		A2	Light green	34
3.	DCM extract	DB	Black	8.7
		DG	Green	11.9
		DY	Yellow	16
4.	Hexane extract	HBG	Blackish green	140
		HYG	Yellowish green	93
		HY	Yellow	87

4.2 Quantitative Phytochemical Analysis

4.2.1 Determination of Total Phenolic Content (TPC)

The total phenolic content of various samples was varied. Among all the samples, the maximum total phenolic content was recorded in M1 sub-fraction (187.58 ± 0.23 µg GAE/mg) followed by A2 sub-fraction (175.89 ± 0.23 µg GAE/mg) (Figure 4.3). The minimum total phenolic content was recorded in the HY sub-fraction (36.22 ± 0.238 µg GAE/mg). The means of the different samples were found to be significantly different at $p \leq 0.05$.

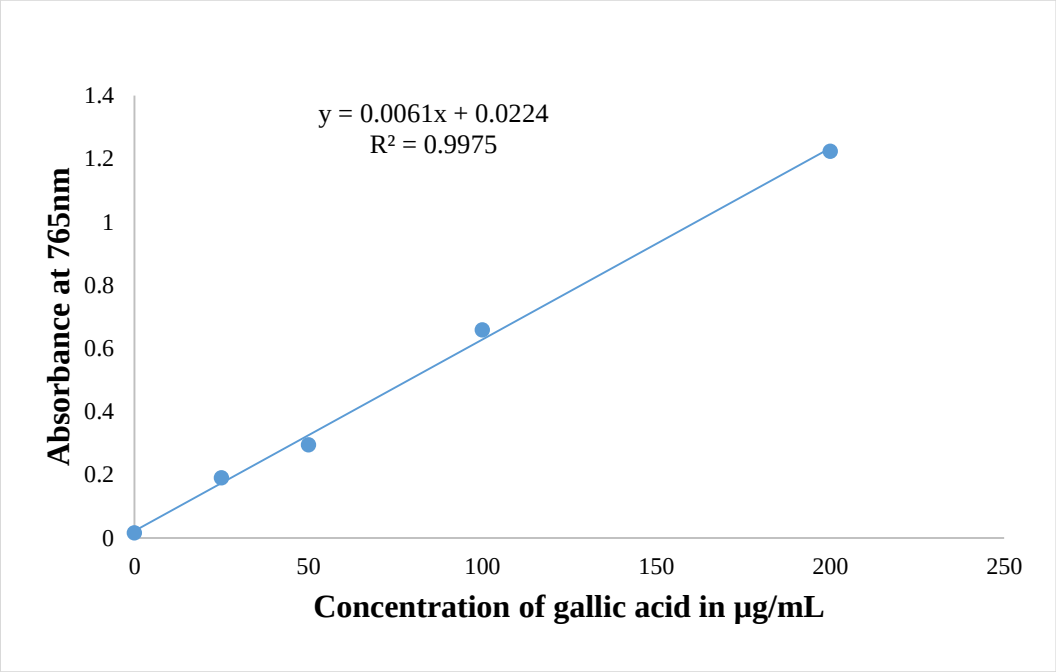

Figure 4.1: Standard curve of gallic acid for TPC determination

4.2.2 Determination of Total Flavonoid Content (TFC)

Among all the samples, the maximum total flavonoid content was recorded in M1 sub-fraction (84.67 ± 0.37 µg QE/mg) followed by A2 sub-fraction (75.43 ± 0.65 µg QE/mg) (Figure 4.3), and the minimum total flavonoid content was recorded in the HY sub-fraction (19.52 ± 0.97 µg QE/mg). The means of the samples were found to be significantly different at $p \leq 0.05$.

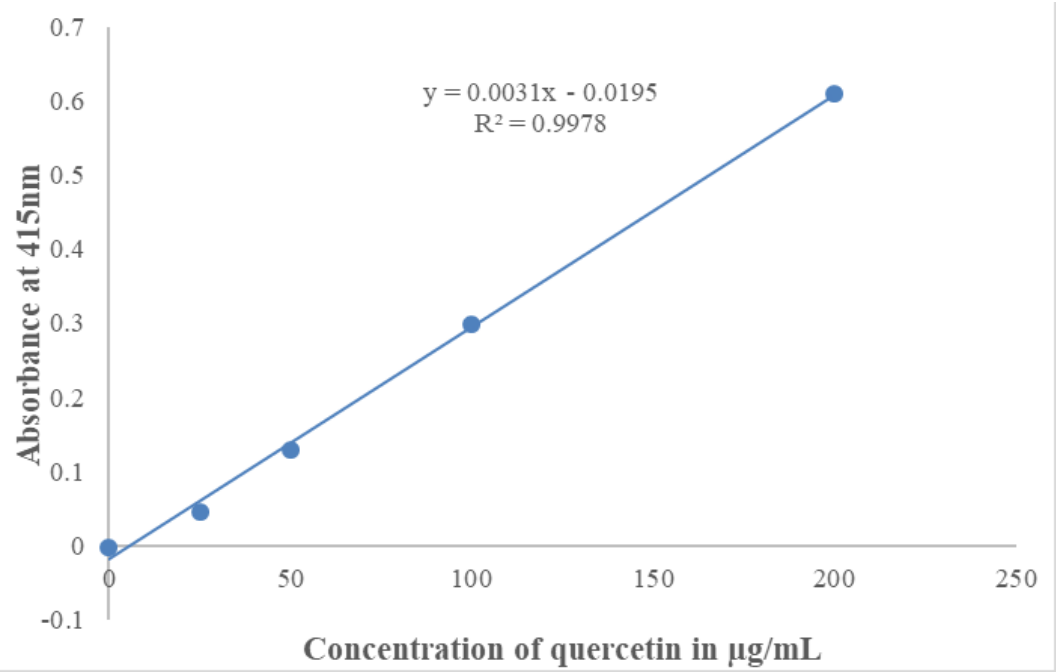

Figure 4.2: Standard Curve of Quercetin for TFC determination

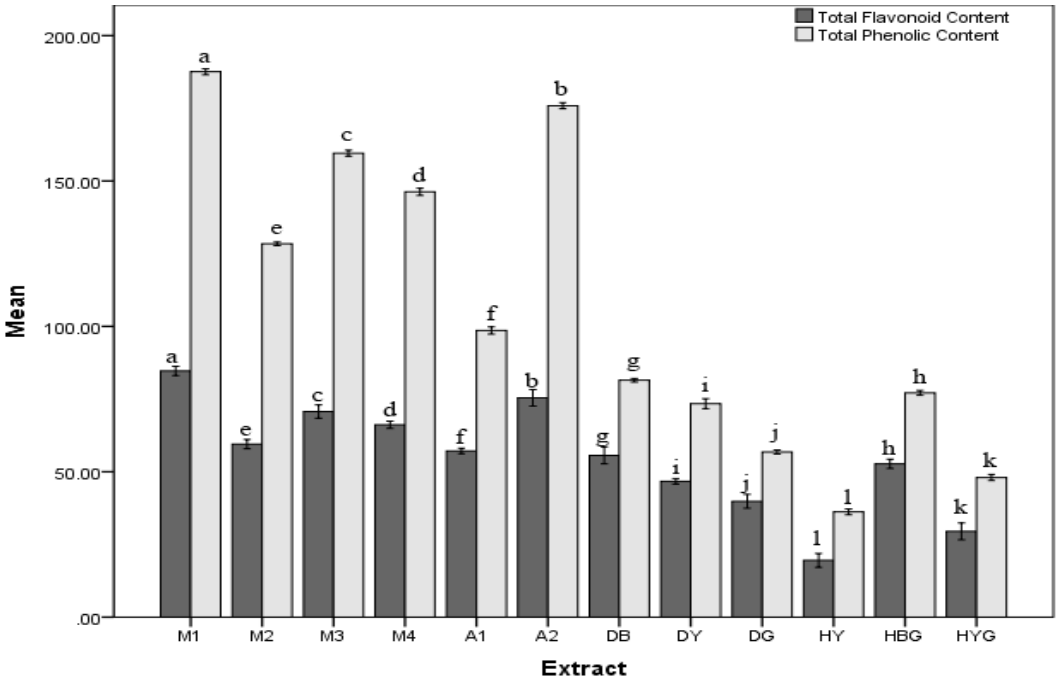

Figure 4.3: Graph showing TPC (µg GAE/mg) and TFC (µg QE/mg) of different tested samples.

Bar with different alphabets within the same group content is significantly different at $p<0.05$ (Duncan multiple range test). The total phenolic and flavonoid contents of the M1 sub-fraction were found to be significantly higher than other samples.

4.3 Determination of Antioxidant Activity

All the sub-fractions showed DPPH free radicals scavenging capacity. The scavenging percentage of DPPH free radicals varied from 3.24±1.29 % for the sub-fraction HY at 25 µg/mL to 70.48±0.62 % for the sub-fraction M1 at 200 µg/mL. The highest DPPH free radical scavenging percentage of M1 sub-fraction at 200 µg/mL was followed by sub-fraction M3 (65.30±0.86 %) at 200 µg/mL. M1 and M3 had the highest DPPH free radicals scavenging percentage at the different concentrations as compared to the other sub-fractions. Therefore, sub-fractions M1 and M3 revealed the highest antioxidant capacity with the highest DPPH free radicals scavenging percentage (Table 4.2).

Table 4.2: Percentage of DPPH radical scavenging activity by sub-fractions at different concentrations.

Sub-fractions	Concentration	Percentage of DPPH radical scavenging activity (Mean±S.E.)
M1	25	20.43±1.09
	50	41.49±1.33
	100	61.36±0.78
	200	70.48±0.62
M2	25	14.29±1.34
	50	26.94±0.86
	100	35.79±1.20
	200	56.51±0.48
M3	25	18.35±1.36
	50	32.94±0.81
	100	57.78±0.62
	200	65.30±0.86
M4	25	16.35±1.26
	50	34.99±1.38
	100	47.29±0.36
	200	54.39±0.52
A1	25	11.74±1.11
	50	20.41±1.54

	100	38.76±1.20
	200	52.55±1.00
A2	25	17.21±1.10
	50	26.49±1.26
	100	48.48±1.07
	200	60.40±0.38
DB	25	9.37±1.44
	50	22.23±1.67
	100	31.66±1.02
	200	46.72±0.99
DY	25	11.06±3.29
	50	24.17±1.67
	100	35.37±0.84
	200	45.10±0.75
DG	25	6.76±1.58
	50	12.71±1.28
	100	18.25±0.86
	200	34.41±0.68
HY	25	3.24±1.29
	50	8.51±1.70
	100	13.20±1.18
	200	21.07±1.93
HBG	25	13.93±4.54
	50	20.29±1.50
	100	38.33±1.45
	200	44.12±0.82
HYG	25	3.59±1.73
	50	9.60±1.57
	100	19.56±1.61
	200	30.28±0.65

DPPH is a stable and free radical dissolved in methanol and showed characteristic colour absorption at 517 nm using a spectrophotometer. Due to the donation of hydrogen molecules, free radicals are scavenged by antioxidant molecules and the purple colour of DPPH changed to light yellow colour causing reduction. The IC_{50} value was evaluated using the linear regression equation obtained from the percentage of scavenging free radicals at different concentrations. Among all 12 sub-fractions, the lowest IC_{50} (99.68 ± 3.31 µg/mL) was noted for the M1 sub-fraction followed by the M3 sub-fraction (118.74 ± 3.36 µg/mL) (Figure 4.4), and the highest IC_{50} value was noted for HY sub-fraction (496.25 ± 27.53 µg/mL). The mean values were significantly different at $p \leq 0.05$.

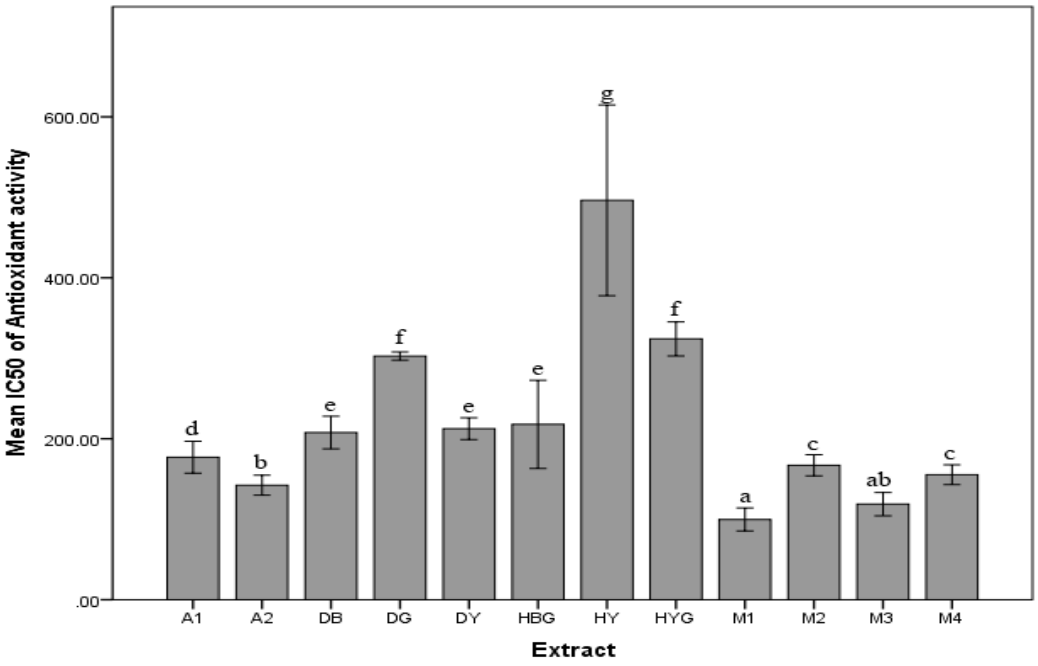

Figure 4.4: Graph showing the mean IC_{50} (µg/mL) of antioxidant activity of different tested samples. Bars with different alphabets are significantly different at $p<0.05$.

4.4 Correlation between TPC, TFC and Antioxidant Capacity

For the correlation study, Pearson's correlation test was performed. All 12 samples with their triplicates i.e., 36 samples were taken for the test, and the total phenolic content (TPC) and total flavonoid content (TFC) were found to be positively correlated. The correlation between total flavonoid content (TFC) and antioxidant capacity (IC_{50}) was significantly strong ($p \leq 0.01$) and negative ($r = -0.931$) as shown in Table 4.3. The negative correlation indicates that an increase in total flavonoid content leads to a decrease in the IC_{50} of antioxidant activity (high antioxidant activity). Similarly, the

correlation between total phenolic content and IC_{50} of antioxidant activity was also found to be significantly strong ($p \leq 0.01$) and negative ($r = -0.832$) (Table 4.3). The increase in phenolic content also leads to a decrease in the IC_{50} of antioxidant activity (high antioxidant activity). Therefore, the presence of high total phenolic and flavonoid content in the sub-fractions has strong antioxidant activity.

Table 4.3: Correlation between TPC, TFC and antioxidant capacity.

		Total Flavonoid Content	Total Phenolic Content	IC_{50} of Antioxidant activity
Total Flavonoid Content	Pearson Correlation	1	0.950**	-0.931**
	Sig. (2-tailed)		0.000	0.000
	N	36	36	36
Total Phenolic Content	Pearson Correlation		1	-0.832**
	Sig. (2-tailed)			0.000
	N		36	
IC_{50} of Antioxidant activity	Pearson Correlation			1
	Sig. (2-tailed)			
	N			36

**. Correlation is significant at the 0.01 level (2-tailed). N= 36

4.5 Anticancer activity

Based on high antioxidant, phenolic contents and flavonoid contents, only the M1, M2, M3, M4, A1 and A2 sub-fractions were tested for their anticancer activities in two different human cancer cell lines namely MCF-7 (human breast cancer) and HeLa (human cervical carcinoma). Anticancer activity of all the six sub-fractions on human cancer cell lines (MCF-7 and HeLa) was determined using MTT [3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The anticancer activity of the sub-fractions against the cancer cell lines is summarized below.

4.5.1 Anticancer activity on MCF-7 cancer cell line

The percentage of growth inhibition of MCF-7 cells by different sub-fractions is shown in Table 4.4. The percentage of growth inhibition of MCF-7 cells was found to vary from 3.58±14.23 % for the sub-fraction A1 at 25 µg/mL to 64.30±1.13% for the sub-fraction M1 at 200 µg/mL. The highest MCF-7 cell growth inhibition by the sub-fraction M1 was followed by the sub-fraction M3 (58.54±5.36%) at 200 µg/mL. The sub-fractions M1 and M3 showed a high inhibition percentage of MCF-7 cell growth at 200 µg/mL as compared to other sub-fractions and the least inhibition percentage was shown by the sub-fraction A1 at 25 µg/mL.

Table 4.4: Percentage of MCF-7 cells growth inhibition by sub-fractions at different concentrations.

Sub-fraction	Concentration	Percentage of MCF-7 cells growth inhibition (Mean±S.E.)
M1	25	39.61±5.88
	50	41.45±1.83
	100	54.55±0.92
	200	64.30±1.13
M2	25	24.53±0.22
	50	37.61±0.71
	100	40.97±0.47
	200	55.03±1.89
M3	25	22.97±4.61
	50	28.15±5.15
	100	36.35±2.91
	200	58.54±5.36
M4	25	20.07±1.36
	50	27.50±3.58
	100	32.17±5.29
	200	49.62±3.46

A1	25	3.58±14.23
	50	3.85±1.06
	100	12.40±15.52
	200	29.47±11.40
A2	25	6.42±2.20
	50	7.27±13.72
	100	8.16±13.95
	200	38.69±7.49

The cell growth inhibition concentration of the sub-fraction by 50% i.e., IC_{50} of the sub-fractions on MCF-7 cells was evaluated using the linear regression equation of the percentage inhibition curve. Among 6 different samples, M1 sub-fraction showed the lowest IC_{50} value of 92.43 ± 9.44 µg/mL followed by M2 sub-fraction with an IC_{50} of 163.02 ± 14.38 µg/mL (Figure 4.5), and the highest IC_{50} was shown by A1 sub-fraction at 402.63 ± 170.22 µg/mL.

4.5.2 Anticancer activity on HeLa cancer cell line

The percentage of growth inhibition of HeLa cells by different sub-fractions is shown in Table 4.5. The percentage of growth inhibition of HeLa cells was found to vary from 10.50±2.73 % for the sub-fraction M2 at 50 µg/mL to 76.03±3.40 % for the sub-fraction M1 at 400 µg/mL. The highest HeLa cell growth inhibition by the sub-fraction M1 was followed by the sub-fraction M3 (66.68±4.82%) at 400 µg/mL. The sub-fractions M1 and M3 showed a high inhibition percentage of HeLa cell growth at 400 µg/mL as compared to other sub-fractions and the least inhibition percentage was shown by the sub-fraction M2 at 50 µg/mL.

Table 4.5: Percentage of HeLa cells growth inhibition by sub-fractions at different concentrations.

Sub-fraction	**Concentration**	**Percentage of HeLa cells growth inhibition (Mean±S.E.)**
M1	50	14.70±4.51
	100	17.54±2.89
	200	22.74±2.38

	400	76.03±3.40
M2	50	10.50±2.73
	100	15.23±4.92
	200	26.72±4.98
	400	54.16±2.32
M3	50	11.38±7.16
	100	24.73±3.20
	200	28.83±2.87
	400	66.68±4.82
M4	50	10.55±2.84
	100	22.79±2.45
	200	32.64±2.63
	400	56.33±6.58
A1	50	17.77±1.51
	100	18.32±7.07
	200	21.75±2.01
	400	52.55±4.17
A2	50	17.20±5.37
	100	21.97±2.42
	200	30.37±6.02
	400	61.10±4.11

The cell growth inhibition concentration of the sub-fractions by 50% i.e., IC_{50} of the sub-fractions on HeLa cells was evaluated by using a linear regression equation derived from the percentage inhibition curve. Among the 6 different samples, M1 sub-fraction showed the lowest IC_{50} value of 283.48 ± 17.55 µg/mL followed by M3 sub-fraction with an IC_{50} of 301.10 ± 18.40 µg/mL (Figure 4.5), and the highest IC_{50} of 418.20 ± 38.17 µg/mL was shown by A1 sub-fraction. The means of the IC_{50} of the samples were found to be significantly different at $p \leq 0.05$.

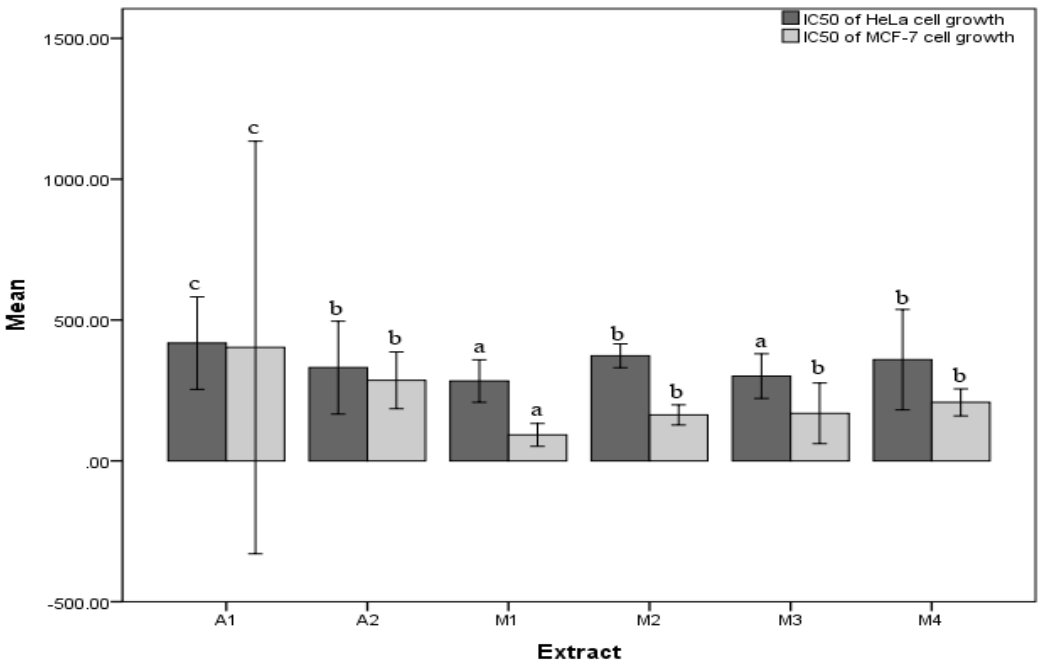

Figure 4.5: Graph showing the IC_{50} of different tested samples on HeLa and MCF-7 cell lines. Bars with different alphabets within the same group content are significantly different at $p<0.05$.

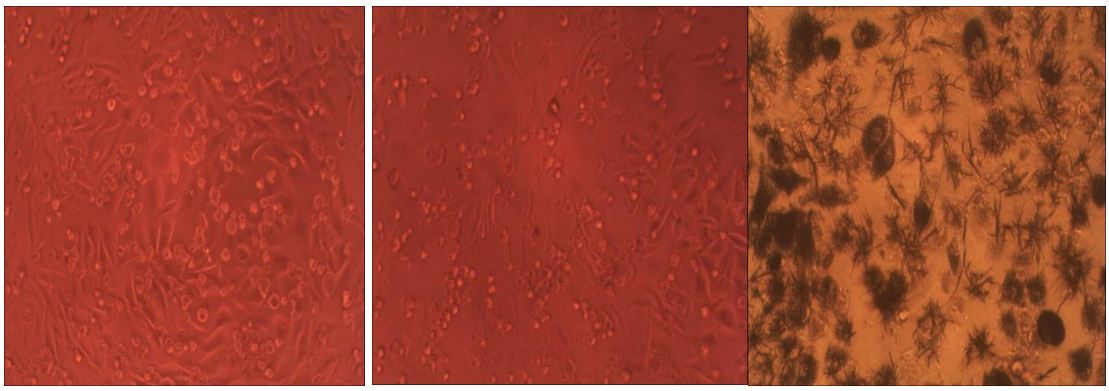

Figure 4.6: Treated HeLa cells (left), treated MCF-7 treated cells (middle), formazan crystals (right).

4.6 Correlation between IC_{50} of antioxidant activity, HeLa and MCF-7 cell growth

All 6 samples taken for the anticancer assay were performed for their correlation to their IC50 of sub-fractions for antioxidant activity by Pearson's correlation (Table 4.6). IC_{50} of sub-fractions for antioxidant and HeLa cell growth inhibition was found to be positively correlated ($r = 0.752$) at a significant level of 0.01. Similarly, the IC_{50} of sub-fractions for MCF-7 cell growth inhibition and the antioxidant capacity of the samples were also found to be positively correlated ($r = 0.524$) at the significance level of 0.05.

There is also a positive correlation between the growth of HeLa and MCF-7 cancer cell lines at a 0.01 level of significance. Therefore, results showed that high antioxidant sub-fractions have high anticancer activities.

Table 4.6: Correlation between IC_{50} of HeLa and MCF-7 cells growth inhibition and antioxidant activities.

		IC_{50} of Antioxidant activity	IC_{50} of HeLa cell growth	IC_{50} of MCF-7 cell growth
IC_{50} of Antioxidant activity	Pearson Correlation	1		
	Sig. (2-tailed)			
	N	18		
IC_{50} of HeLa cell growth	Pearson Correlation	0.752**	1	
	Sig. (2-tailed)	0.000		
	N	18	18	
IC_{50} of MCF-7 cell growth	Pearson Correlation	0.524*	0.600**	1
	Sig. (2-tailed)	0.026	0.008	
	N	18	18	18

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

*. Correlation is significant at the 0.05 level (2-tailed).

CHAPTER FIVE: DISCUSSION

5.1 Total Phenolic and Flavonoid Content

In the current study, different sub-fractions of hexane, DCM, methanol and hexane extracts prepared from *in vitro*-grown protocorms of *Dendrobium longicornu* were taken for the quantitative analysis of the phenolic content (TPC) and total flavonoid content (TFC).

Among all the samples of *D. longicornu* protocorms (Table 1), the M1 sub-fraction has the highest total phenolic and flavonoid content, followed by the A2 sub-fraction. In contrast to our findings, the highest TPC and TFC were found in an acetone extract of the *Dendrobium longicornu* plant (Paudel *et al*., 2017). The results of these two studies may differ due to the use of different solvents.

The current study was also influenced by Klongkumnuankarn *et al.* (2015), who isolated various phenolic compounds from a methanolic extract of *Dendrobium brymerianum*. Huda-Faujan *et al*. (2009), also supported this study that methanolic extracts of some plants had the highest phenolic content. Similarly, the methanolic extract of *Marrubium peregrinum* also had the highest phenolic and flavonoid content (Stankovic, 2011) which supports the present research. Saeed *et al*. (2012) also reported that the methanol extract of *Phyllanthus* species has a significant concentration of phenols and flavonoids (Zain and Omar, 2018) which supports this study. The phenolic and flavonoid components are dissolved in the high polarity solvent due to the reason, the M1 sub-fraction has high contents of total phenol and flavonoid. The previous supporting literature reported the phenolic and flavonoid content in the naturally growing plants, whereas, in the present research, *in vitro*-developed protocorms were used as materials to estimate the total phenolic and flavonoid contents, the nutritional environment for the growth of protocorms may enhance the synthesis of phenolic and flavonoid components.

5.2 Antioxidant activity

The antioxidant activity of a total of 12 samples i.e. 4 sub-fractions of methanol extract, 3 sub-fractions of DCM extract, 3 sub-fractions of hexane and 2 sub-fractions of aqueous extract was determined using DPPH radicals scavenging assay.

Among all the sub-fractions, the M1 sub-fraction was found to scavenge 50% DPPH free radicals at 99.68 ± 3.31 µg/mL of its concentration followed by the M3 sub-fraction. This outcome could be explained by the fact that the M1 sub-fraction has the highest phenolic and flavonoid components when compared to other sub-fractions. Plant phenolic compounds scavenge free radicals because of their hydroxyl groups (Saeed *et al*., 2012). Plants produce a variety of phenolic and flavonoid compounds which have strong antioxidant properties (Nunes *et al*., 2012). The function of antioxidants is that they interact with oxidative free radicals and scavenge them. For example, the 2,2-diphenyl-1-picrylhydrazyl (deep violet) reduces to 2,2-diphenyl-1-picrylhydrazine (yellow) when interacting with antioxidant riched components. The discolouration of 2,2-diphenyl-1-picrylhydrazyl indicated that the extract's high polyphenol and flavonoid content can scavenge free radicals (Mensor *et al*., 2001; Brand-Williams *et al*., 1995). Paudel *et al.* (2017) revealed that the highest TPC and TFC containing acetone extract of *Dendrobium longicornu* had strong antioxidant activity by scavenging the DPPH radicals. Similarly, according to Stankovic (2011), the methanolic extract of *Marrubium peregrinum* with the highest phenolic and flavonoid contents exhibited significant antioxidant activity, supporting the findings of the present study. This study is also supported by the discovery that *Dendrobium officinale* exhibits a strong positive correlation between total flavonoid content and DPPH radical scavenging rate (Zhang *et al*., 2017). In this study, protocorms were used to test antioxidant capacity and found that M1 sub-fraction of the protocorms extract has a strong antioxidant activity which is also supported by the previous study of Paudel *et al.* (2020) in which methanol extract of protocorms of *Dendrobium longicornu* showed strong antioxidant activity.

5.3 Cytotoxicity

The human body produces reactive oxygen species and free radicals which cause cancer (Halliwell 2014; Nimse and Pal, 2015). The presence of compounds that are rich in antioxidant properties in plant products scavenges these types of radicals and prevents the development of cancer (Sayin *et al*., 2014; Gali-Muhtasib *et al*., 2015). These substances cause apoptotic bodies and cell cycle arrest to stop cancer. The cytotoxic activity of 6 samples i.e., 4 sub-fractions of methanol extract and 2 sub-fractions of aqueous extract of protocorms of *D. longicornu* against MCF-7 and HeLa cancer cell lines were studied by MTT assay. Among the samples, M1 sub-fraction showed a

higher percentage inhibitory effect on the proliferation of MCF-7 (human breast cancer) cells with the lowest IC_{50} value of 92.43 ± 9.44 µg/mL followed by M2 with an IC_{50} of 163.02 ± 14.38 µg/mL. While in HeLa (human cervical carcinoma) cell line, M1 sub-fraction also showed a higher percentage inhibitory effect on the growth of HeLa cells with the lowest IC_{50} value of 283.48 ± 17.55 µg/mL.

This finding suggests that the M1 sub-fraction of methanol extract has a higher concentration of bioactive components (phenolics and flavonoids) that inhibit the proliferation of cancer cells. The findings can be validated by the works that explained that the whole plant of *D. longicornu* contains bibenzyl and phenanthrene, and some amounts of monoaromatics, steroid and flavonoid derivatives due to which it performs anticancer activity (Hu *et al*., 2008; Hu *et al*., 2010; Chen *et al*., 2010; Li *et al*., 2009).

The high amount of phenolics and flavonoids in the M1 sub-fraction may be the cause of cytotoxicity on cancer cell lines. Many earlier researchers have noted the cytotoxic activity of phenolics and flavonoids (Patel and Patel, 2011; Jeune *et al*., 2005).

Many isolated bioactive compounds from plants are being vigorously tested for their anticancer properties. According to Klongkumnuankam *et al*. (2015), the methanolic extract of *D. brymerianum* exhibited notable cytotoxicity against human cancer cell lines. This study lends support to our work because in the current study also, the M1 sub-fraction of the methanol extract displayed strong cytotoxic effects.

The highest cytotoxic effect of the M1 sub-fraction among the sub-fractions may also be attributed to the highest antioxidant activity, which may be connected to the presence of phenolics and flavonoids. The total phenolic and flavonoid contents and cytotoxicity of the sample were also found to be positively correlated. This also validates the fact that phenolics and flavonoids have cytotoxic activity.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, *in vitro*-grown protocorms of *D. longicornu* have synthesized the phenolics and flavonoids which have potent antioxidant and anticancer activities. The M1 sub-fraction of protocorms has scavenged the DPPH free radicals which have shown a potential antioxidant effect due to the presence of phenolics and flavonoid content. Due to the presence of bioactive components, M1 sub-fraction also inhibited the proliferation of MCF-7 and HeLa cancer cell lines indicating the anticancer activity on these cell lines.

6.2 Recommendations

The isolation and purification of the bioactive compounds in the sub-fraction can lead to the discovery of drugs in the future. So, the bioactive compounds in the sub-fractions should be further isolated and purified. Additional comprehensive investigations are required to unravel the underlying mechanisms that account for the antioxidant and anticancer effects of the sub-fractions obtained from the *in vitro*-grown protocorms of *D. longicornu*.

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APPENDICES

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Antioxidant and Anticancer Activities of The Bioactive Components Riched Protocorms of *Dendrobium longicornu*

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ABSTRACT

Dendrobium longicornu is a traditionally used medicinal orchid that has many bioactive compounds such as bibenzyl and phenanthrenes, monoaromatics, steroid, and flavonoid derivatives. This study aimed to investigate the total phenolic and flavonoid contents, and the antioxidant and cytotoxic activity of the different sub-fractions obtained from the *in vitro*-grown protocorms. The total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of all the 12 sub-fractions was determined using Folin-Ciocalteu phenol reagent colorimetric method, Aluminum Chloride (AlCl3) colorimetric method and DPPH free radical scavenging assay respectively. The cytotoxic activity of only 6 sub-fractions M1, M2, M3, M4, A1, and A2 was evaluated against human breast cancer cells (MCF-7) and cervical carcinoma cells (HeLa) using MTT assay. TPC and TFC were found to be the highest in the M1 sub-fraction with the value of 187.58 ± 0.23 mg GAE/gm and 84.68 ± 0.37 mg QE/g respectively. The highest antioxidant activity against DPPH free radical with the lowest IC50 value of 99.68 ± 3.31 µg/ml was shown by the M1 sub-fraction. M1 sub-fraction showed the highest inhibitory effect on the growth of both MCF-7 and HeLa cells with the lowest IC50 value of 92.43 ± 9.44 µg/ml and 283.48 ± 17.55 µg/ml respectively.
Keywords: Anticancer; Antioxidant; Protocorms; TFC; TPC

INTRODUCTION

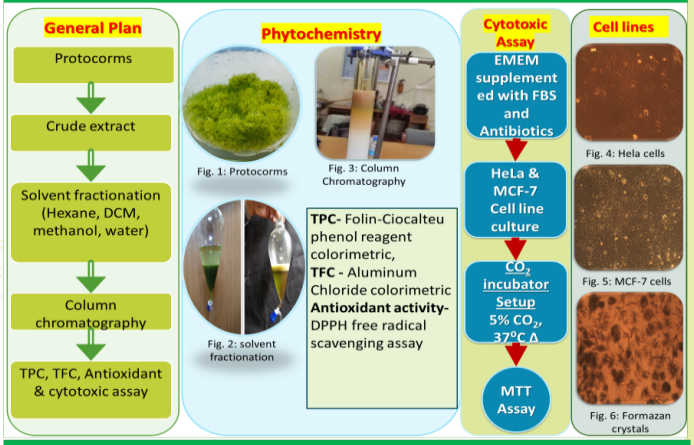
D. longicornu is a medicinally important orchid which is greatly used for the treatment of coughs and fever (Manandhar, 1995). In addition to these, this plant's biological activities have not yet been documented. Reactive nitrogen and oxygen species, such as singlet oxygen, hydrogen peroxide, superoxide anion, hydroxyl radical, and nitrous oxide, are frequently produced in living things as byproducts of biological reactions and are recognized to play a significant part in oxidative cellular damage. These free radicals' oxidative stress contributes significantly to the manifestation of many ailments, including aging, diabetes, cancer, and cardiovascular diseases (Hu et al., 2008; Chen et al., 2010). Natural antioxidants produced by *Dendrobium longicornu* are a powerful source of novel, beneficial chemicals with biological activity.

The main goals of the current study are to quantify the overall polyphenol & flavonoid contents in the various sub-fractions of *in vitro*-grown protocorms of this plant, as well as to assess the antioxidant and cytotoxic properties of the sub-fractions.

DISCUSSION

M1 sub-fraction of methanol extract was found to scavenge more DPPH free radicals (highest antioxidant activity) with the lowest IC50 value. This outcome could be explained by the fact that M1 has the largest phenolic and flavonoid levels when compared to other sub-fractions. The M1 sub-fraction also showed a higher percentage inhibitory effect on the growth of both MCF-7 and the HeLa cell lines with the lowest IC50 value. The high cytotoxicity of the M1 sub-fraction might be attributable to the presence of a high amount of flavonoid molecules. Flavonoids have been reported for their cytotoxic effect by many researchers. Methanolic extracts have been reported for their high cytotoxicity in different cell lines by many researchers (Paudel et al., 2020). The methanolic extract of *Dendrobium moniliforme* has also shown the highest cell growth inhibition of HeLa cells (Paudel et al., 2018). It has been reported that the methanolic extract of species of *Dendrobium* species has shown cytotoxic effects against different human cell lines.

METHODS AND MATERIALS



RESULTS

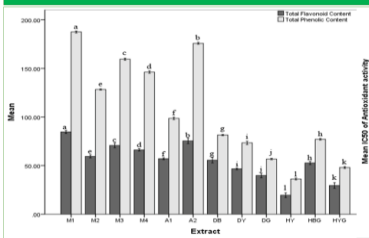


Fig. 7: Graph showing TPC & TFC of different tested samples.

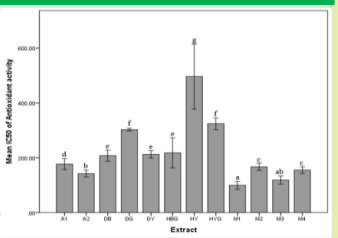


Fig. 8: Graph showing mean IC50 of antioxidant activity of different tested samples

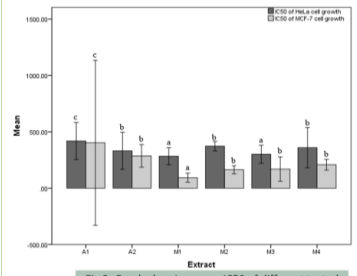


Fig 3: Graph showing mean IC50 of different tested samples on HeLa & MCF-7 cell lines

Based on the color of the bands in column chromatography, a total of 12 sub-fractions obtained from different solvent extracts were used for the tests.

- ✓ TPC (of 187.58 ± 0.23 mg GAE/gm) & TFC (84.68 ± 0.37 mg QE/g) were found to be the highest in the M1 sub-fraction of methanol extract.
- ✓ Antioxidant activity was shown to be the highest in the M1 sub-fraction with the lowest IC50 (of 99.68 ± 3.31 µg/ml) value among all the tested samples.
- ✓ Overall high cell inhibition was observed in the M1 sub-fraction of methanol with the lowest IC50 value in both MCF-7 (92.43 ± 9.44 µg/ml) & HeLa (283.48 ± 17.55 µg/ml) cell lines.

CONCLUSIONS

- ➢ *In vitro*-grown protocorms of *D. longicornu* have the potential to exhibit antioxidant and anticancer properties.
- ➢ *In vitro* developed protocorms of the *D. longicornu* synthesize phenolic compounds, flavonoid compounds, and other bioactive compounds which are potent antioxidant and anticancer agents.
- ➢ The sub-fraction M1 is found to be the best among all the sub-fractions tested.

FUTURE DIRECTIONS

- ➢ The bioactive compounds in these sub-fractions should be further isolated and purified.
- ➢ The isolation and purification of the bioactive compound in the sub-fraction M1 can lead to the discovery of drugs in the future.

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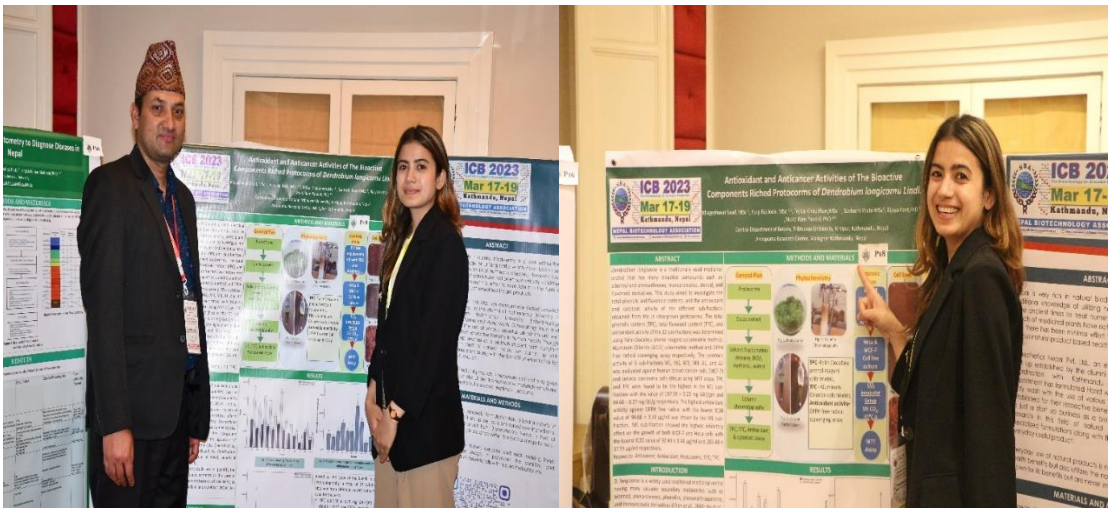
A poster presented in the 4th International Conference on Biotechnology (March 17-19, 2023).

A certificate of achievement received in the 4th International Conference on Biotechnology (March 17-19, 2023).



A photograph with my supervisor (left) and showing the poster (right) in the 4th International Conference on Biotechnology (March 17-19, 2023).

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Total Flavonoid Content	M1	3	84.6774	.64516	.37248	83.0747	86.2801	84.03	85.32
	M2	3	59.5161	.64516	.37248	57.9135	61.1188	58.87	60.16
	M3	3	70.6989	.93121	.53763	68.3857	73.0122	70.16	71.77
	M4	3	66.1828	.49275	.28449	64.9587	67.4069	65.65	66.61
	A1	3	57.1505	.37248	.21505	56.2252	58.0758	56.94	57.58
	A2	3	75.4301	1.13287	.65406	72.6159	78.2443	74.35	76.61
	DB	3	55.6452	1.16308	.67151	52.7559	58.5344	54.35	56.61
	DY	3	46.7204	.37248	.21505	45.7951	47.6457	46.29	46.94
	DG	3	39.8387	.96774	.55873	37.4347	42.2427	38.87	40.81
	HY	3	19.5161	.96774	.55873	17.1121	21.9201	18.55	20.48
	HBG	3	52.7419	.64516	.37248	51.1393	54.3446	52.10	53.39
	HYG	3	29.5161	1.16308	.67151	26.6269	32.4054	28.23	30.48
	Total	36	54.8029	18.31237	3.05206	48.6069	60.9989	18.55	85.32
Total Phenolic Content	M1	3	187.5847	.41256	.23819	186.5598	188.6096	187.15	187.97
	M2	3	128.4044	.25041	.14458	127.7823	129.0264	128.13	128.62
	M3	3	159.5519	.41256	.23819	158.5271	160.5768	159.11	159.93
	M4	3	146.2732	.50083	.28915	145.0291	147.5173	145.84	146.82
	A1	3	98.5683	.50083	.28915	97.3242	99.8124	98.13	99.11
	A2	3	175.8907	.41256	.23819	174.8659	176.9156	175.51	176.33
	DB	3	81.4645	.25041	.14458	80.8424	82.0865	81.25	81.74
	DY	3	73.4317	.68251	.39405	71.7362	75.1272	72.89	74.20

	DG	3	56.8197	.28394	.16393	56.1143	57.5250	56.66	57.15
	HY	3	36.2186	.41256	.23819	35.1937	37.2434	35.84	36.66
	HBG	3	77.1475	.32787	.18930	76.3331	77.9620	76.82	77.48
	HYG	3	48.0219	.41256	.23819	46.9970	49.0467	47.64	48.46
	Total	36	105.7814	50.48336	8.41389	88.7003	122.8625	35.84	187.97
	M1	3	99.6774	5.74036	3.31420	85.4175	113.9372	93.05	103.14
	M2	3	167.0960	5.28301	3.05015	153.9722	180.2197	162.05	172.58
	M3	3	118.7382	5.83014	3.36603	104.2553	133.2211	112.10	123.05
	M4	3	155.3529	4.98981	2.88087	142.9575	167.7483	149.89	159.67
	A1	3	177.0180	7.96265	4.59724	157.2377	196.7984	167.86	182.30
	A2	3	142.2967	4.99967	2.88656	129.8768	154.7166	136.55	145.67
IC50 of Antioxidant activity	DB	3	207.6950	8.14842	4.70449	187.4532	227.9368	198.50	214.02
	DY	3	212.4444	5.45783	3.15108	198.8864	226.0024	206.77	217.66
	DG	3	302.6521	2.05664	1.18740	297.5431	307.7611	300.92	304.93
	HY	3	496.2516	47.69316	27.53566	377.7752	614.7279	441.54	529.02
	HBG	3	217.8565	22.07333	12.74404	163.0233	272.6897	202.62	243.17
	HYG	3	324.0668	8.53027	4.92495	302.8764	345.2571	316.97	333.53
	Total	36	218.4288	107.90867	17.98478	181.9178	254.9398	93.05	529.02
	M1	3	283.4771	30.41398	17.55952	207.9246	359.0296	249.22	307.29
	M2	3	373.0814	17.03755	9.83663	330.7578	415.4050	353.81	386.15
	M3	3	301.1058	31.88381	18.40812	221.9020	380.3096	274.98	336.63
IC50 of HeLa cell growth	M4	3	359.2226	71.73695	41.41734	181.0181	537.4270	314.69	441.98
	A1	3	418.2065	66.12478	38.17716	253.9434	582.4696	366.16	492.61
	A2	3	331.0394	66.44083	38.35963	165.9912	496.0875	272.03	403.00
	DB	0	.	.	.	.	.	.	.

	DY	0	.	.	.	.	.	.	.
	DG	0	.	.	.	.	.	.	.
	HY	0	.	.	.	.	.	.	.
	HBG	0	.	.	.	.	.	.	.
	HYG	0	.	.	.	.	.	.	.
	Total	18	344.3555	63.75159	15.02639	312.6525	376.0584	249.22	492.61
	M1	3	92.4265	16.35474	9.44242	51.7990	133.0539	75.31	107.89
	M2	3	163.0191	14.38691	8.30629	127.2801	198.7582	150.64	178.81
	M3	3	168.7879	43.28256	24.98920	61.2680	276.3077	135.02	217.58
	M4	3	207.6464	19.42048	11.21242	159.4033	255.8896	185.74	222.75
	A1	3	402.6344	294.83285	170.22183	-329.7711	1135.0398	226.06	743.00
	A2	3	285.9212	40.56263	23.41885	185.1581	386.6844	244.24	325.26
IC50 of MCF-7 cell growth	DB	0	.	.	.	.	.	.	.
	DY	0	.	.	.	.	.	.	.
	DG	0	.	.	.	.	.	.	.
	HY	0	.	.	.	.	.	.	.
	HBG	0	.	.	.	.	.	.	.
	HYG	0	.	.	.	.	.	.	.
	Total	18	220.0726	146.04340	34.42276	147.4469	292.6983	75.31	743.00