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Phytochemical screening, estimation of total phenols, total flavonoids and determination of antioxidant activity in the methanol extract of *Dendrobium denudans* D. Don stems

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Abstract

The aim of the present study was to investigate the phyto-constituents present within the methanol extract of *Dendrobium denudans* D. Don stem and to estimate total phenols, total flavonoids and antioxidant activity. Phytochemical studies confirmed the presence of carbohydrates, phenols and flavonoids. The amount of total phenols and flavonoids were analyzed using a spectrophotometric technique, based on Folin-Ciocalteu reagents and aluminium chloride colorimetric assay, respectively. Gallic acid was used as standard compound for total phenols and catechin for total flavonoids. Furthermore, the antioxidant activity was determined by using two *in-vitro* models namely, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power methods. In both the methods, ascorbic acid was used as reference compound. The presence of antioxidant compound, rutin in the methanol extract was determined by High Performance Liquid Chromatography (HPLC) analysis.

The total phenols and flavonoids were found to be 129.02 ± 0.865 mgGAE/g and 108.333 ± 1.155 mg CAT/g respectively. IC_{50} of methanol extract was found to be 153.509 ± 3.476 μ g/mL and 10.052 ± 2.365 μ g/mL for DPPH and reducing power assays respectively.

Keywords: *Dendrobium denudans*, total phenols, total flavonoids, Folin-Ciocalteu, aluminium chloride, antioxidant, reducing power, rutin.

1. Introduction

Dendrobium species (orchidaceae), locally known as “Shihu” or “Huangcao” in China are widely distributed throughout Asia, Europe and Australia by more than 1100 species. There are 74 species and 2 variations of *Dendrobium* plants found in China and about 30 species of them are used in traditional or folk medicine for anti-pyretic, eyes-benefiting, immunomodulatory purposes, etc [1].

The traditional Chinese crude drug ‘Shihu’, derived from the dried or fresh stems of several *Dendrobium* species (Orchidaceae), is widely used as both traditional Chinese and folk medicines for the treatment of various diseases, such as chronic atrophic gastritis, diabetes, skin aging and cardiovascular diseases, which are believed to be closely associated with the metabolic disorders of reactive oxygen species (ROS) and the promotion of body fluid in the human body [2]. Free radicals of different forms are constantly generated for specific metabolic requirement and quenched by an efficient antioxidant network in the body. When the generation of these species exceeds the levels of antioxidant mechanism, it leads to oxidative damage of tissues and biomolecules, eventually leading to disease conditions, especially degenerative diseases [3]. Previously, EtOAc-soluble fraction of a 60% EtOH extract of the stems of *D. nobile* was found to exhibit antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay which led to the isolation of bibenzyl derivatives, with significant antioxidant activity higher than or equivalent to vitamin C [4]. In another research, the methanol extracts of *D. tosaense* Makino and *D. moniliforme* SW showed antioxidant activity. Alkyl ferulates and quercetin were isolated from *D. moniliforme* and *D. tosaense* respectively [5].

However, there is no report so far on the phytochemicals, total phenols, total flavonoids and antioxidant activity of *Dendrobium denudans*. Hence, the aim of the present study was to determine the phytochemical constituents, total phenols, total flavonoids and antioxidant activity of *Dendrobium denudans* D. Don.

2. Materials and Methods**2.1 Chemicals and reagents**

Catechin, quercetin and gallic acid were obtained from Sigma-Aldrich. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), sodium hydroxide, sodium nitrite, disodium hydrogen phosphate,

sodium dihydrogen phosphate, aluminium chloride, potassium ferricyanide, trichloroacetic acid, ferric chloride and rutin were purchased from Himedia. Ascorbic acid was obtained from SLR, Folin-Ciocalteu phenols reagent were purchased from Merck. Methanol, acetonitrile and acetic acid for HPLC were obtained from Rankem. All other chemicals and solvents were of analytical grade.

2.2 Plant materials

The stem of *Dendrobium denudans* were collected from Willong Khunou region of Senapati district, Manipur in the month of February and maintained in the green house. It was identified by Y. Nanda Devi of Centre of Orchid Gene Conservation of Eastern Himalayan Region (COGCEHR), Manipur. A voucher specimen with accession No. 00065 is preserved in this institute.

2.3 Preparation of plant extract

Stems of *Dendrobium denudans* were washed and shade dried, then coarsely grounded. The shade-dried stems of *D. denudans* (214g) were subjected to cold extraction with analytical grade methanol in a round bottom flask. The solvent was evaporated under reduced pressure using a rotary evaporator. This process yields 10g of dried methanol extract which is stored at $\pm 20^{\circ}\text{C}$ until use.

2.4 Phytochemical screening

Phytochemical tests were carried out to identify the phytochemical constituents in the methanol extract of *D. denudans* stems [6, 7, 8].

2.4.1 Test for alkaloids

Extract was dissolved in dilute HCl and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows:

A) Mayer's test

The filtrate was mixed with few drops of Mayer's reagent. A white or creamy precipitate indicates the test as positive.

B) Wagner's test

To the filtrate, few drops of Wagner's reagent were added. A reddish-brown precipitate confirms the test as positive.

C) Hager's test

The filtrate was mixed with few drops of Hager's reagent. A prominent yellow precipitate indicates the test as positive.

2.4.2 Test for carbohydrates

Extract was dissolved in few mL of water and filtered. The filtrate was subjected to the following tests:

A) Barfoed's test

Barfoed's reagent (1mL) was added to the filtrate (1mL) and heated on a boiling water bath for 2 mins. Red precipitate indicates presence of sugar.

B) Fehling's test

The filtrate (1mL) was boiled on a water bath with 1mL each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.

2.4.3 Glycosides

A few mg of extract was hydrolysed with concentrated HCl on a water bath for 2 hours and filtrated. The filtrate was subjected to following tests:

A) Borntrager's test

To 2 mL of filtrate, 3 mL of chloroform were added and shaken. Chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicates the presence of glycosides.

B) Legal's test

50 mg of the extract was dissolved in pyridine, sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. Presence of glycoside was indicated by pink colour.

2.4.4 Saponins

20 mg of the extract was diluted with distilled water and made-up to 10 mL. The suspension was shaken in a graduated cylinder for 15 mins. A prominent layers of foams indicates the presence of saponins.

2.4.5 Proteins and amino acids

The extract was dissolved in distilled water and filtered. The filtrate was subjected to test for proteins and amino acids.

A) Millon's test

The filtrate was mixed with few drops of Millon's reagent. A white precipitate indicates the presence of proteins.

B) Ninhydrin test

The filtrate was mixed with few drops of Ninhydrin solution. A characteristic purple colour indicates the presence of amino acids.

2.4.6 Phenols compounds

Extract was dissolved in distilled water and filtered. The filtrate was subjected to following test.

A) Ferric chloride test

Few drops of neutral 5% ferric chloride solution were added to the filtrate. A dark green colour indicates the presence of phenols compounds.

B) Few drops of 10% lead acetate solution were added to the filtrate. A bulky white precipitate indicates the presence of phenols compounds.

2.4.7 Flavonoids

Extract was dissolved in distilled water and filtered. The filtrate was subjected to following test.

A) Alkaline reagent test

The filtrate was treated with 10% ammonium hydroxide solution. A yellow fluorescence indicates the presence of flavonoids.

2.4.8 Gums and Mucilages

A) Alcohol 95% test

B) Extract was dissolved in distilled water and filtered. The filtrate was treated with few mL of absolute alcohol with constant stirring. A characteristic white or cloudy precipitate indicates the presence of gums and mucilages.

2.5 Total phenols

The total phenols in methanol extract of *D. denudans* stems were estimated by Folin-Ciocalteu reagent [9]. Different concentration of gallic acid 10, 20, 40, 50, 60, 80, 100 µg/mL were prepared from the standard stock solution of gallic acid (1mg/mL) in several test tubes and make up the volume to 1mL with distilled water. To the standard solution or extract (1mL), 5mL of Folin-Ciocalteu reagent (1:10 with distilled water) were added and incubated for 5 mins. After 5mins, 4mL of 7.5% sodium carbonate solution were added to the test tubes sequentially. A control was prepared using distilled water. Absorbance was measured after 2hrs of incubation at 760nm. The average absorbance values obtained at different concentration of gallic acid were used to plot the calibration curve. The total phenols of the extract were expressed as milligram gallic acid equivalents (GAE) per gram dry weight of extract.

The total phenols was calculated by using the formula;

$TPC = C \times V/m$; Where, TPC = Total phenols content in mg/g;
C = concentration of gallic acid established from the calibration curve in mg/mL;

V = volume of extract in mL; m = weight of plant extract in g;
GAE = gallic acid.

2.6 Total flavonoids

The total flavonoids were measured by the method of the aluminium chloride colorimetric method [10]. An aliquot of 1mL of methanol extract or standard solution of catechin (20, 40, 60, 80 and 100 µg/mL) was added to 10 mL volumetric flask containing 4mL of distilled water. To the flask was added 0.3mL of 5% sodium nitrite. After 5 mins, 0.3mL of 10% aluminium chloride was added. After another 6 mins, 2mL of 1M sodium hydroxide was added and total volume made up to 10mL with distilled water followed by incubation for 15 mins at room temperature. Absorbance was measured at 510nm with a UV-Visible spectrophotometer (Multiskan spectrum, Thermo Scientific). Total flavonoids were expressed as milligram catechin equivalents (CAT) per gram dry weight of extract through the calibration curve with catechin.

The total flavonoids was calculated by using the formula;

$TFC = C \times V/m$;

Where, TFC = Total flavonoids content in mg/g;

C = concentration of catechin established from the calibration curve in mg/mL;

V = volume of extract in mL; m = weight of plant extract in g;
CAT = catechin.

2.7 Antioxidant activity

The DPPH free radical scavenging was assessed according to the method [11] with slight modification. 0.1mM DPPH radical solution in methanol was prepared and 1mL of this solution was added to 1 mL of different concentration of methanol extract (50, 100, 150, 175 and 200 µg/mL) or standard solution of ascorbic acid (1, 2, 5, 8 and 9 µg/mL). A control was prepared with methanol. After 30 mins, the absorbance was measured at 517nm on UV-Visible spectrophotometer (Multiskan spectrum, Thermo Scientific). Decrease in absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. The radical scavenging

activity, expressed as percentage of inhibition was calculated and this activity was expressed as an inhibition concentration 50 (IC50). The percentage of inhibition was calculated by using the equation:

$$\% \text{ Inhibition} = [(A-B)/A] \times 100$$

Where A is the absorbance of control (DPPH solution without the sample), B is the absorbance of DPPH solution in the presence of the sample.

2.8 Reducing power

The reducing power was determined according to the method [12]. Different concentration of methanol extract (5, 10, 20, 30, and 40 µg/mL) and standard solution of ascorbic acid (1, 2, 4, 5, 6, 8 and 10 µg/mL) are prepared in different test tubes and made up to 1mL by adding distilled water. A control was prepared with distilled water. To this test tubes, 2.5mL of phosphate buffer (0.2M, pH 6.6) and 2.5mL potassium ferricyanide (1%) were added and incubated at 50 °C for 20min. After incubation, 2.5mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10min. The upper layer of the solutions (2.5mL) was mixed with 2.5mL distilled water and 0.5mL ferric chloride (0.1%). Absorbance was measured at 700nm on UV-Visible spectrophotometer (Multiskan spectrum, Thermo Scientific). Increased in absorbance of the reaction mixture indicates increase in reducing power. Percentage of reducing power inhibition was calculated and this activity was expressed as an inhibition concentration 50 (IC50). The percentage reducing power was calculated by using the formula.

$$\% \text{ Reducing power} = [(B-A)/A] \times 100$$

Where B is the absorbance of sample and A is the absorbance of control.

2.9 High performance liquid chromatography (HPLC) analysis

HPLC method has been used for determination of flavonoid compound in the methanol extract according to the method [13]. The methanol extract of *D. denudans* and different concentration of rutin 10, 50, 100, 200 and 500 (µg/mL) prepared from the stock solution of rutin (1mg/mL) were subjected to reverse Phase HPLC equipped with a photo-diode array detector (RP-HPLC-PDA). It was performed on a Waters HPLC system equipped with reversed phase X-bridge™ C18 (4.6 mm × 250 mm i.d. 5 µm) column (Waters). The mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid in isocratic mode. The flow rate and injection volume were 1mL/min and 20µl respectively. A UV-detector (Photo-Diode Array, Waters Model No. 2996) detects the compound, rutin at 254nm wavelength. The chromatographic peak of rutin in the methanol extract was confirmed by comparing the retention time with the reference standard. Calibration curve of rutin was constructed by plotting peak area under curve against different concentration of rutin. The concentration of rutin in the extract was calculated from the corresponding calibration plot [14].

3. Results and Discussion

3.1 Phytochemical Screening

Our observations revealed that methanol extracts of *Dendrobium denudans* stem contain carbohydrates, phenols and flavonoids qualitatively, whereas alkaloids, glycosides, saponins, proteins, amino acids, gum and mucilages are absent in the methanol extract. The preliminary phytochemical screening results are shown in Table 1.

Table 1: Preliminary phytochemical screening of methanol extract of *Dendrobium denudans* stems.

Sl.No.	Test	Test applied/Reagent used	Inference
1.	Alkaloids	a) Mayer's reagent b) Wagner's reagent c) Hager's reagent	- - -
2.	Carbohydrates	a) Barfoed's test b) Felhing's test	+ +
3.	Glycosides	a) Borntrager's test b) Legal's test	- -
4.	Saponins	Foam test	-
5.	Proteins and Amino acids	a) Millon's test b) Ninhydrin reagent	- -
6.	Phenols	a) Ferric chloride test b) Lead acetate test	+ +
7.	Flavonoids	Alkaline reagent test	+
8.	Gums and Mucilages	Alcohol 95% test	-

(+) = present, (-) = absent

3.2 Estimation of total Phenols and flavonoids content

From the standard curve equation of gallic acid; $y = 0.0081x + 0.0098$, $R^2 = 0.9997$ (Fig. 1) and catechin; $y = 0.0004x - 0.0058$, $R^2 = 0.9977$ (Fig. 2).

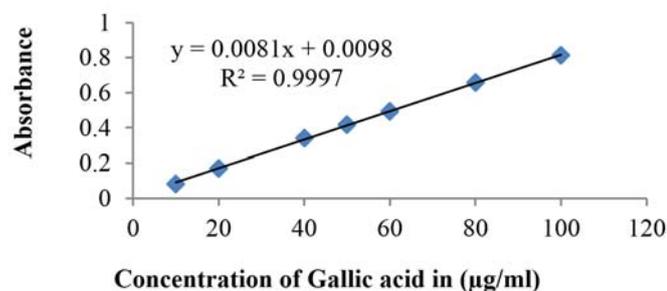


Fig 1: Calibration curve of gallic acid

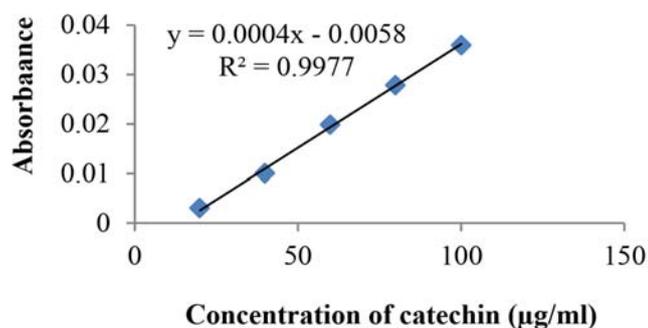


Fig 2: Calibration curve of catechin

The total phenols and total flavonoids content of methanol extract of *Dendrobium denudans* are found to be 129.02 ± 0.865 mgGAE/g and 108.333 ± 1.155 mgCAT/g respectively (Table 2).

Table 2: Total phenols and flavonoids content of methanol extract of *D. denudans* stems.

Extract	Total phenols content (mg of GAE/g of extract)	Total flavonoids content (mg of CAT/g of extract)
Methanol	129.02 ± 0.865	108.333 ± 1.155

Plant flavonoids and phenols are highly effective free radical scavengers and antioxidants. They are used for the prevention and cure of various diseases which are mainly associated with free radicals [15]. It has been reported that the antioxidant activity of flavonoids is affected by the location of hydroxyl group in the B-ring of the molecule. Hydroxylation at the para position alone in the B-ring shows strong antioxidative action [16]. The mechanisms of action are through scavenging or chelating process [17, 18].

3.3 Antioxidant activity

3.3.1 DPPH free radical scavenging assay

The study revealed that methanol extract of *Dendrobium denudans* shows a decrease in absorbance of DPPH solution. The decrease in absorbance of DPPH solution is because of reaction between antioxidant molecules present in methanol extract with DPPH, by hydrogen donation [19].

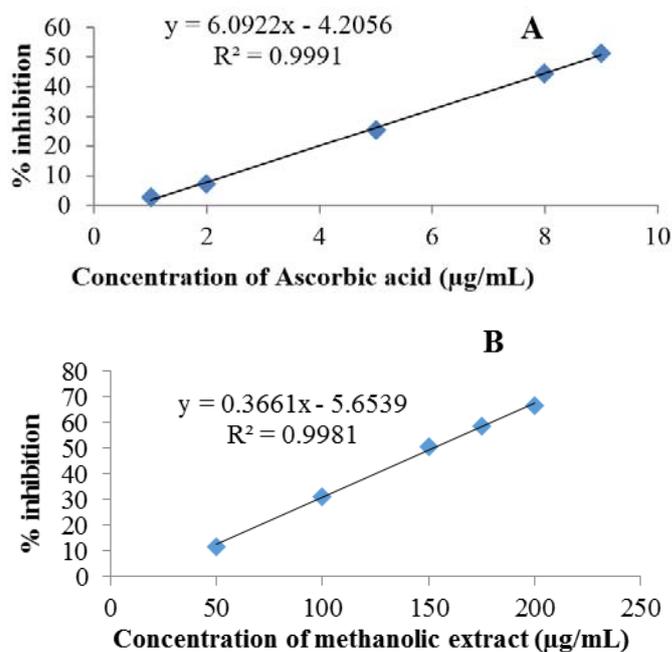


Fig 3: Percentage inhibition at different concentrations in DPPH assay (A) Ascorbic acid, (B) methanol extract of *Dendrobium denudans* stem.

The scavenging activity was determined as percent inhibition of DPPH radical and expressed as IC_{50} value of the extract [20]. The IC_{50} value for methanol extract was found to be 153.509 ± 3.476 $\mu\text{g/mL}$ (Fig. 3B and Table 2). Ascorbic acid was taken as reference which shows IC_{50} value of 8.459 ± 0.393 $\mu\text{g/mL}$ (Fig. 3A and Table 2)

3.3.2 Reducing power assay

Similarly, the methanol extract shows an increased in absorbance with increasing concentration of the extract indicating reductive ability of methanol extract of *Dendrobium*

denudans. The IC₅₀ value of methanol extract was found to be 10.052±2.365 µg/mL (Fig. 4B and Table 2) which is comparatively close to the IC₅₀ values of standard ascorbic acid 3.880±0.366 µg/mL (Fig. 4A and Table 2).

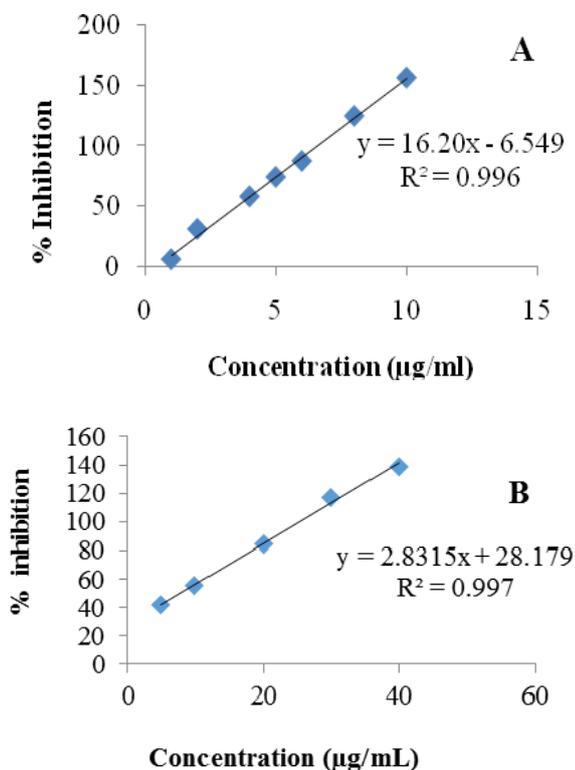


Fig 4: Percentage inhibition at different concentrations in reducing power assay (A) Ascorbic acid, (B) methanol extract of *Dendrobium denudans* stem.

Table 2: IC₅₀ value of *in-vitro* antioxidant activity of methanol extract of *D. denudans* stem.

SL. No.	Sample	IC ₅₀ value (µg/mL) of <i>in-vitro</i> antioxidant Activities	
		Reducing power	DPPH
1.	Ascorbic acid	3.880±0.366	8.459±0.393
2.	Methanol extract	10.052±2.365	153.509±3.476

3.4 HPLC Analysis

HPLC analysis confirmed the presence of rutin in the methanol extract of *D. denudans*. Rutin has been known to possess antioxidant activity that includes an affinity to scavenge free radicals [21].

From the peak area of rutin (Fig 5), the concentration of rutin in the methanol extract of *D.denudans* was calculated and found to be 52.904±3.217µg/mL.

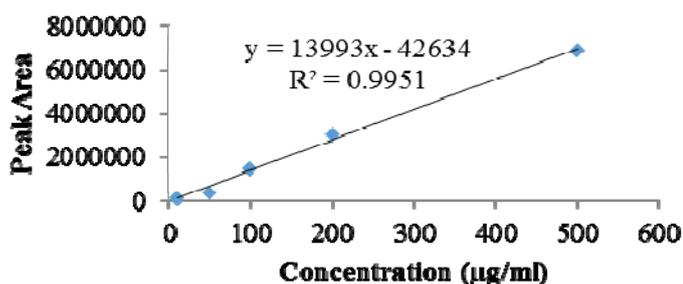


Fig 5: Peak area of rutin at different concentrations.

4. Statistical Analysis

All the experiments were carried out in triplicate and the results are expressed as mean ± standard deviation.

5. Conclusion

Our observations revealed that methanol extract of *Dendrobium denudans* contains carbohydrates, phenols and flavonoids qualitatively, whereas alkaloids, glycosides, saponins, proteins, amino acids, gum and mucilages are absent. The extract also shows strong antioxidant activity which may be due to presence of high phenols and flavonoids content. The findings of the present study suggest that *Dendrobium denudans* could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the oxidative damage of tissues and biomolecules, eventually leading to disease conditions, like degenerative diseases.

Flavonoids are the major bioactive compounds useful against free radical derived oxidative stress and presence of rutin in *D. denudans* confirmed its antioxidant potential. A detailed study on the role of different phytoconstituents which influences the antioxidant activities are further required to be investigated.

6. Acknowledgements

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