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ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF *DOLICHANDRONE ATROVIRENS* USING VARIOUS *IN VITRO* ASSAY MODELS

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ABSTRACT

In the present study, the antioxidant and free radical scavenging activities of methanolic leaf and bark extracts of *Dolichandrone atrovirens* was evaluated by different *in vitro* antioxidant assay models. The total phenol and flavonoid content was also determined in the extracts. The plant extracts exhibited strong antioxidant and radical scavenging activity on ABTS radical cation, DPPH free radical, hydrogen peroxide, superoxide radical and hydroxyl radical. Both extracts showed strong activity in total reducing power assay. The antioxidant and free radical scavenging activities of the extracts were comparable to standard antioxidants used such as ascorbic acid and rutin. The extracts had good phenol and flavonoid contents. The antioxidant and radical scavenging activity of the plant extracts may be due to the rich amount of phenols and flavonoids. Therefore, the plant could be considered as a very good antioxidant source with therapeutic potential.

Key words: *Dolichandrone atrovirens*, Antioxidant, Free radical scavenging activity, Flavonoids, Total phenol content, Total flavonoid content.

INTRODUCTION

Oxidation and reduction reactions are essential to many living organisms for the production of energy to biological purposes. However, oxygen free radicals and other reactive oxygen species (ROS) which are continuously produced in vivo, result in cell death and tissue damage. These species can react with biological substrates such as DNA and proteins, leading to several diseases including cancer, diabetes, cardiovascular diseases, aging, arthritis and atherogenesis (Halliwell and Gutteridge, 2007; Willcox *et al.*, 2004). Antioxidants are vital substances which provide protection to living organisms from damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking (Ghosal *et al*., 1996; Ozsoy *et al*., 2008). Several anti-inflammatory, antinecrotic, neuroprotective, chemopreventive and hepatoprotective drugs have recently been shown to have antioxidant and radical scavenging mechanism as part of their activity (Lin and Huang, 2000; Repetto and Llesuy, 2002).

There is an increased interest in natural antioxidants present in medicinal and dietary plants, which might help to prevent oxidative damage (Silva *et al*., 2005). *Dolichandrone atrovirens* is a deciduous tree, belongs to the family Bignoniaceae., distributed throughout India. The pods of the plant are used to treat abdominal pain and leaves for tooth ache. It has long been used by tribes and native medical practitioners to treat various chronic disorders including diabetes. Literature review revealed that no phytochemical and pharmacological studies have been carried out in this plant. Based on these details, the present study is aimed to evaluate the antioxidant and free radical scavenging potential of methanolic leaf and bark extracts of

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Dolichandrone atrovirens using various *in vitro* assay models.

MATERIALS AND METHODS

Chemicals

2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Sigma Aldrich Co., St. Louis, USA. Rutin and p–nitroso dimethyl aniline (*p*–NDA) were obtained from Acros Organics, New Jersy, USA. Ascorbic acid and nitro blue tetrazolium (NBT) were obtained from S.D. Fine Chem, Ltd., Biosar, India. 2- Deoxy–D-ribose was from Hi–Media Laboratories Ltd., Mumbai. All other chemical used were of analytical grade.

Collection and Extraction

The leaves and bark of *Dolichandrone atrovirens* were collected from Chitheri hills, Salem in the month of November 2009. The plant was then authenticated by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India, Coimbatore, Tamilnadu, India, where a voucher specimen is preserved for further reference. The shade dried coarse powders of the plant material (1.5 kg) were extracted with 80 %v/v aqueous methanol by maceration at room temperature for 72 h. After completion of each extraction, the extracts were filtered, concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50 °C). The residues were then stored in vacuum dessicator.

Preliminary phytochemical screening

Prepared plant extracts of *D. atrovirens* were analyzed for the presence of various phytochemical constituents employing standard procedures. Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

Preparation of test and standard solutions

The methanolic leaf and bark extracts of *D. atrovirens* and the standard antioxidants (ascorbic acid and rutin) were dissolved in distilled dimethyl sulfoxide (DMSO separately and used for the *in vitro* antioxidant assays except the hydrogen peroxide method because it interferes with the method. For hydrogen peroxide method, the extracts and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain the lower concentrations.

Estimation of total phenol content

About 0.1 ml of each extract (10 µg/ml) was mixed with 0.5 ml of Folin-Ciocalteu reagent (diluted 1:10 ratio with distilled water) and 1.5 ml of sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml with double distilled water. The mixture was allowed to stand for 2 hrs. The absorbance was measured at 750 nm using PerkinElmer Lambda 25 UV-Visible spectrophotometer. Using the gallic acid standard curve (2 – 10 µg/ml), the total phenol content was obtained. The total phenol content was expressed as gallic acid equivalent in mg/g of the extract (Kumaran and Karunakaran, 2007).

Estimation of total flavonoid content

About 0.5 ml of each plant extract (100 µg/ml) was mixed with 1.5 ml of methanol (75% v/v), 0.1 ml of aluminium chloride (10 %w/v), 0.1 ml of potassium acetate (1 M) and 2.8 ml of double distilled water. The reaction mixture was allowed to incubate for 30 min at room temperature before the absorbance was taken at 435 nm. Water (0.1 ml) was used to substitute aluminium chloride for blank. Rutin was used as a standard for the calibration curve. The result was expressed as rutin equivalent mg/g of extract (Hsu, 2008).

In vitro antioxidant activity

The methanolic leaf and bark extracts of *D. atrovirens* were tested for its *in vitro* antioxidant activity using the standard methods. In all these methods, a particular concentration of the extract or standard solution was used which gave a final concentration of 1000-15.625 µg/ml after all the reagents were added. Absorbance was measured against a blank solution containing the extract or standards, but without the reagents. A control test was performed without the extract or standards. Percentage scavenging and IC_{50} values ± SEM were calculated.

ABTS radical scavenging activity

In a final volume of 1 ml, the reaction mixture comprised 950 µl of ABTS · + solution and 50 µl of each plant extract at various concentrations. The reaction mixture was homogenized and incubated for 20 min. absorbances of these solutions were measured spectrophotometrically at 734 nm (Re *et al*., 1999).

DPPH radical scavenging activity

The DPPH assay method depends on the reduction of purple DPPH to a yellow colored diphenyl picrylhydrazine and the remaining DPPH which showed maximum absorption at 517 nm was measured. About 2 ml of various concentrations of the plant extract or standards were added to 2 ml of DPPH solution (0.1 mM, 2 ml). After 20 min of incubation at 37 °C in the dark, the absorbance was recorded at 517 nm (Shirwaikar *et al*., 2006).

Superoxide radical scavenging activity by alkaline DMSO method

In this method, superoxide radical is generated

by the addition of sodium hydroxide to air saturated dimethyl sulfoxide. The generated superoxide remains stable in solution, which reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature and that can be measured at 560 nm. Briefly, to the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and 0.3 ml of the extract in freshly distilled DMSO at various concentrations, added 0.1 ml of NBT (1 mg/ml) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm (Elizabeth and Rao, 1990).

Hydrogen peroxide radical scavenging method

In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically at 230 nm. To 1 ml of various concentrations of extract or standard in methanol was added to 2 ml of hydrogen peroxide (20 mM) in phosphate buffer saline. After 10 min the absorbance was measured at 230 nm (Jayaprakasha *et al*., 2004).

Hydroxyl radical scavenging activity

***p*-NDA method**

Various concentration of the extract or standards in 0.5 ml of distilled DMSO were added to a solution mixture containing 0.5 ml of ferric chloride (0.1 mM), 0.5 ml of EDTA (0.1 mM), 0.5 ml of ascorbic acid (0.1 mM), 0.5 ml of hydrogen peroxide (2 mM) and 0.5 ml of *p*-NDA (0.01 mM) in phosphate buffer (pH 7.4, 20 mM) to produce a final volume of 3 ml. Absorbance was measured spectrophotometrically at 440 nm (Srinivasan *et al*., 2007).

Deoxyribose method

To the reaction mixture containing deoxyribose (0.2 ml, 3 mM), ferric chloride (0.2 ml, 0.1 mM), EDTA (0.2 ml, 0.1 mM), ascorbic acid (0.2 ml, 0.1 mM) and hydrogen peroxide (0.2 ml, 2 mM) in phosphate buffer (pH, 7.4, 20 mM), added 0.2 ml of various concentrations of extract or standard in freshly distilled DMSO to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37 °C. After incubation, ice–cold trichloro acetic acid (0.2 ml, 15 %w/v) and thiobarbituric acid (0.2 ml, 1 %w/v), in 0.25 N HCl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm (Srinivasan *et al*., 2007).

Iron reducing power assay

To 1 ml of the plant extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 min. after incubation period, 2.5 ml of 10% trichloroacetic acid was added and the reaction mixture was centrifuged at 1000 rpm for 10 min. The upper 2.5 ml layer was mixed with 2.5 ml of deionized water and 0.5 ml of ferric chloride and thoroughly mixed. The absorbance was measured spectrophotometrically at 700 nm. A higher absorbance indicates a higher reducing power (Chung *et al*., 2005).

RESULTS

Preliminary qualitative analysis

Preliminary qualitative analyses of the leaf and bark extracts of *D. atrovirens* were done for the detection of various phytochemical constituents. Both the plant extracts showed the presence of a variety of phytochemicals. The preliminary qualitative analysis report is displayed in Table 1.

Total Phenol Content and Total Flavonoid Content

The total phenol content of the plant extracts were determined using the Folin-Ciocalteu reagent, calculated from the regression equation of calibration curve (y = 0.102x-0.041, r2 = 0.990) and is expressed as gallic acid equivalents. Total phenol content of the leaf extract was found to be 71.95 ± 0.82 mg/g of extract and in bark extract it was found to be 93.51 ± 0.61 mg/g of the extract. The total flavonoid content was estimated by aluminium chloride method using rutin as standard. The amount was calculated from the regression equation of calibration curve (y = 0.010 *x* - 0.001, r2 = 0.991) and is expressed as rutin equivalents. The amount of total flavonoids was found to be 44.11 ± 3.18 mg/g and 56.16 ± 2.04 mg/g of leaf and bark extracts, respectively.

ABTS and DPPH Radical Scavenging Assay

ABTS and DPPH radical scavenging activity of leaf and bark extracts of *D. atrovirens* are shown in Table 2. The extracts showed potent radical scavenging activity in concentration dependent manner. Among the extracts, bark extracts showed good activity when compared to leaf extract. However, both extracts exhibited good radical scavenging activity against the tested models. The results obtained were comparable with the standards used and the IC_{50} values are presented in Table 2.

Superoxide and Hydrogen peroxide radical Scavenging Activity

Superoxide radical scavenging activity of leaf and bark extracts of *D. atrovirens* were assessed by alkaline DMSO method. The plant extracts moderately inhibit the superoxide radical generation. In hydrogen peroxide radical scavenging assay, the extracts were found to be equipotent with ascorbic acid but less potent when compared to rutin. The values were tabulated in Table 3.

Hydroxyl radical Scavenging Assay

Hydroxyl radical scavenging activity of leaf and bark extracts of *D. atrovirens* was measured by p-NDA method and deoxyribose method. In p-NDA method, the extracts showed potent activity when compared to standards used. In deoxyribose method, the plant extracts showed moderate activity when compared to the standard rutin. The IC_{50} values were presented in Table 4.

Iron Reducing Power Assay

Fig.1 shows the reductive ability of leaf and bark extracts of *D. atrovirens* compared to ascorbic acid and rutin. Absorbance of the extracts was increased when the concentration increased. A higher absorbance indicates a higher reducing power.

Table 1. Preliminary Phytochemical screening of leaf and bark extract of *D. atrovirens*

S. No	Phytochemical constituents	Leaf	Bark
01	Carbohydrates	+	+
02	Alkaloids	-	-
03	Glycosides	+	+
04	Proteins & Aminoacids	+	+
05	Tannins & Phenolics	+	+
06	Flavonoids	+	+
07	Terpenoids	+	+
08	Saponins	+	+
09	Gums & Mucilages	_	-
10	Fixed oils & Fats	+	-
11	Phytosterols	_	-

Note: + = Presence; - = Absence

Table 2. Effect of leaf and bark extracts of *D. atrovirens* on ABTS and DPPH Methods

Extracts/ Standards	IC_{50} (µg/ml)* by method	
	ABTS	DPPH
Leaf Extract	10.24 ± 0.17	15.1 ± 0.72
Bark Extract	7.11 ± 0.13	12.7 ± 0.81
Ascorbic Acid	12.25 ± 0.49	5.01 ± 0.05
Rutin	0.62 ± 0.04	6.15 ± 0.13

*Average of three determinations; Data are expressed as mean ± SEM

Table 3. Effect of leaf and bark extracts of *D. atrovirens* on Superoxide Radical Scavenging and Hydrogen peroxide Scavenging Methods

Extracts/ Standards	IC_{50} (µg/ml)* by method	
	Superoxide radical scavenging	H_2O_2 radical scavenging
Leaf Extract	234.76± 6.98	137.28 ± 1.36
Bark Extract	211.37± 4.78	116.12 ± 0.82
Ascorbic Acid	>1000	187.41 ± 3.92
Rutin	>1000	41.63 ± 1.20

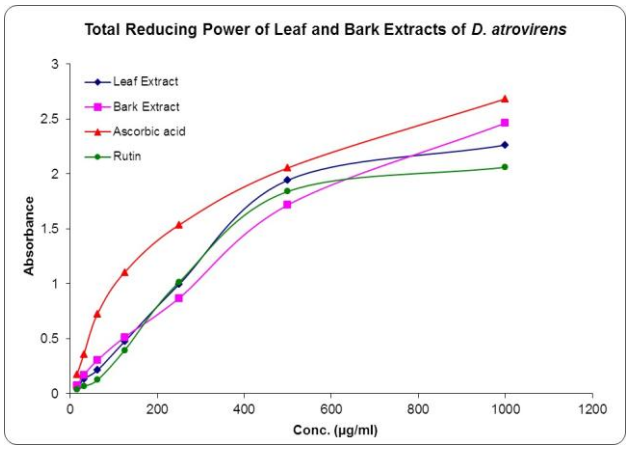
*Average of three determinations; Data are expressed as mean ± SEM

Table 4. Effect of leaf and bark extracts of *D. atrovirens* on Hydroxyl Radical Scavenging Assay

Extracts/ Standards	IC_{50} (µg/ml)* by method	
	Hydroxyl Radical Scavenging	
	p-NDA Method	Deoxyribose Method
Leaf Extract	289.3 ± 9.34	312.4 ± 9.61
Bark Extract	241.13 ±8.13	256.2 ± 6.21
Ascorbic Acid	>1000	-
Rutin	>1000	28.32 ± 1.63

*Average of three determinations; Data are expressed as mean ± SEM

Fig.1 Total Reducing Power of Leaf and Bark Extracts of *D. atrovirens*



DISCUSSION

In the last two decades there has been an explosive interest in the role of oxygen free radicals, more generally known as "reactive oxygen species" (ROS) and of "reactive nitrogen species" (RNS) in experimental and clinical medicine (Halliwell and Gutteridge, 2007). ROS/RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems (Valko *et al*., 2004). Beneficial effects of ROS involve physiological roles in cellular responses to noxia, as for example in defense against infectious agents and in the function of a number of cellular signaling systems. One further beneficial example of ROS at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids termed as oxidative stress (Poli *et al*., 2004). Oxidative stress has been implicated in the pathology of many diseases such as inflammation, cancer, diabetes, neurodegenerative disorders and aging.

Reactive oxygen species and reactive nitrogen species such as superoxide anions, hydroxyl radical and nitric oxide inactivate enzymes and damage intracellular components causing injury through covalent binding and lipid peroxidation. Antioxidants are compounds that hinder the oxidative processes and thereby delay or prevent oxidative stress (Rajkapoor *et al*., 2010).

The harmful effects of ROS are balanced by the antioxidant enzymes. Despite the presence of the cell's antioxidant defense system to counter act oxidative damage from ROS, oxidative damage accumulates during the life cycle, and radical-related damage to DNA, to proteins and to lipids has been proposed to play a key role in the development of age dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions (Halliwell and Gutteridge, 2007). The antioxidant and free radical scavenging activity of the leaf and bark extracts of *Dolichandrone atrovirens* was investigated against various *in vitro* models. Since, free radicals are of different chemical entities, it is essential to test the extracts against many free radicals to prove their antioxidant activity. Hence, a large number of *in vitro* methods were used for the screening. IC^{50} values obtained were compared with the standards used, that is, ascorbic acid and rutin.

ABTS radical scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS • + for the estimation of antioxidant activity (Nenadis te al., 2004). The extracts showed potent antioxidant activity in ABTS method which is comparable to the standard used. Here, the extracts radical scavenging activity showed a direct role of its phenolic compounds in free radical scavenging.

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants (Sancehz-Moreno, 2002). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soare *et al*., 1997). The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The experimental data of the extracts revealed that the extracts are likely to have the effects of scavenging free radicals. From the result we observe that a dose dependent relationship in the DPPH radical scavenging activity. The involvement of free radicals, especially their increased production, appears to be a feature of most of the human diseases including cardiovascular diseases and cancer (Deighton *et al*., 2000). It has been found that cysteine, glutathione, ascorbic acid, tocopherols, flavonoids, tannins and aromatic amines reduce and decolorize the DPPH by their hydrogen donating ability. Flavonoids and phenolic compounds of leaf and bark extracts of *D. atrovirens* are possibly involved in its radical scavenging activity.

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species (Halliwell and Gutteridge, 2007). The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide via dismutation reaction. Moreover, the conversion of superoxide and hydrogen peroxide into more reactive species. The extracts are found to be an efficient scavenger of superoxide radical generated in alkaline DMSO system. The result clearly indicates that the plant extracts have a noticeable effect as scavenging superoxide radical.

Hydrogen peroxide itself is not very reactive, but sometimes is toxic to cell because it may give rise to hydroxyl radical in the cells (Halliwell, 1991). Therefore, removing of hydrogen peroxide is very important for antioxidant defense in cell system. Polyphenols have also been shown to protect mammalian cells from damage induced by hydrogen peroxide, especially compounds with the orthohydroxy phenolic compounds like quercetin, gallic acid, caffeic acid and catechin (Nakayama, 1994). Therefore, the phenolic compounds of the leaf and bark extracts of *Dolichandrone atrovirens* may probably be involved in scavenging hydrogen peroxide.

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Sakanaka *et al*., 2005). In the present study, the hydroxyl radical scavenging activity of leaf and bark extracts of *D. atrovirens* was assessed by the inhibition of p-NDA bleaching method and deoxyribose degradation method. In p-NDA method, the hydroxyl radical is generated through Fenton reaction. In this reaction, iron-EDTA complex reacts with hydrogen peroxide in presence of ascorbic acid to generate hydroxyl radical which can bleach p-NDA specifically. The extracts show potent scavenging activity by inhibition of bleaching of p-NDA. In deoxyribose method, the sugar is degraded on exposure to hydroxyl radical generated Fenton reaction. The resulting complex mixture of products is heated under acid condition; malondialdehyde (MDA) is formed and detected by its ability to react with thiobarbituric acid to form a pink chromogen. In the deoxyribose method, the plant extracts shows good hydroxyl radical scavenging activity which can be comparable with the standards used. The scavenging activity may be due to the presence of various phytochemicals including polyphenols and flavonoids in the extracts.

In the measurement of the reducing ability, it has been investigated from the Fe^{3+} - Fe^{2+} transformation. Fe^{3+} reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action and can be strongly correlated with other antioxidant properties (Dorman *et al*., 2003). The reducing properties of the plant extracts are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The data obtained in the present study suggest that it is likely to contribute significantly towards the observed antioxidant effects. However, the antioxidant activity has been attributed by various mechanisms, like prevention of chain initiation, binding of transition metal ion catalysts, prevention of continued hydrogen abstraction, reductive capacity, radical scavenging activity and decomposition of peroxides. Like the antioxidant activity, the reducing power of the extracts increases with increasing concentration.

The systemic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers. Therefore, it is necessary to determine the total amount of phenols and flavonoids in the plant extracts chosen for the study. Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. The content of total phenols and flavonoids were estimated by the standards curves and expressed as gallic acid equivalents for total phenols and rutin equivalents for flavonoids. The extracts contains considerable amount of total flavonoids and phenols. Previous literatures showed that high phenol and flavonoid content increases the antioxidant activity (Holasova *et al*., 2002) and there is a linear relation between the phenol and flavonoid contents and antioxidant activity (Gheldof and Engeseth, 2002).

Phenolic compounds are commonly found in both edible and medicinal plants and they have been reported to have various biological effects including antioxidant activity. The antioxidant activities of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Osawa, 1994). The methanolic leaf and bark extracts of *Dolichandrone atrovirens* showed strong antioxidant activity in various *in vitro* systems tested. The antioxidant effect of *D. atrovirens* is may be due to the phenolic compounds present in it. To our knowledge this is the first report on the antioxidant and radical scavenging potential of *Dolichandrone atrovirens.*

CONCLUSION

The results from various free radicals scavenging systems reveal that methanolic leaf and bark extracts of *Dolichandrone atrovirens* have significant antioxidant activity. The extracts are found to have different levels of antioxidant activity in all the methods tested. IC_{50} values obtained were comparable with that of the standards used, that is, ascorbic acid and rutin. Since, free radicals are of different chemical entities, it is essential to test the extracts against many free radicals to prove their antioxidant activity. Hence, a large number of *in vitro* methods were used for the screening. However, the difference in the activity in extracts may be due to the different chemical entities of the free radicals and the diverse chemical nature of the extracts. According to this

study, a significant and linear relationship was found between the antioxidant activity and total phenol and flavonoid contents, indicating that these compounds could be major contributors to antioxidant activity. Further studies in our laboratory are in progress for the isolation and identification of phytochemical compounds and to ensure the medicinal properties of the plant *in vivo* correlate with its antioxidant activity.

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