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Research

Antioxidant activity and total phenolic and flavonoid contents of *Dendrobium amoenum* Wall. ex Lindl.

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Abstract

Dendrobium amoenum has biologically active phytoconstituents valued for traditional Chinese or folk medicine as tonic. Total phenolic and flavonoid contents of its stem extract was estimated by using Folin-Ciocalteu's reagent and Aluminium chloride methods respectively. Antioxidant activity was determined by DPPH free radical scavenging method. Total phenolic content found in acetone (134.34 µg GAE/mg extract) and chloroform (101.55 µg GAE/mg extract) extract was significantly higher than other solvent extracts. Similarly, these two extracts had significantly high flavonoid content (acetone: 115.73 µg QE/mg extract, and chloroform: 84.16 µg QE/mg extract). Presence of high phenolic and flavonoid contents in these extracts showed the highest antioxidant activity. Highest antioxidant activity of these extracts was determined by their lowest IC_{50} value (acetone: 53.19 µg/ml and chloroform: 36.48 µg/ml). Significant negative relationship was found between phenolic content and IC_{50} (R^2 = 0.209, $p < 0.01$) and flavonoid content and IC_{50} (R^2 = 0.389, $p < 0.01$), which indicates high antioxidant activity due to high phenolic and flavonoid contents. This result revealed that *D. amoenum* act as an antioxidant agent due to its free radical scavenging activity which plays a crucial role in the development of new chemotherapeutic agents.

Key-words: *Dendrobium amoenum*, DPPH, extract, flavonoid, IC_{50}, phenolic

Introduction

Dendrobium is the second largest genus in the family Orchidaceae comprising 1100 species in the world (Cameron *et al*. 1999; Dressler 1983) and 30 species in Nepal, most of them are categorized as endangered and listed in Appendix II of the CITES (Rokaya *et al*. 2013; Rajbhandari 2014). They are highly collected species among the orchids for traditional medicines and horticultural uses. Their stems are highly valued in traditional Chinese medicines to reduce fever, produce bodily fluid and nourish stomach (Ng *et al*. 2012; Xu *et al*. 2013). *Dendrobium amoenum* Wall. ex Lindl. is one of the most popular epiphytic orchids in South and Southeast Asia, and valued for its attractive flowers and medicinal uses. Dried stems of this species are used as tonic (Pant and Raskoti 2013). *Dendrobium amoenum* has been reported to have many important compounds, such as bibenzyl derivatives, phenanthrenes and sesquiterpenoids (Majumder *et al*. 1999; Majumder and Bandyopadhyay 2010). However, little information exists on the biological activity of such naturally occurring compounds. The bibenzyl derivative (isoamoenylin), extracted from *D. amoenum*, has shown moderate antioxidant activity (Venkateswarlu *et al*. 2002). Besides

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that of isoamoenylin, antioxidant activities of different solvent extracts of this species are still not known.

Generally, phenolic and flavonoid compounds present in higher plants show marked antioxidant activities by reducing the oxidative damage of free radicals and other reactive oxygen species produced during metabolic reactions of biomolecules (Katalinic *et al*. 2006). The present study aims to evaluate the natural antioxidants, especially plant phenolics and flavonoids of different solvent extracts of *D. amoenum*. This study will help to understand potentiality of *D. amoenum* for maximum utilization, because antioxidant constituents could play a crucial role in the development of new chemotherapeutic agents for the treatment of some of the important diseases suffered by humanity.

Materials and Method

PLANT MATERIALS

Healthy and mature stems of *D. amoenum* were collected from Daman, Makawanpur District, Central Nepal at 1600 m asl. Taxonomic identity of the species was confirmed at National Herbarium and Plant Laboratory, Lalitpur, Nepal. The collected plant materials were air-dried in shade at room temperature (20°C). The dried stems were grinded to fine powder and stored in tight-seal dark container until use.

CHEMICALS

Acetone, chloroform, ethanol, hexane and methanol used for extraction were purchased from Qualigens. Standards of phenolics (gallic acid) and of flavonoids (quercetin hydrate), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Himedia Lab, India. The Folin-Ciocalteu's phenol reagent and aluminium chloride ($AlCl_3$) were purchased from Qualigens. All solvents and chemicals used in this study were of analytical grade.

PREPARATION OF PLANT EXTRACTS

Plant extracts were prepared according to a standard protocol (Moin *et al*. 2012; Mukherjee *et al*. 2012; Tatiya *et al*. 2012; Sahaya *et al*. 2013) using Soxhlet extractor. Grinded stem powder (35 g) was transferred to thimble and extraction was done with different solvents of increasing polarity, from hexane, chloroform, acetone, ethanol to methanol, successively in the ratio of 1:10 (w/v). The solvent was evaporated under room temperature to obtain dry extract. The extracts were kept in sterile vials dissolved in ethanol and stored at 4°C.

ESTIMATION OF TOTAL PHENOLIC CONTENT

The concentration of phenolics in different plant extracts was determined using Folin-Ciocalteu's reagent method (Stankovic 2011). Ethanolic solution of each extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of ethanolic solution of extract, 2.5 ml 10% Folin-Ciocalteu's reagent and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 ml ethanol, 2.5 ml 10% Folin-Ciocalteu's reagent and 2.5 ml 7.5% of $NaHCO_3$. The samples were thereafter incubated in room temperature for 45 min. Absorbance was determined using spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. The content of phenolics in extracts expressed in terms of gallic acid equivalent (µg of GAE/mg of extract) was calculated using the following equation:

$$C = (c \times V)/m$$

where, C is the total content of phenolic compounds (µg/mg plant extract in GAE), c is the concentration of gallic acid established from the calibration curve (µg/ml), V is the volume of extract in ml, and m is the weight of crude plant extract in mg.

ESTIMATION OF TOTAL FLAVONOID CONTENT

The content of flavonoid in the examined plant extracts was determined using aluminium chloride method (Stankovic 2011). The sample contained 1 ml ethanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in ethanol. The samples were incubated for an hour at room temperature.

Absorbance was determined using spectrophotometer at 415 nm. The samples were prepared in triplicate for each analysis. The same procedure was repeated for the standard solution of quercetin hydrate and the calibration line was construed. The content of flavonoid in extracts expressed in terms of quercetin equivalent (µg of QE/mg of extract) was calculated using the following equation:

$$C = (c \times V)/m$$

where, C is the total content of flavonoid (µg/mg plant extract in QE), c is the concentration of quercetin established from the calibration curve (µg/ml), V is the volume of extract in ml, and m is the weight of crude plant extract in mg.

DETERMINATION OF ANTIOXIDANT ACTIVITY

The antioxidant activity of different extracts of *D. amoenum* was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method. The DPPH assay is widely used to evaluate the properties of plant extract for scavenging free radicals (Zhao *et al*. 2006). The free radical scavenging activities of five solvent extracts, *viz.* hexane, chloroform, acetone, ethanol and methanol were assayed by using DPPH. A freshly prepared DPPH solution exhibits deep purple colour, which generally fades when antioxidant molecules quench DPPH free radicals and convert it into light yellow colour forming 2,2-diphenyl-1-picrylhydrazine. An aliquot of 1.5 ml of 0.25 mM DPPH solution in ethanol and 1.5 ml of extract at various concentrations (50, 100, 200, 400 and 800 µg/ml) were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm with a Genesys UV-visible spectrophotometer. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging rate} = [1- (A_1 - A_2) / A_0] \times 100\ \%$$

where, A_0 is the absorbance of the control (DPPH, without extract), A_1 is the absorbance of the extract with DPPH, and A_2 is the absorbance without DPPH.

The antioxidant activity of each extract was expressed in IC_{50} (µg/ml). Ascorbic acid and quercetin were used as standards. IC_{50} values of each extract were calculated using non-linear regression equation, where abscissa represents the concentration of tested extracts and ordinate represents the triplicate percentage of antioxidant activity of each concentration.

STATISTICAL ANALYSIS

All the experimental measurements were carried out in triplicate and are expressed as mean of three analyses. Phenolic and flavonoid contents of each extract were compared using one-way ANOVA with Duncan multiple range test. Equation for standard gallic acid and quercetin were obtained from linear regression model and the triplicate values were analyzed by F-test at $P \leq 0.05$. Non-linear regression equation obtained from percentage antioxidant activity was used to calculate IC_{50}. IC_{50} of each extract was compared with that of ascorbic acid and quercetin standards using one-way ANOVA with Duncan multiple range test. The relationship between phenolic and flavonoid content and IC_{50} was done using linear regression. All analyses were done using statistical software SPSS version 20.

Results

EXTRACT YIELD

The highest yield of dry extract was obtained using ethanol as extraction solvent (Table 1). Increasing polarity of solvents was used in extraction process. Solvent with lowest and highest values of polarity yield less amount of extract.

Table 1. Dry extract yield from 35 g dry stem powder of *Dendrobium amoenum*.

Extraction solvent	Dry extract yield (mg)
Hexane	920
Chloroform	970
Acetone	1835
Ethanol	2568
Methanol	192

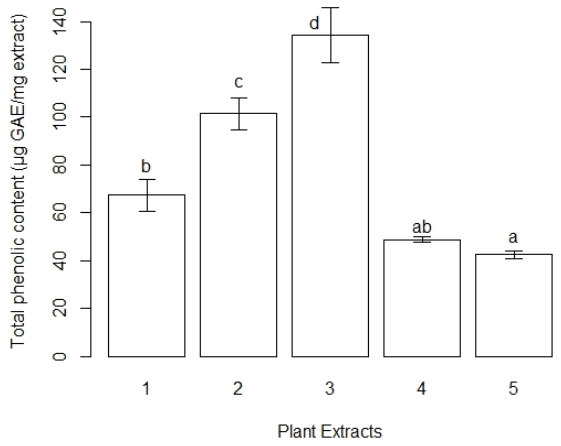

Figure 1. Total phenolic content of *D. amoenum* stem extracts (1-hexane, 2-chloroform, 3-acetone, 4-ethanol, and 5-methanol). The bar represents mean ± SE (n = 3). Values with different alphabet are significantly different ($F_{4,10}$ = 33.35, p < 0.0001).

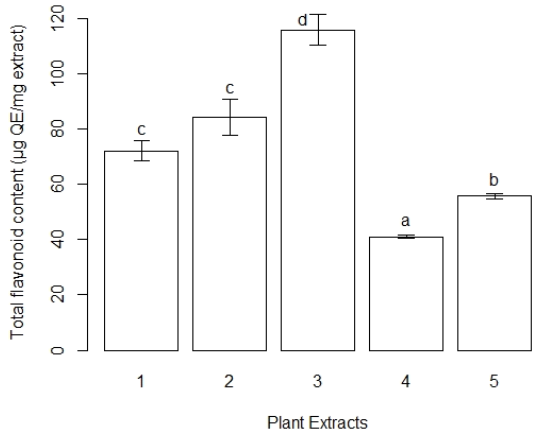

Figure 2. Total flavonoid content of *D. amoenum* stem extracts (1-hexane, 2-chloroform, 3-acetone, 4-ethanol, and 5-methanol). The bar represents mean value ± SE (n = 3). Values with different alphabet are significantly different ($F_{4,10}$ = 46.18, p < 0.0001).

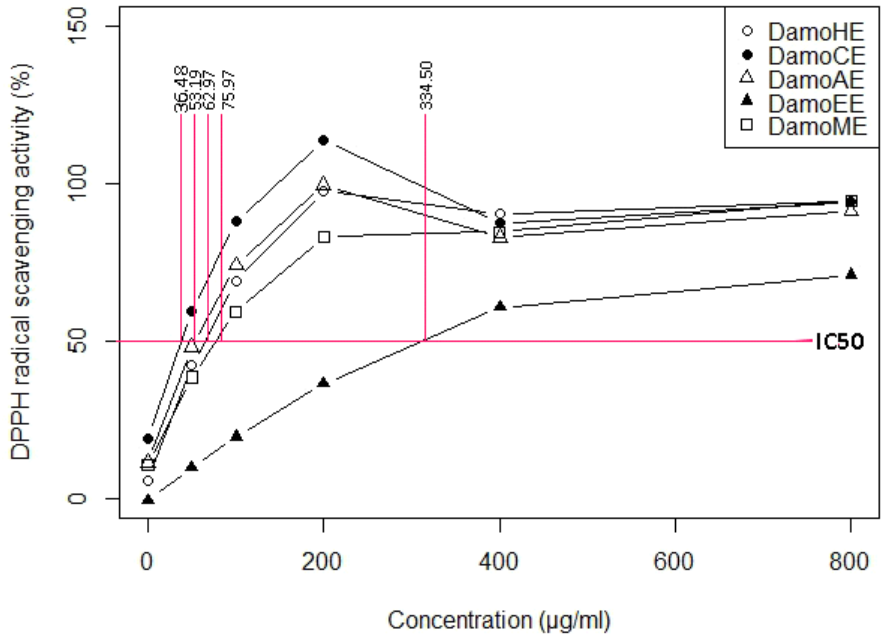

Figure 3. Mean percentage DPPH free radical scavenging activity of various concentration of different extract of *D. amoenum* stem extracts (DamoHE - *D. amoenum* hexane extract; DamoCE - *D. amoenum* chloroform extract; DamoAE - *D. amoenum* acetone extract; DamoEE - *D. amoenum* ethanol extract; DamoME - *D. amoenum* methanol extract) (n = 3).

TOTAL PHENOLIC AND FLAVONOID CONTENT

Total phenolic content was measured and expressed in terms of gallic acid equivalent. Equation of standard gallic acid (y = 0.0154x - 0.3285, R^2 = 0.989) obtained from linear regression model was statistically significant (F = 194.3, p = 0.005). The highest phenolic content was found in acetone extract (134.34 µg GAE/mg extract), followed by chloroform extract (101.55 µg GAE/mg extract) (Figure 1). Total phenolic content of acetone and chloroform extracts differed significantly with that of other extracts (p < 0.01).

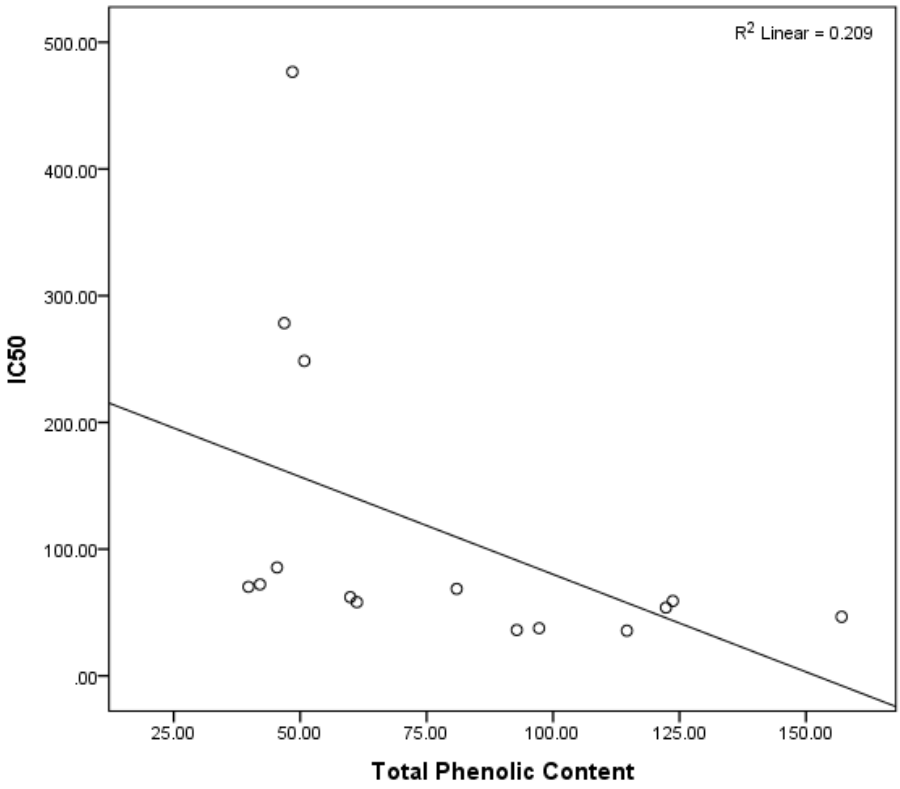

Figure 4. Relationship between phenolic content and IC_{50}.

Similarly, total flavonoid content was measured and expressed in terms of quercetin equivalent. Equation of standard quercetin obtained from linear regression model ($y = 0.0242x - 0.1845$, $R^2 = 0.976$) was also significant ($F = 82.49$, $p = 0.012$). Like phenolic content, the highest flavonoid content was also found in acetone (115.73 µg QE/mg extract), followed by chloroform (84.16 µg QE/mg extract) (Figure 2). Total flavonoid content of acetone extract was significantly higher than other extracts ($p < 0.01$).

ANTIOXIDANT ACTIVITY

As shown in Figure 3, all extracts reacted directly with and quenched DPPH radicals. Highest percentage of DPPH scavenging activities were shown by chloroform, acetone and hexane extracts and lowest IC_{50} was shown by acetone (53.19 µg/ml) and chloroform (36.48 µg/ml) extracts (Figure 3). Comparison of IC_{50} value of extracts with that of standards revealed significant difference between ethanol (334.50 µg/ml) and other solvent extracts at $p < 0.05$. Significant negative relationship was found between phenolic content and IC_{50} ($y = -1.539 x + 234.0$, $R^2 = 0.209$, $p = 0.006$; Figure 4) and flavonoid content and IC_{50} ($y = -2.867 x + 324.1$, $R^2 = 0.39$, $p = 0.001$; Figure 5).

Discussion

The phenolic and flavonoid contents in extracts depend on the polarity of solvents used. Solvents between low and high polarity quantified high amount of phenolic and flavonoid contents. Low amount of phenolic and flavonoid contents were estimated in the ethanol and methanol extracts, respectively. The result was in accordance with that of *Dendrobium speciosum* (Moretti *et al*. 2013) and *D. macraei* (Prajapati and Patel 2013). Various compounds of bibenzyl, stilbenoids and sesquiterpene derivatives of alkaloid group were isolated

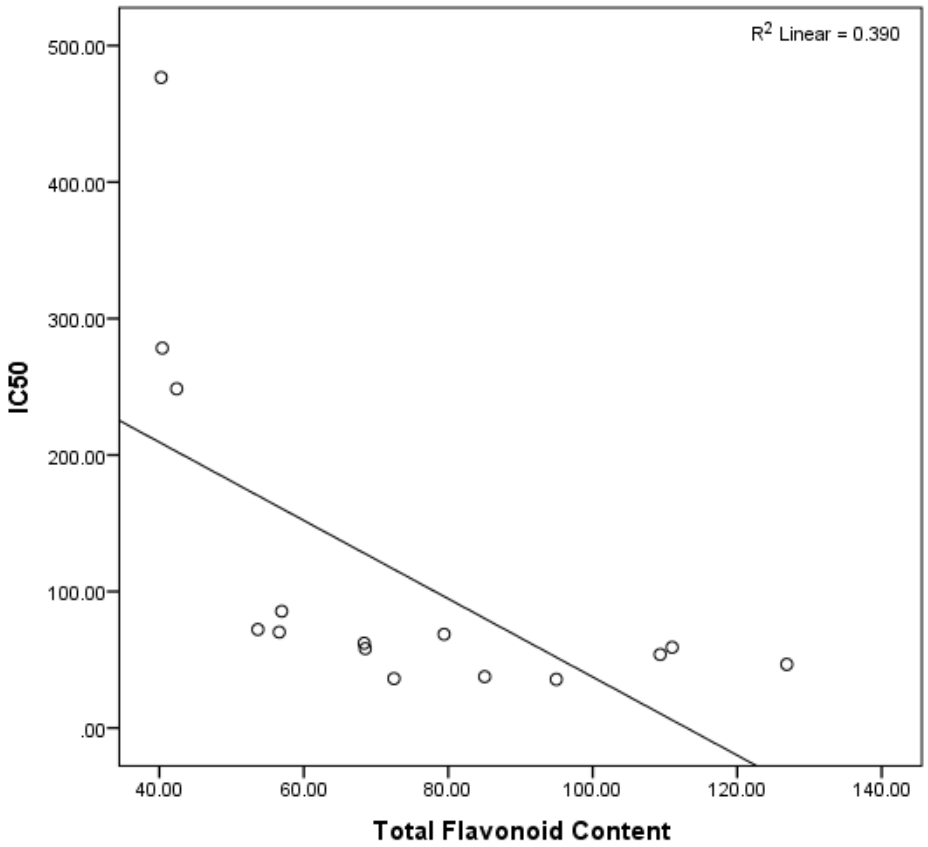

Figure 5. Relationship between flavonoid content and IC_{50}.

previously from *D. amoenum* (Majumder *et al*. 1999; Venkateswarlu *et al*. 2002; Majumder and Bandyopadhyay 2010). However, the compounds of phenolic and flavonoid groups are still unknown in this species. This study explores the amount of phenolic and flavonoid content in the stem of *D. amoenum* which help to identify more compounds from these groups.

Phenolic and flavonoid compounds are the most abundantly reported phytochemicals in plants having ability to destroy free radicals. They play an important role in antioxidant activity by giving up their hydrogen atoms from their hydroxyl groups to free radicals and form stable phenoxyl radicals. There are many reports on antioxidant activity of different species of *Dendrobium* other than *D. amoenum* (Fan *et al*. 2009; Luo and Fan 2011; Luo *et al*. 2011; Mukherjee *et al*. 2012; Moretti *et al*. 2013; Xing *et al*. 2013). Antioxidant activity of only isoamoenylin isolated from *D. amoenum* has been reported previously (Venkateswarlu *et al*. 2002). In the present study, we first time report the total phenolic and flavonoid contents in different solvent extracts of *D. amoenum* stem and determine their antioxidant activity. The acetone and chloroform extracts with high phenolic and flavonoid content shows high antioxidant activity indicating that these compounds contribute to the strong antioxidant activity of *D. amoenum*.

It is concluded that *D. amoenum* is a natural source of antioxidant substances of high importance. There is high potentiality of this species in the pharmacological uses and drug discovery.

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