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Biological activities of protocorms and stems extracts of *Dendrobium transparens*

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ABSTRACT

This study assessed the phenolic and flavonoid contents, and antioxidant, antibacterial, as well as cytotoxic properties of the protocorm extract of *Dendrobium transparens* and compared it to its wild equivalents. Methanol was used to extract compounds from the stems (DTSE) and protocorms (DTPE). DTSE contained 61.889 mg flavonoid and 82.00 mg phenolic content per gram extract equivalent to quercetin and gallic acid, respectively. At a concentration of 191.23 µg/mL, DTSE exhibited a 50% DPPH radical scavenging efficiency. Compared to the 3T3 cell line (2108.87 µg/mL), the DTPE's cytotoxic ability against the HeLa (229.30 µg/mL) and U251 (213.90 µg/mL) cell lines was found to be significantly stronger. However, the U251 cell line was strongly cytotoxic to DTSE (75.84 µg/mL). At a dose of 2000 mg/kg, neither DTSE nor DTPE caused any discernible harm in mice. They could inhibit the growth of *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Based upon the experimental results, the wild stems and protocorms were found to be alternatives suitable for creating pharmacologically bioactive substances.

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1. Introduction

Medicinal plants have long been recognized for their remarkable ability to produce a diverse array of biologically active compounds that exhibit a wide range of therapeutic effects (El Jabboury et al., 2023; Kota et al., 2023). These natural products, often the result of millions of years of evolutionary adaptation, have the potential to treat various ailments, from inflammation and pain to cancer and infectious diseases (Hapuarachchi et al., 2022). By harnessing the power of medicinal plants, researchers and healthcare professionals can develop novel drugs and treatments that are both effective and safe, offering hope to those suffering from debilitating conditions (Mohammadhosseini et al., 2019; Mohammadhosseini et al., 2021). As we continue to explore the vast and largely untapped potential of these botanical wonders, we move closer to unlocking the secrets of nature's pharmacy and improving the quality of life for people around the world.

Dendrobium transparens (common name: Translucent Dendrobium) is a medium to large-sized rare orchid growing on trees, with slender, basally swollen, erect to pendulous stems. Translucent Dendrobium is found in the Himalayas, from Kumaon, Nepal to Bhutan, NE India, Laos, and Myanmar at altitudes of 800-2000 m (eFloras, 2020). It is a horticulturally important orchid. Besides horticulture, it has been widely important in traditional medicine to treat fractured, dislocated bones and geriatric diseases (Gutierrez, 2010). The wild resource of this orchid is depleting day by day due to habitat loss, and collection from hobbyists to large-scale illegal trade. The rate of vegetative multiplication of this orchid is extremely slow producing 2-4 shoots per year, however, its seed germination process is very complex as it needs a specific mycorrhiza due to the lack of

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endosperm, and about 2.0-5.0% are germinated (Otero et al., 2002; Pant et. al., 2017). Plant tissue culture has overstated the endeavours in plant conservation and would be the most appropriate substitute to reduce the burden on wild orchids (Pfab and Scholes, 2004; Pant, 2013, 2014), thus assisting in their sustainable utilization. Furthermore, it is useful for delivering plant-derived bioactive components (Bourgaud et al., 2001; Vanisree et al., 2004; Hussain et al., 2012; Ochoa-Villarreal et al., 2016; Espinosa-Leal et al., 2018). Orchid protocorms are unique, highly organized tissues that emerge from *in vitro* seed cultures and undergo differentiation into mature plants. These tissues are characterized by the accumulation of a high concentration of secondary metabolites (Paudel et al., 2020; Pant et al., 2021). The major purpose of orchid protocorm development is to form plantlets and the isolation of bioactive secondary metabolites (Yeung, 2017).

This research was assumed on Translucent Dendrobium to (i) Perceive the effect of different phytohormones on seed germination, protocorms formation, shoot and root initiation, (ii) Determine the phenolic and flavonoid contents of wild plants and tissue culture-raised protocorms, and (iii) Evaluate the antioxidant, antibacterial and cytotoxicity of wild plant and *in vitro* developed protocorms.

2. Experimental

2.1. Plant materials

The mature capsule and stems of Translucent Dendrobium were collected from the Bhaktapur district of central Nepal (27.6451° N, 85.4427° E) in June and July 2018. The voucher specimen was deposited at the Tribhuvan University Central Herbarium (TUCH) (voucher number P08), Kathmandu, Nepal. The seeds were used as explants in the production of protocorms and plantlets.

2.2. Sterilization of capsule

The capsule was sterilized with 1-2 drops of Tween-20 and rinsed for at least half an hour under running tap water. The capsule was submerged in a sodium hypochlorite solution (1.0%) for 15 minutes and in ethanol (70%) for 10 minutes. Then, it was washed with sterile water.

2.3. Culture medium and culture conditions

Full and half strengths of Murashige and Skoog (MS) basal medium were used for protocorm development from seeds. BAP (6-benzylaminopurine) and NAA (1-naphthaleneacetic acid) were used as organic additives. The pH of the medium was adjusted to 5.8 before being sterilized in an autoclave at 121 °C and 1 kPa for 20 minutes. The cultures were kept in a room at 25 ± 2 °C under a 16/8-hour (light/dark) photoperiod of 30 µmol/m^2/s intensity.

2.4. Induction of protocorms and plant regeneration

For protocorm formation, mature seeds were placed on a hormone-free MS solid medium and supplemented with BAP (0.5-2.0 mg/L) and NAA (0.5-1.0 mg/L). Protocorms were cultivated on hormone-free MS medium as control and on MS medium comprising NAA, BAP or kinetin with concentrations ranging from 0.5 to 2.0 mg/L for shoot initiation. For subsequent growth, healthy shoots were sub-cultured on the basal MS medium. MS medium with NAA, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) at 1-1.5 mg/L as well as coconut water (100 mL/L) were used for root initiation.

2.5. Biomass determination and extraction

In-vitro growth protocorms were removed by mesh filtering and the biomass was quantified after surface drying. The biomass of protocorms was dried up at room temperature. The *in vitro* developed protocorms and wild plant stems were soaked in methanol for 48 hours and centrifuged at 4000x for 15 minutes. The supernatant was collected and dried in a rotary evaporator. Both protocorms extract (DTPE) and stem extract (DTSE) of Translucent Dendrobium were stored at 4 °C until further use.

2.6. Determination of total phenolic content (TPC)

Total phenolic content of DTPE and DTSE was determined using the Folin-Ciocalteu (FC) reagent colorimetric method. In brief, 25 µL of extract (1.0 mg/mL) was mixed with 25 µL of FC reagent (10.0%) followed by 75 µL of distilled water in a 96-well plate. A similar procedure was applied for a gallic acid standard (25-200 mg/mL). The blank was prepared by replacing the plant sample with absolute methanol. After a steady reaction, 100 µL of Na_2CO_3 (1.0 M) was added to each sample. The plate was covered and incubated in the dark for 90 minutes. The absorbance of each solution was then read at 765 nm using a microplate reader (Azure Biosystems Microplate Reader). The experiment was done in triplicate. Total phenolic content was stated in terms of milligram of gallic acid equivalent per gram of plant extract (mg GAE/g).

2.7. Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) of DTPE and DTSE was determined using the aluminium chloride ($AlCl_3$) colorimetric method. In summary, 25 µL of extract (1 mg/mL) was mixed with 75 µL of $AlCl_3$ (10.0%) and then 50 µL of potassium acetate (1.0 M) in a 96-well plate. A similar procedure was applied for the quercetin as the TFC standard (25-200 mg/mL). The blank was prepared by replacing the plant sample with absolute methanol. The plate was then covered and incubated at room temperature for 30 minutes. Finally, the absorbance was recorded at 415 nm using a microplate reader (Azure Biosystems Microplate Reader). The experiment was done in triplicate and the TFC was stated in terms of a milligram of quercetin equivalent per gram of plant extract (mg QE/g).

2.8. Determination of antioxidant activity

The antioxidant activity of DTPE and DTSE was determined by the DPPH free radicals scavenging method (Babili et al., 2022). Briefly, 50 µL of extract at a concentration ranging from 25 to 200 µg/mL was mixed up with 150 µL of DPPH (0.2 mM) in a 96-well plate. The blank was prepared by replacing the same volume of plant sample with absolute methanol. After 30 minutes of incubation in the dark at room temperature, the absorbance of the plate was read at 517 nm using a microplate reader (Azure Biosystems Microplate reader). The antioxidant capacity of the extract (IC_{50}) was calculated using a polynomial regression equation derived from the percentage of DPPH scavenging activity at various concentrations of the extract.

2.9. Determination of cytotoxic activity

The cytotoxic activity of DTPE and DTSE was determined by employing the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay in HeLa, U251 and 3T3 cell lines. Briefly, 1 x10^4 cells were transferred to a 96-well plate with 100 µL of DMEM (Dulbecco's Modified Eagle Medium) including 10.0% fetal bovine serum (FBS), penicillin/streptomycin (1.0%) and L-glutamine (1.0%). The plate was incubated in a 5.0% CO_2 incubator at 37 °C to reach a confluence of 80-90%. The extract at 50, 100, 200, and 400 µg/mL concentrations was put into the well and incubated for 48 hours. After incubation, the supernatant was substituted with 150 µL of medium and 50 µL of MTT. Purple formazan crystals appeared after 4 hours of incubation and were dissolved by DMSO (0.1%). The absorbance of the plate was read at 595 nm using a microplate reader (Azure Biosystems Microplate Reader) with a commercially available cisplatin serving as a positive control. The cytotoxic capacity of extract (IC_{50}) was calculated using a polynomial regression equation obtained from percentage cell growth inhibition at different concentrations of extract.

2.10. Acute toxicity assay

Twenty albino mice, approximately 8-10 weeks old, were selected, with 10 males and 10 females, weighing 28 ± 4 g. The mice were kept under control conditions for five days according to OECD (2008) test guidelines. Before dosing, the mice were fasted overnight for approximately 16 hours. They were then orally administered a dose of 20 mL/kg of DTPE and DTSE extracts at a concentration of 2000 mg/kg body weight. The control group consisted of five mice that were fed an equivalent volume of sterile water. After dosing, the mice were fed their regular diet. Furthermore, both groups were closely monitored for any signs of abnormality for 14 days.

2.11. Determination of antibacterial activity

The pure culture of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii* in nutrient broth (HiMedia, India) was incubated in a shaker at 37 °C and 120 rpm overnight. Each bacterial strain was inoculated on the Mueller-Hinton agar plate. The antibacterial activity of the extracts was determined by using the well diffusion method. Five wells of 7 mm diameter were made on a nutrient agar plate. Three wells were filled with 100 µL of each DTSE and DTPE (2.5, 5, 10 and 15 mg/mL), one with DMSO (negative control) and one with antibiotics (positive control). After incubation overnight at 37 °C, the zone of inhibition was measured in millimetres (mm).

2.12. Statistical analysis

The data were presented as mean ± SD of three replicates. One-way analysis of variance was applied to compare the values using SPSS version 20.

3. Results and Discussion

3.1. Results

The seeds of Translucent Dendrobium (Fig. 1) were cultured on full-strength MS medium and full-strength MS medium supplemented with BAP (0.5 to 2.0 mg/L) and NAA (1 mg/L). The seeds were germinated and developed into protocorms on full-strength MS medium as early as 4 weeks of culture (Table 1). The full-strength MS medium supplemented with 2.0 mg/L BAP and 1 mg/L NAA also induced protocorm formation after 6 weeks of seed culture, and these protocorms developed into shoots and roots within 8 and 11 weeks of culture, respectively (Table 1, Fig. 1). The protocorms and wild stem extracts (DTPE and DTSE) were evaluated for their total phenolic and flavonoid contents and antioxidant activity. Accordingly, DTSE demonstrated comparable high total phenolic content (82.00 mg GAE/g) and total flavonoid content (61.889 mg QE/g) as that of DTPE (Table 2). In view of the obtained findings of this study, DTSE was capable of scavenging 64.91% of DPPH radical, while DTPE scavenged only 53.79-56.10% at 100-200 µg/mL concentration. However, ascorbic acid (as a positive control) scavenged 62.15-74.58% at the same concentration (Fig. 2). The DTSE performed the highest DPPH radical scavenging activity and thus showed strong antioxidant capacity by scavenging 50% DPPH radicals at 191.23 µg/mL as compared to DTPE (Table 2).

The cytotoxic activity of DTSE and DTPE was evaluated against two cancer cell lines, namely HeLa and U251 and a normal cell line, namely 3T3. DTPE exhibited a significantly high percentage of cell growth inhibition in HeLa (56.63%) and U251 (60.29%) cell lines compared to the 3T3 cell line (7.6%) at a concentration of 400 µg/mL (Fig. 3 and Fig. 4). The cytotoxic capacity (IC_{50}) of DTPE against HeLa (229.30 µg/mL) and U251 (213.90 µg/mL) cell lines was found to be significantly stronger than that against the 3T3 cell line (2108.87 µg/mL) (Table 3). Similarly, DTSE showed significantly strong cytotoxic capacity towards both cancer cell lines, whereas it was least cytotoxic towards normal cell lines (data retrieved from our previous publication, Joshi et al. 2020). However, commercially available cisplatin exhibited

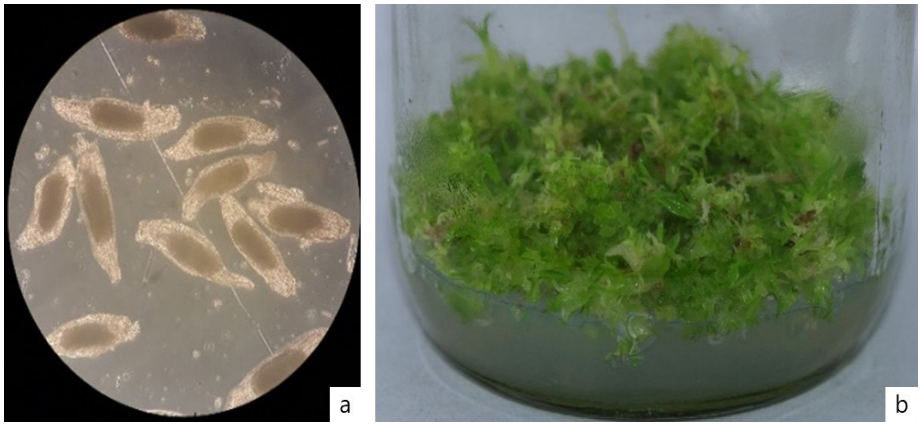

Fig. 1. Seed germination and protocorms development: Ocular view of seeds under inverted microscope at x200 magnification before culture (a), Protocorms developed on full strength MS medium with 2 mg/L BAP and 1 mg/L NAA (b).

Table 1

Effect of different strengths of MS medium with or without BAP and NAA for *in vitro* culture of seeds of Translucent Dendrobium.

S.N.	Medium	PGRs		Initiation of events observed in a week			
		BAP	NAA	Germination	Protocorms	Shoot	Root
1	HMS	0	0	5±0.3	7±0.6	10±0.5	12±0.7
2	FMS	0	0	4±0.4*	6±0.5*	9±0.7	∞±0
3	FMS	0.5	1	7±0.5	9±0.3	∞±0	∞±0
4	FMS	1	1	6±1.6	7±0.5	∞±0	∞±0
5	FMS	1.5	1	6±1.1	7±0.7	10±0.2	14±0.7
6	FMS	2	1	5±0.4	6±1.5*	8±0.3*	11±0.2*

*The values with an asterisk (*) are significantly different at $p < 0.05$.

Table 2

Total phenolic, total flavonoid, and antioxidant activity of Translucent Dendrobium extracts.

S.N.	Extract	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)	Antioxidant capacity (IC_{50}) (µg/mL)
1	Wild stem (DTSE)	82	61.89	191.23
2	Protocorms (DTPE)	72.33	56.89	295.83
3	Ascorbic acid	-	-	33.72

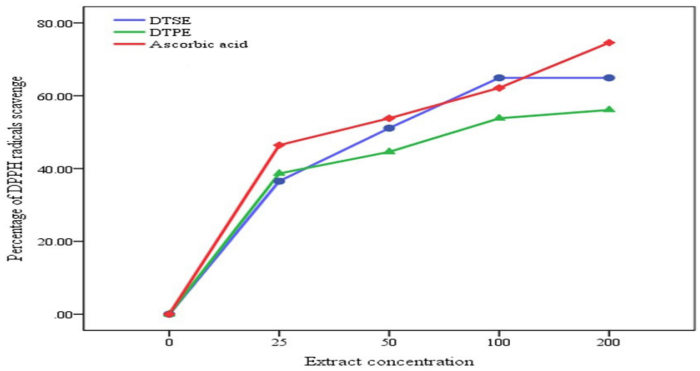

Fig. 2. Percentage of DPPH radical scavenging by Translucent Dendrobium extracts and Ascorbic acid.

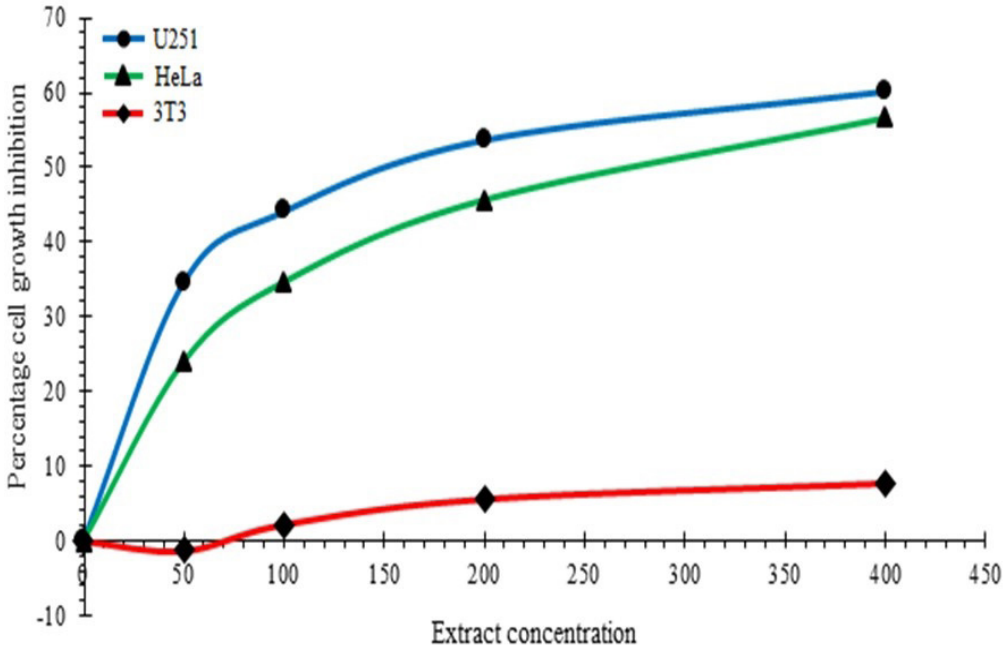

Fig. 3. Percentage of cell growth inhibition by Translucent Dendrobium protocorm extract (DTPE).

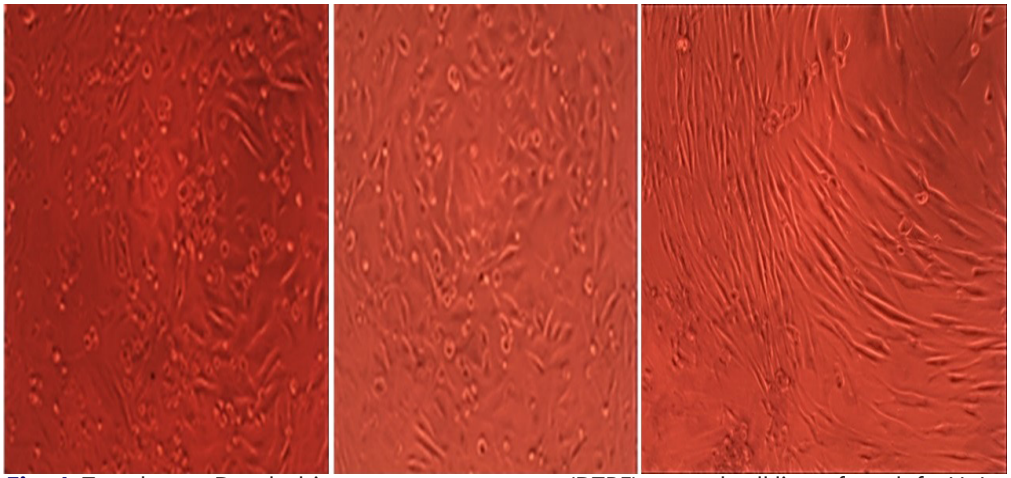

Fig. 4. Translucent Dendrobium protocorm extract (DTPE) treated cell lines; from left- HeLa, U251 and 3T3.

significantly strong anticancer activity towards both cancer cell lines compared to the extracts tested (Table 3). The acute toxicity of DTSE and DTPE was assessed in albino mice. It was observed that all tested animals survived until the 14-day observation period, and there was no significant change compared to the control group of mice. The lethal dose (LD) value ranged above 2000 mg/kg body weight for both extracts (Table 4).

Antibacterial activity of the DTSE and DTPE was evaluated against the five ATCC-type bacteria, namely *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii* (Fig. 5). Regarding the experimental findings, both of the extracts were capable of inhibiting the growth of all the bacterial strains used, but only the higher concentration of extracts inhibited the growth

Table 3
Cytotoxic capacity (IC_{50}) of Translucent Dendrobium extracts and cisplatin towards HeLa, U251 and 3T3 cell lines.

S.N.	Extract/Control	IC_{50} for HeLa cells (µg/ mL)	IC_{50} for U251 cells (µg/ mL)	IC_{50} for 3T3 cells (µg/ mL)
1	Wild stem (DTSE)	382.14*	75.84*	2329.17
2	Protocorms (DTPE)	229.30*	213.90*	2108.87
3	Cisplatin drug	25.00**	25.00**	-

Table 4
Acute toxicity test in albino mice.

S.N.	Extract	LD_{50} value	Remarks
1	Wild stem (DTSE)	>2000 mg/kg BW	Acute oral toxicity test according to OECD TG 425 Up and down procedure, 2008
2	Protocorms (DTPE)	>2000 mg/kg BW	

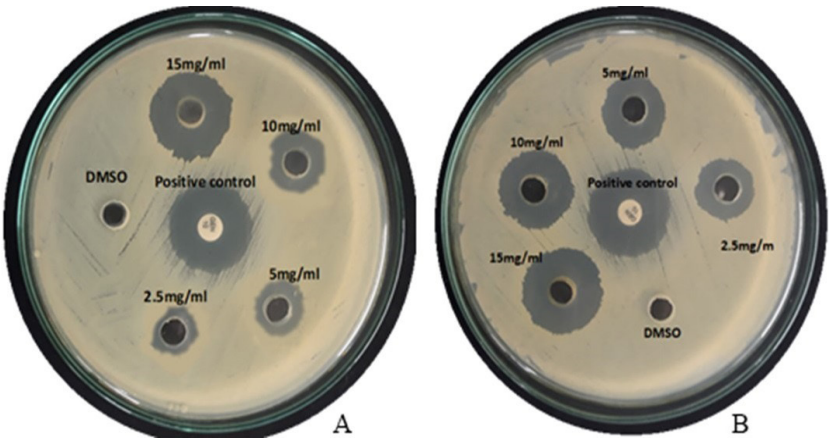

Fig. 5. Inhibition of growth of *Acinetobacter baumannii* by DTSE (A) and DTPE (B).

of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Table 5). However, commercial antibiotics (gentamycin) have significantly inhibited the growth of bacterial strains.

3.2. Discussion

Orchids are gaining popularity as a means of preventing and treating a variety of ailments. They are considered safe and free of major side effects, encouraging consumers to use natural treatments (Pariyani et al., 2015). Orchids are also equally important in the field of horticulture. However, their large-scale consumption from natural habitats has led to a decrease in their population. To address this, orchids are being conserved through plant tissue culture techniques, which can synthesize bioactive secondary metabolites in their tissues. Protocorms, highly organized tissues produced through seed culture, are particularly noteworthy as they accumulate these valuable bioactive secondary metabolites. Protocorm cultures have been accepted for the mass propagation of valuable orchid species (Park et al., 2000, 2020; Paudel et al., 2020) and the production of bioactive compounds including polysaccharides (Zha et al., 2007). In this study, a full-strength MS medium was found to produce protocorms for further biological assays. Production of the metabolite from *in vitro* cultures is dependent on the composition of media, pH, the density of inoculum used, and environmental conditions like temperature, light density, and aeration because of which conditions need to be optimized for efficient metabolite production (Pant 2014; Ochoa-Villarreal et al., 2016; Isah et al., 2018). Macronutrients and micronutrients, vitamins, sugars, amino acids

and plant hormones in a culture medium might have influenced the metabolite formation in protocorms (Cardoso et al., 2019), due to which protocorms have subsequently shown better antioxidant, antibacterial and cytotoxic activity including a significant number of polyphenols comparable to its wild counterpart.

Previous studies by Park et al. (2000), Zha et al. (2007), and Cui et al. (2015) support our claim that protocorms can be a proper source for isolating bioactive components with decent biological activity. In this study, we produced biomass from *in vitro*-developed protocorms for the synthesis of bioactive secondary metabolites and evaluated their antioxidant, antibacterial, and cytotoxic activities.

Our results show that the wild sample exhibited slightly superior antioxidant, antibacterial, and cytotoxic activity compared to the protocorm extract, which had higher flavonoid and phenolic content. Antioxidant-rich substances found in plants can scavenge free radicals and reactive oxygen species produced in the human body, potentially preventing cancer. These antioxidant-rich substances can halt the cell cycle and induce apoptosis, as reported by Pham-Huy et al. (2008) and Roleira et al. (2015). In a previous study, *in-vitro*-developed protocorms from *Dendrobium densiflorum* were also found to have significant cytotoxicity and antioxidant activity compared to their wild counterpart (Pant et al., 2021). A similar result was also reported in *Coelogyne stricta* with cytotoxic activity against HeLa cells (Thapa et al., 2020). The existence of bioactive compounds varies depending on which section of the plant is inoculated, and variations in the nutrient components of organic supplements have a significant impact on the accumulation of such compounds (Zha et al., 2007; Cui et al., 2015). Similar mechanisms of action of antioxidant-rich substances found in wild counterparts and protocorms were seen in *Dendrobium longicornu*, *D. amoenum, D. crepidatum, D. moniliforme, D. chryseum* and *D. densiflorum* on the HeLa and U251 cell lines (Sayin et al., 2014; Gali-Muhtasib et al., 2015; Paudel et al., 2017, 2018, 2019; Pant et al., 2021). Many folk medicinal plants have been reported to exhibit toxic effects (Roleira et al., 2015; Saleem et al., 2016). Initial toxicological evaluation is crucial for confirming the safety of herbal medications. However, acute oral toxicity studies are required to determine the appropriate dosage to manage the clinical signs and symptoms of the drugs (Saleem et al., 2016). Mice were used in the present study to collect data on lethal doses as they are more suitable for anticipating toxic effects (Walum et al., 1995). The acute toxicity test revealed that the extracts of Translucent Dendrobium (DTSE and DTPE) are non-toxic and reliable, with no evidence of toxicity observed up to a dose of 2000 mg/kg body weight.

4. Concluding remarks

On a full-strength MS medium containing 2.0 mg/L BAP and 1.0 mg/L NAA, *D. transparens protocorms* were effectively replicated *in vitro*. *D. transparens* wild stems extract displayed comparable high total phenolic content and total flavonoid content, therefore, scavenged at least 50% DPPH radicals at its 191.23 µg/mL concentration. However, *D. transparens* wild stems and protocorms extracts exhibited cytotoxic effects against HeLa and U251 cell lines, but not normal cell lines (3T3). These extracts showed no overt harmful effects in mice. These extracts also have antibacterial activity against certain ATCC-type bacteria. Therefore, *D. transparens* wild stems and *in-vitro*-raised protocorms may be ideal for synthesizing pharmacologically bioactive substances. Protocorms alone in pharmacology would be used sustainably to lessen the strain on this species' natural population.

Abbreviations

BAP: 6-Benzylaminopurine; **DMEM: Dulbecco's Modified Eagle Medium; DTPE:** *Dendrobium transparens* Protocorms Extract; **DTSE:** *Dendrobium transparens* Stem Extract; **FBS: Fetal Bovine Serum; FC:** Folin-Ciocalteu; IAA: Indole-3-Acetic Acid; **IBA:** Indole-3-Butyric Acid; **LD:** Lethal Dose; **MS:** Murashige and Skoog; **MTT:** (3-[4,5-Dimethylthiazole-2-Yl]-2,5-Diphenyl-Tetrazolium Bromide)**; NAA:** 1-Naphthaleneacetic Acid; **TPC:** Total Phenolic Content; **TUCH:** Tribhuvan University Central Herbarium.

Conflict of interest

The authors declare that there is no conflict of interest.

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