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Comparative analysis of nutraceutical potential phytochemicals and antioxidant activities in different parts of wild and *in vitro* regenerated plantlets of *Dendrobium heterocarpum* Wall. ex Lindl.: A medicinal orchid

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Abstract
A comparative study on certain nutraceutical compounds *viz*., total phenol, flavonoid, tannin, alkaloid and antioxidant activities in different wild plant parts and *in vitro* regenerated plants of *Dendrobium heterocarpum* revealed presence of differential quantity of nutraceutical compounds. Higher total phenolic content was in 3 years old pseudobulbs (15.98 GAE/g) followed by tissue culture plants (14.18 mg GAE/g); flavonoid content in tissue culture regenerates (38.38 mg QE/g) followed by young leaves (27.27 mg QE/g); tannin from tissue culture plants (41.48 mg TA/g) followed by 3 years old pseudobulbs and alkaloid in tissue culture plants (8.78 mg AE/g) followed by 1 year old pseudobulbs (7.57 mg AE/g). The antioxidant activities of different extracts in 3 years old pseudobulbs and tissue culture plants were comparable. Total phenol and flavonoid correlations with DPPH, FRAP, and ABTS showed the strongest correlation between TPC and ABTS (y=-0.017x+20.29, R^2=0.933); TPC and DPPH (y =-5.383x+21.97, R^2=0.928).

Keywords: antioxidant activity, *Dendrobium heterocarpum*, medicinal orchids, phytochemical content, secondary metabolites, tissue culture regenerates

1. Introduction
Plant bases medicines and nutraceuticals are being used throughout the world including India. In most cases these plants are being extracted from the wild as there are few species cultivated for commercial purposes by the concerned commercial organizations/companies. The sources of these nutraceutical and medicinally important compounds and or bio-molecules from different parts of the plant body and the quality and quantity vary with different season, age and also growth phase. But the ruthless commercial exploitation of these plants from the wild includes uprooting the whole plants of all ages, resulting in restriction in multiplication of the species, thus pushing the species in threatened categories. Nagaland is a hilly Indian state where ethnic tribes rely on plant-based remedies and knowledge of plant medicinal properties has been passed down through generations. Ethno medicinal plants used by different indigenous tribes of Nagaland have been documented by different workers [1-3]. For sustainable uses of these important bioresources and to ensure conservation of these species one viable alternative is to produce these plants *in vitro* as the *in vitro* raised regenerate promises production of these biomolecules/secondary metabolites in significant quantities and in many cases higher than the plant organs harvested from the wild [4-5]. Production of *in vitro* clonal planting materials not only ensure large scale production of plants, conservation of source plants, the harvesting calendar of target organ for extraction of these nutraceutical compounds/ secondary metabolites can also be prepared for timely extraction for commercial purpose.
Early documentations from all around the world show orchids being utilized for restorative and therapeutic purposes. Orchids are prized for their horticultural and medicinal virtues, as well as their ecological significance [6]. Nagaland is a small state of north-eastern region of India and home for large number of wild orchid species due to its suitable agro-climatic conditions [7, 1]. Deb and Imchen [7] reported 396 orchid species under 92 genera, including numerous therapeutic orchids. Excessive harvest, habitat damage, and climate change are common threats for exploitation of orchids. The decline of orchid populations in the wild necessitates conservation and effective mass multiplication strategies, such as *in vitro* culture

and reintroduction into their native habitat, in order to avoid the species' vulnerable status. In today's world, where extinction, morphological, and genetic changes are all too frequently, taxonomic documenting of plants and saving the genetic information of orchids have become a requirement.

Dendrobium ranks as the third largest genera with 900 species many of which are used as stomachics, tonics, aphrodisiacs, anti-pyretic, diaphoretics, and anti-inflammatory drugs [8]. The Chinese Pharmacopoeia records about 40 species of *Dendrobium* species, which are use in Chinese traditional medicine [9]. Treatment for many diseases like problems related to skin, digestion, tumours pain, reproductive organs and fever using orchids have been reported around the world through ethno-pharmacological studies [1, 10]. Many *Dendrobium* species have been reported to be smuggled out of Nagaland to surrounding countries with no records or understanding of their usage.

Antioxidants found in many medicinal plants, primarily polyphenols and flavonoids, have been linked to the prevention of degenerative diseases such as cardiovascular and neurological ailments, oxidative stress malfunctions, and cancer. In the last decade, researchers have been looking for effective antioxidants to reduce oxidative stress in cells and treat a variety of human diseases. Several compounds extracted from the *Dendrobium* orchid have been shown to have strong and moderate antioxidative characteristics [11]. *In vitro* propagation technology has advanced significantly, and it is now widely used for rapid and mass production. Modern orchid cultivation techniques have been established by the creation of Knudson B medium [12] and success in asymbiotic germination methods of orchids without fungal association. Plants regenerated through *in vitro* techniques have been shown to increase the production of a variety of chemical compounds with pharmacological importance, such as alkaloids, saponins, carotenoids, anthocyanins, and polyphenols [4-5]. Secondary metabolite production in *in vitro* produced plantlets of *Coelogyne ovalis* Lindl. [5], *Dendrobium nobile* Lindl. [4]. *Dendrobium heterocarpum* Wall. ex Lindl. like many *Dendrobium* species is known to produce pharmaceutically important secondary metabolites. However, the species is endangered due to overexploitation by traditional medicinal healers and smugglers for worldwide illegal trade.

Recognizing the species' value, the current study aimed to *in vitro* propagation and comparative quantification of certain nutraceutical potential secondary metabolites and antioxidant activity of different parts of *Dendrobium heterocarpum* from wild and *in vitro* regenerated plantlets to identify the right stage for harvesting plant parts for isolation of different secondary metabolites and also alternative of wild plant source.

2. Materials and methods

2.1 Sample collection

Dendrobium heterocarpum was collected from Sumi Settsu village, Zunheboto district, Nagaland, India. Plant identification was done using morphological traits, followed by molecular identification for confirmation. Plant materials were maintained in the Orchidarium, Department of Botany, Nagaland University, India (Figure 1 A).

2.2 DNA extraction and amplification

Genomic DNA was isolated using CTAB protocol [13] with slight modifications. For molecular characterization of the species plant *matk*-1-F and *matk* 1-R, rbcl-M-3-F and *rbcL*-M-13-R, and ITS 1 and ITS 2 primers were used. The PCR amplicons were confirmed and sequenced from 1st Base, Singapore. Sequences result were searched for homologous sequences using BLAST algorithm and submitted at NCBI for ITS and NCBI Bankit for *mat* K and *rbcL* (Table 1).

2.3 *In vitro* plant regeneration

About 8 months old immature green pods of *Dendrobium heterocarpum* was collected from the Orchidarium and sterilized appropriately and immature embryos from these pods was scooped out and cultured on Murashige and Skoog (MS) medium [14] fortified with sucrose (3%, w/v) and indole-3-acetic acid (IAA, 6 µmol/L) at 25±2ºC temperature under fluorescent light (40 µmol $m^{-2}s^{-1}$ illumination) at 12/12 hr photo period. Four to six week old resultant protocorm-like bodies (PLBs) from *D. heterocarpum* germinated embryos (Figure 1 B) were cultured on MS medium containing sucrose (3%) and α-naphthaleneacetic acid (NAA, 3 µmol/L) and Kinetin (KN, µmol/L) in combination, and PLBs differentiated to young plantlets within 4-5 weeks of culture (Figure 1 C). On subsequent subculture as many as 9 shoots developed with fully open leaves. About 2.5 cm long regenerated plantlets with ~1.1 cm pseudobulb and ~1.3 cm long leaves (Figure 1 D) was resulted. Young plantlets with three to four leaves were used in the phytochemical assessment.

2.4 Plant material and preparation of extract

Young leaves from the naturally grown donor plant, pseudobulbs of different ages (1, 2 and 3 years old) (Figure 1 E) and young *in vitro* regenerated plantlets of about 10-12 week old (Figure 1 D) were collected and oven dried at 60 °C till the consistent weight of the samples are ensured. About 200 mg of each dry material was crushed and dissolved in 2, 3 and 5 ml of 80% (v/v) methanol followed by incubation in a water bath (60-70 °C) for 30 min. The supernatant was filtered using Whatman's filter paper No. 1 and the final volume was made to 10ml with 80% methanol after centrifugation at 10,000 rpm for 10 min. This methanol extract was used for analysis. The Thermo Scientific UV-Visible Evolution 201 and Thermo Scientific Multiskan Spectrophotometer were used to measure phytochemicals and antioxidant activity respectively.

Fig 1: A. *D. heterocarpum* plant with flowers;
B. Asymbiotic immature embryo germination and production of PLBs;
C. Shoot formation from differentiated PLBs;
D. Regenerated plantlets;
E. Different parts of naturally grown donor plant used for photochemical analysis

2.4.1 Biochemical analysis

2.4.1.1 Quantification of total phenol content

Modified Folin-Ciocalteu method [15] was used to estimate total phenolic content. 100 µL of methanol extract was added to 2.9 ml of deionised water, 2 ml of 7% (w/v) sodium carbonate and 0.5 ml of 10% (v/v) Folin-Ciocalteu reagent were added in a test tube. The mixture solutions were incubated in dark room temperature for 90 min. The absorbance of the reactions was measured at 765 nm. Total phenolic content was quantified as mg of gallic acid equivalent (GAE) per gram sample (dry weight basis, D/W).

2.4.1.2 Quantification of total flavonoid content

Total flavonoid was quantified following the method of Tan [16]. For quantification, 50 µL of methanol extract was mixed with 0.15 ml of 0.5M sodium nitrate, 0.15 ml 0.3M aluminium chloride and volume was adjusted to 4.0 ml with 30% methanol). After a gap of 5 min 1 ml of 1M sodium hydroxide was added and absorbance measured at 510 nm. The resultant absorbance were used to determine the concentration of flavonoid against standard quercetin, expressed as mg of quercetin equivalent (QE) per gram of sample (D/W).

2.4.1.3 Quantification of total tannin content

Folin-Dennis method [4] was used to estimate total tannin content. Reaction mixture was prepared by mixing 50 µL of methanol extract, 3.95 ml of deionised water, 0.5 ml each of Folin-Dennis reagent, 20% sodium carbonate and absorbance was measured at 775 nm. The total tannin content was obtained from calibration curve of tannic acid and expressed as mg of tannic acid equivalent (TAE) per gram sample (D/W).

2.4.1.4 Quantification of total alkaloid content

The total alkaloids were determined following protocol described by Patel *et al*. [17] with minor modifications. In a soxhlet apparatus, raw sample was filtered in 80% methanol and the extract was condensed and then evaporated to dryness. Residue obtained was dissolved adding 2N hydrochloric acid (HCl). In a separating funnel 1 ml of test solution was transferred, to which 5 ml phosphate buffer of *p*H 4.7 and 10^{-4} M bromocresol green was added. Chloroform (1, 2, 3 and 4 ml) were added to the mixture and shaken and complex formed were collected in 10 ml volumetric flask, final volume was made 10 ml with chloroform. The absorbance of complex was measured at 470 nm. Total alkaloids content was estimated as mg of atropine equivalents (AE) per gram of sample (D/W).

2.4.1.5 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging assay

Radical scavenging activity of the sample extracts was determined by DPPH assay according to Brand-William *et al*. [18]. Sample extract at different concentrations were added to 3 ml of DPPH reagent (0.1mM in 80% methanol). Final volume was made to 4ml with 80% methanol and incubated for 30 min in dark and the absorbance of the mixture was measured at 517nm. The percentage inhibition of DPPH free radicals was calculated using the following equation:

$$\text{Free radical inhibition \%} = \frac{\text{Control Abs} - \text{Sample Abs}}{\text{Control Abs}} \text{ x } 100$$

2.4.1.6 Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity estimation through FRAP assay was determined base on ability of antioxidants to reduce ferric (Fe^{3+}) ion to ferrous (Fe^{2+}) ion in FRAP reagent with 2, 4, 6-tri-(2-pyridyl)-5-triazine (TPTZ) [19]. Freshly prepared 10 mM TPTZ solution, 20 mM ferric chloride and 300 mM sodium acetate buffer (*p*H 3.6) were added in the ratio of 10:1:1 at 37 °C water bath to prepare FRAP reagent. Sample extract and 3 ml of FRAP reagent was added to test tube and final volume made to 4 ml by double distilled water. After incubation of 30 minutes in dark at 37ºC, absorbance was measured at 593 nm. Standard curve was made with ferrous sulphate and sample absorbance was calculated to determine FRAP unit in mmol Fe^{2+} per gram of dry sample.

2.4.1.7 2, 2-Azino-(3-ethyl) benzothiazoline)-6-sulfonic acid diammonium salt (ABTS) radical cation scavenging assay

The antioxidant capacity of the sample extract was conducted by ABTS assay using modified Babbar *et al*. [20] method. An ABTS stable stock solution incubated in dark room temperature for 16 h was made with 7 nmol/L aqueous ABTS solution and 2.45 mmol/L potassium persulfate. Absorbance of the solution was adjusted with ethanol to 0.70± 0.02 AU at 734 nm before use. Appropriate volume sample extract was added to 3 ml of ABTS working solution with control as methanol. After incubation in dark for 30 min, absorbance was taken at 734 nm.

3. Results and Discussion

3.1 Species identity conformation

As morphological features of orchids are known to be altered by environmental conditions, molecular markers are regarded reliable tool to authenticate orchid identification [21]. In the present study, to enable the correct identification and provide a genetic marker for *D. heterocarpum* found in Nagaland, partial plastid and nuclear genes from *D. heterocarpum* were amplified and sequenced. Before sending the template for sequencing, the PCR amplicon results of the template with *mat K*, *rbcL*, and ITS primers were confirmed for quality. Using the blast algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequence was examined for similar strains at NCBI GenBank. Species identification was validated by comparing query coverage percentages, determining GC content, and assigning unique accession numbers (Table 1).

Table 1: *D. heterocarpum* sequence details and genBank accession number

Primer	Query coverage (%)	GC contain (%)	Voucher I.D.	GenBank Accession Nos.
matK-1-F & R	100	32.05	NU-BOT-TBL-CRDEB-DH1F	ME000349
rbcl-M-13-F & R	100	43.35	NU-BOT-TBL-CRDEB-RM13R	MW036488
ITS 1 & 2	94-97	52.84	NU-BOT-TBL-CRDEB-IT1	MW032194

3.2 Photochemical screening

Dendrobium species are highly valued in traditional medicines and its medicinal significances are attributed to the secondary metabolites [9]. As a result, identifying the appropriate developmental stage and part(s) of the plant in relation to the photochemical content can aid in exploiting its nutraceutical and medicinal aspect. The present study evaluated certain secondary metabolites *viz.*, total phenol, flavonoid, tannin, and alkaloid content from different parts of the plant (Figure 1 E) and *in vitro* generated plantlets (Figure 1 D). Results revealed that the secondary metabolites content varied significantly in different parts of the mother plant and *in vitro* plantlets (Table 2). The total phenolic content in 3 years old pseudobulbs was found to be highest (15.98 mg GAE/g), followed by young *in vitro* raised plants (14.18 mg GAE/g), and the least in 1 year old pseudo bulb (9.71 mg GAE/g). The total flavonoid of the extracts observed the highest concentration in *in vitro* grown plants (38.38 mg QE/g) and comparatively 3 years old pseudobulbs (27.27±0.5 mg GAE/g) showed more TFC than other parts of the mother plant. Differences in hormone levels, specific changes in metabolic and various endogenous physiological activities in plant systems may be the cause of phenolic and flavonoid concentration variation in various parts of the plant [23].

Table 2: Total phenolic, flavonoid, tannin and alkaloid contents quantified from different parts of donor plant and *in vitro* plantlets of *D. heterocarpum*

Sample	TPC (mg GAE/g)	TFC (mg QE/g)	Total Tannin (mg TA/g)	Total Alkaloids (mg AE/g)
Young leaves	13.93±0.3	25.2±1.4	12.64±0.6	5.32±0.05
1 year old PBL	9.71±0.3	11.6±0.6	12.03±0.3	7.57±0.12
2 years old PBL	12.51±0.2	18.6±1.2	9.47±0.2	5.51±0.06
3 years old PBL	15.98±0.5	27.27±0.5	17.13±0.7	6.83±0.06
In vitro raised regenerates	14.18±0.9	38.38±1.9	41.48±0.6	8.78±0.18

* PBL-Pseudobulb, ±-standard error; All data are presented in dry weight basis

Very high total tannin content was found in *in vitro* regenerated plantlets (41.48 mg TA/g) against significantly lower concentration in mother plant extract (highest in 3 years old psuedobulbs, 17.13 mg TA/g). Total alkaloid content was also highest in *in vitro* plantlets (8.78 mg AE/g) followed by 1 year old pseudobulb (7.57 mg AE/g) and least in young leaves (5.32 mg AE/g). The use of cytokinins during propagation has been shown to significantly increase the levels of secondary metabolites in the *in vitro* cultured plantlets [22-23]. Therefore, this effect might have attributed to the higher deposition of tannin and alkaloids in the *in vitro* propagated plantlets as compared to the various parts of the mother plant in the current study. Thus, efficient multiplication of orchids by *in vitro* culture can be a viable alternative to large-scale production of medicinally important orchids, decreasing the exploitation of orchids in their natural habitat.

3.3 Antioxidant capacities

Antioxidants are important in protecting the human body against damage by reactive oxygen species molecule, as it prevents the oxidation of other molecules such as free radicals or reactive oxygen species. Antioxidants protect the human body against the damaging effects of reactive oxygen species molecules by preventing the oxidation of other molecules such as free radicals and reactive oxygen species [24]. Disorders like ageing, degenerative diseases and cancer related to oxidative stress treatment have been reported to be effective with plant base natural antioxidants. Due to the complex reactive nature of phytochemicals, determining antioxidant activity of extracts necessitates the use of multiple antioxidant techniques, as findings vary between tests [25]. As a result, scavenging activity was assessed in this work utilising the DPPH, FRAP, and ABTS techniques (Table 2). The percentage inhibition of concentrations was used to calculate the radical scavenging activity of *D. heterocarpum* extracts using the DPPH method, as shown in figure 2, table 3. The antioxidant activity of the extracts was evaluated using inhibition percentages, with 3 years old PBL having the greatest (IC50 1.18 mg/ml) and 1 year old PBLs having the lowest (IC50 2.18 mg/ml). Similarly, using the ABTS method, 3 years old PBL had higher antioxidant activity (IC50 0.240 mg/ml) and least in 1 year old PBL (IC50 0.610 mg/ml). The ferric reducing assay revealed that 1 year old psuedobulbs (94.99 mM Fe^{2+}/g) and 2 years old psuedobulbs (107.76 mM Fe^{2+}/g) have higher reducing power.

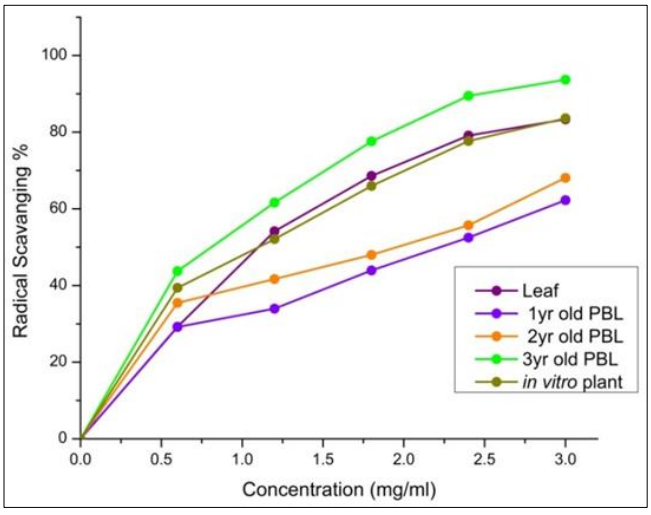

Fig 2: Scavenging activities of different parts of *D. heterocarpum* and *in vitro* plantlets by DPPH assay

Table 3: Antioxidant activities of *D. heterocarpum* extract in DPPH, ABTS and FRAP methods

Sample	DPPH IC_{50} value (mg/ml)	ABTS IC_{50} (mg/ml)	FRAP (mM Fe^{2+}/g)
Leaf	1.41±0.01	0.37±0.03	164.65±2.9
1 yr old PBL	2.18±0.02	0.61±0.08	94.99±1.6
2 yrs old PBL	1.93±0.05	0.43±0.06	107.76±1.1
3 yrs old PBL	1.18±0.02	0.24±0.02	290.15±1.7
In vitro raised regenerates	1.39±0.01	0.42±0.03	165.09±1.4
Trolox	0.01±0.04	0.008±0.0001	7.32±0.06

*****PBL-Pseudobulb, ±-standard error; All data are presented in dry weight basis

3.4 Correlation study of antioxidant activity with Total phenolic and flavoniod content

Many medicinal plants are known for its natural antioxidants, which have been linked to the content of total phenol and flavonoid [26]. Association between higher antioxidant activities to their total phenolic compounds have been reported in many medicinal plants [19]. Analysis of correlation established between reducing capacity to that of total phenol and flavonoid are shown in table 4. The total phenolic content was shown to have a significant linear relationship with the DPPH and ABTS scavenging assays. The strongest correlation was found between TPC and ABTS (y=-0.017x+20.29, R^2=0.933); TPC and DPPH (y =-5.383x+21.97, R^2=0.928) (Table 4). Total flavonoids in relation to scavenging activity as measured by DPPH, ABTS, and FRAP, on the other hand, revealed that flavonoid concentration had no influence on antioxidant activity. The findings confirm the existence of bioactive chemicals in *D. heterocarpum* and a link between increased phenol content and antioxidant activity of plant extracts.

Table 4: Linear correlation of antioxidant activity with total phenol and flavonoid content of *D. heterocarpum*

	DPPH	ABTS	FRAP
TPC	y = -5.383x+21.97, (R^2=0.928)	y =-0.017x+20.29 (R^2=0.933)	y =0.026x+8.902 (R^2=0.765)
TFC	y =-19.44x+55.66 (R^2=0.659)	y =-38.95x+40.34 (R^2=0.323)	y =0.055x+15.04 (R^2=0.263)

*TPC: Total phenolic content, TFC: Total flavonoid content

4. Conclusion

Morphological identification *D. heterocarpum* identification was supported by the molecular markers and unique accession numbers for the species found in Nagaland were obtained. Quantitative phytochemical estimation from the different extracts revealed the presence of different biological important secondary metabolites in significant concentrations. In this study, the potential plant parts and growth age at which the plant organ can be taken for separation of these metabolites instead of the entire plant was determined. Of the different plant parts of different age studies, 3 years of old pseudobulb was the ideal sources for all the nutraceutical potential compounds studies along with highest antioxidant activity. Interestingly it was found that the *in vitro* grown young plants were also potent source for the studies compounds and in some cases higher quantities was found in *in vitro* regenerated plants. Prolonging the growth period of *in vitro* plantlets might be a viable solution to increase the production for commercial exploitation. This will, in one hand relieve the pressure on the natural population and will aid in species conservation, on the other hand will resolve difficulties metabolites contamination by different microbes when isolated from the naturally grown plants.

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5.1 Conflict of Interest: Authors declare that there is no conflict of interest.

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