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Antioxidant capacity of crude extract and fractions from *Woodfordia fruticosa* flower

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SUMMARY

Woodfordia fruticosa Kurz. (Lythraceae), commonly known as Dhayero, is used in the treatment of various ailments in Nepal. In this study, the antioxidant capacity of crude extract and different polarity fractions of *Woodfordia fruticosa* flowers were assayed for their scavenging abilities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide radicals, competitive β-carotene bleaching, reducing power, metal chelating ability and total phenolic content. Crude extract and polar fractions showed stronger antioxidant capacity and contained very high level of total phenolics. They exhibited strong DPPH radical scavenging, nitric oxide scavenging and reducing power, medium β-carotene bleaching and poor metal chelating capacity. Positive correlation obtained between total phenolic content and antioxidant capacity assays, indicated the contribution of phenolics towards antioxidant capacity.

Key words: *Woodfordia fruticosa*; Antioxidants; Radical scavenging; Phenolic content

INTRODUCTION

In recent years, free radicals and related species have attracted a great deal of attention as they have been implicated in aging and a number of human diseases. Free radicals and reactive oxygen species (ROS) can adversely alter lipids, proteins and DNA. Damage induced by ROS includes DNA mutation, protein oxidation and lipid peroxidation, contributing to the development of cancer, diabetes, atherosclerosis, inflammation, and premature aging (Finkel *et al.*, 2000). The oxidation of lipids in foods is responsible for the formation of off-flavours and undesirable chemical compounds which may be detrimental to health (Brand-Williams *et al.*, 1995) and the addition of antioxidants to food products has therefore become popular as a means of increasing shelf life and to reduce wastage and nutritional losses by inhibiting and delaying oxidation (Tsuda *et al.*, 1994).Recent studies on free radicals have confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers (Gerber *et al.*, 2002; Kris-Etherton *et al.*, 2002) and neurodegenerative diseases, like Parkinson's and Alzheimer's diseases (Di Matteo and Esposito, 2003).Therefore, there is growing interest in antioxidants as dietary supplements and/or food preservatives. Moreover, natural antioxidants, especially that of plant origin are becoming more popular because of the safety and toxicity problems of synthetic antioxidants.

Herbal medicine represents one of the most important fields of traditional medicine in Nepal.

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A great number of traditional medicinal plants have been used in folk medicine to treat a wide range of physical ailments such as asthma, bronchitis, rheumatism, inflammatory diseases and others. *Woodfordia fruticosa* Kurz (Lythraceae), commonly known as Dhayero, is widely distributed through out Nepal below 1500 m and is used in the treatment of various ailments. Flowers are used in haemorrhage, dysentery, menorrhagia, stomach troubles, leucorrhoea (IUCN Nepal, 2000) disorders of the mucous membrane, liver diseases and safe stimulant in pregnancy (Medicinal plants of Nepal, 1997). Leaf decoction is orally administered in malarial fever; pounded bark is used to cure wounds (Siwakoti and Siwakoti, 2000). Flowers of *Woodfordia fruticosa* are reported to have immunomodulatory activity (Kroes *et al.*, 1993). Moreover, hydrolysable tannins isolated from the plant inhibited DNA topoisomerase II (Kadota *et al.*, 1990) and shown antitumor activity (Yoshida *et al.*, 1991).

Medicinal plants with folklore reputation should be studied in order to promote their proper use and to determine their potential as sources for new drugs and food preservatives. In search for sources of novel antioxidants, medicinal plants have been extensively studied in many parts of the world. However, little is known about the antioxidant property of Nepalese plants and to our best knowledge, there has been no report on antioxidant capacity of *Woodfordia fruticosa*. Here, we reported the antioxidant capacity and phenolic content of ethanolic crude extract and different polarity fractions from *Woodfordia fruticosa* flower.

MATERIALS AND METHODS

Chemicals

Octadecyl-functionalized silica gel, butylated hydroxy anisole (BHA), β-carotene, α-tocopherol, 1,1-diphenyl-2-picrylhydrazyl (DPPH) linoleic acid, gallic acid (GA), ascorbic acid (AA), quercetin (Q), tween-40, ferrozine, potassium ferricyanide, trichloro acetic acid (TCA), ferrous chloride, sodium phosphate (monobasic and dibasic), sodium chloride, sodium carbonate, sodium nitroprusside, sulfanilamide, phosphoric acid, *N*-(1-naphthyl) ethylenediamine dihydrochloride, Folin-Ciocalteu reagent were purchased from Sigma-Aldrich Co. (St. Louis). Aluminium sheets (Silica gel 60 F_{254}) were from Merck, Germany. Ethylenediaminetetraacetic acid (EDTA) was purchased from Yakuri Pure Chemicals, Osaka, Japan, ferric chloride from Junsei Chemical Company, Japan. All other reagents were of analytical grade. Recordings were made in a UV-vis Diode Array Spectrophotometer, Hewlett Packard 8453.

Plant material

Woodfordia fruticosa flowers, in their natural habitat, were collected and identified by one of us (L.R. Bhatt) from Dhading district, Central Nepal in March 2004 and authenticated by Dr. Lokendra R. Sharma and Mr. Puran Pd. Kurmi, Department of Plant Resources, Ministry of Forest & Soil conservation. The voucher specimen was deposited in Natural Product Research Laboratory, Wonkwang University, Korea. The plant was chosen based on its reported uses in the literature and by conducting ethno botanical survey.

Preparation of extracts and fractions

The freshly picked flowers were air-dried at room temperature for two weeks, with no direct sunlight and then subjected to extraction. The air- dried and powdered flowers (30.72 g) were extracted in ethanol at room temperature for 24 h, thrice. The obtained organic solution was filtered using Whatman no 42 and evaporated, under vacuum below 40°C to dryness (yield, 28.22%) and stored at 4°C until use. During our initial screening of 18 ethanol extracts from Nepalese medicinal plants (data not shown), ethanol extract of *Woodfordia fruticosa* flower showed the strongest activity against DPPH radical and was, therefore further partitioned using reverse phase flash column chromatography. Ethanol extract of *Woodfordia fruticosa* flower (8.24 g)

was coated on C_{18} silica gel (16.48 g) and loaded onto a glass column already containing 82.4 g of C_{18} silica gel. The column was developed in a stepwise manner starting with H_2O and followed by H_2O:MeOH, MeOH, MeOH:$CHCl_3$, $CHCl_3$ and hexane Altogether, 10 fractions viz. aqueous (Fr. 1, yield, 6.27 g), aqueous methanol 9: 1 (Fr. 2, yield 0.8 g), aqueous methanol 3:1 (Fr. 3, yield, 0.09 g), aqueous methanol 1:1 (Fr. 4, yield, 0.08 g), aqueous methanol 1:3 (Fr. 5, yield, 0.06 g), aqueous methanol 1:9 (Fr. 6, yield, 0.05 g), methanol (Fr. 7, yield, 0.08 g), methanol chloroform 3:1 (Fr. 8, yield, 0.06 g), chloroform (Fr. 9, yield, 0.09 g) and hexane fraction (Fr.10, yield, 0.14 g) were obtained.

DPPH radical-scavenging capacity

The scavenging capacity of crude extract and fractions was determined, using DPPH, as a stable free radical (Yen and Chen, 1995). In brief, 500 μl of various concentrations of the samples in methanol was added to 500 μl of 0.12 mM methanol solution of DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. Radical scavenging activity was expressed as the percentage of DPPH elimination after 30 min and calculated as follow.

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

Where, A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the test compound. BHA and á-tocopherol were used as positive controls.

Antioxidant assay using a β-carotene-linoleate model system

The antioxidant activity of extract was evaluated by the β-carotene-linoleate model system (Miller, 1971). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two milliliters of this solution was added to a 100 ml round-bottom flask containing 40 mg of linoleic acid and 400 mg of tween 40 (polyoxyethylene sorbitan monopalmitate) emulsifier. After removing the chloroform under vacuum below 40°C, 100 ml of oxygenated distilled water was added to flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion was transferred into test tubes containing different concentrations of the samples (0.2 ml) and zero time absorbance was determined immediately against the blank, consisting of above emulsion without â-carotene on a spectrophotometer at 470 nm. The tubes were placed, at 50°C, in a water bath and absorbance was recorded after 2 h. BHA and α-tocopherol, were used as references. Antioxidant activity was calculated using the following equation: Antioxidant activity = [β-carotene content after two h of assay/initial β-carotene content] × 100.

Nitric oxide (NO) scavenging capacity

The scavenging effects of extracts and fractions on NO were measured (Marcocci *et al.*, 1994). In brief, 10 mM Sodium nitroprusside solution in phosphate buffered saline (PBS), pH 7.4 was prepared immediately before the experiment. Sodium nitroprusside (final concentration 5 mM) in PBS was mixed with samples, diluted in PBS and incubated at 25°C for 150 min. After incubation, samples (0.5 ml) were removed and diluted with 0.5 ml of greiss reagent (1% sulfanilamide, 2% phosphoric acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride) and the absorbance was read at 546 nm. The inhibition of NO generation was estimated by comparing the absorbance values of control with that of treatments. The same reaction mixture without the sample or standard but the equivalent amount of PBS served as the control.

Metal chelating capacity

The ferrous ions chelating capacity of crude extract and fractions were estimated (Dinis *et al.*, 1994). Briefly, different concentrations (100 - 1,000 μg/ml) of extracts in 0.4 ml methanol were added to a 50 μl solution of $FeCl_2$ (2 mM). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and total

volume was adjusted to 4 ml with methanol. Then the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula:

Metal chelating effect (%) = $[(A_0 - A_1)/A_0] \times 100$

Where, A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the sample and standards. The control contains $FeCl_2$ and ferrozine, complex formation molecules.

Reducing power determination

The reducing power of samples and standards was determined following Oyaizu (1986). Different amounts of samples (50 - 200 μg/ml) in 1 ml methanol were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 1,000 *g* for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Total phenolic content

The total phenolic content was determined following the Folin-Ciocalteu method (Slinkard and Singleton, 1977). The reaction mixture containing 20 μl of sample, 1.58 ml water and 100 μl of the Folin-Ciocalteu reagent was mixed thoroughly. After one minute, 300 μl of 20% sodium carbonate solution was added and shaken well to mix. After 2 h incubation at room temperature the absorbance was measured at 765 nm against the blank (solution contained all the reaction reagents except the sample). Gallic acid (0 - 500 mg/l) was used for calibration of a standard curve. Total phenolic content was determined as Gallic acid equivalents (GAE) and values were expressed as mg of acid/g of plant material (in GAE).

Statistical analysis

The data are results of triplicate experiments. Microsoft Excel was used to compute means, standard deviation, correlation and regression. The significance of difference was calculated by Student's *t*-test and values < 0.05 were considered to be significant.

RESULTS

DPPH radical-scavenging capacity

As illustrated in Fig. 1, the crude extract and different polarity fractions (except hexane fraction) of *W. fruticosa* exhibited a concentration-dependent DPPH radical scavenging capacity. Crude extract and polar fractions (Frs. 1-4) exhibited strong DPPH radical-scavenging capacity, higher than the positive controls, at all tested concentrations. Aqueous/methanol (3:1) fraction was the most active, however at 15 μg/ml concentrations, no significant difference was obtained among the activity of crude extract and Frs. 1-4. Among the ten different polarity fractions, the hexane fraction revealed no activity while the chloroform, chloroform/methanol and methanol fractions showed poor activity (Fig. 1).

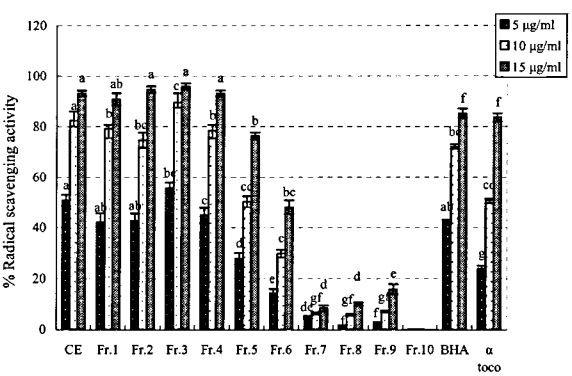

Fig. 1. DPPH radical-scavenging activity of *Woodfordia fruticosa* extract and fractions. The values represent the mean ± SD (n = 3), Values within same concentration bar with different letters differ significantly ($P < 0.05$), α-Toco: α-Tocopherol; BHA: Butylated hydroxyanisole.

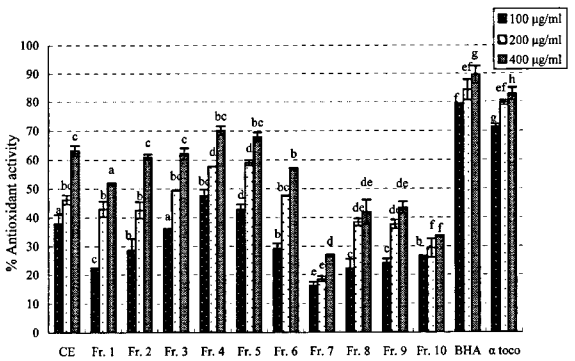

Fig. 2. Antioxidant activity of extract (CE) and fractions (1 - 10) of *Woodfordia fruticosa* in β-carotene/linoleate system. Each value is mean ± SD (n = 3), Values within same concentration bar with different letters differ significantly ($P < 0.05$), α-Toco: α-Tocopherol.

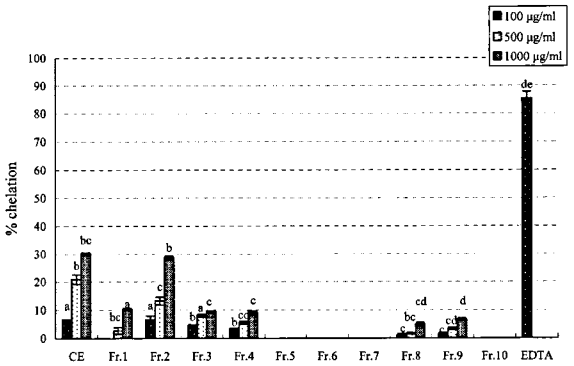

Fig. 4. Ferrous ion chelating activity of crude extract (CE) and fractions (1 - 10) of *Woodfordia fruticosa*. Values represent the mean ± SD (n = 3), Values within same concentration bar with different letters differ significantly ($P < 0.05$).

β-carotene-linoleic acid assay

Crude extracts and different polarity fractions showed concentration-dependent bleaching activity. Aqueous and aqueous-methanol fractions and crude extract were able to suppress discoloration of β-carotene in comparison to control, showing stronger bleaching activity than methanol and non-polar fractions (Fig. 2).

NO scavenging capacity

In present study, crude extract and the different solvent fractions, except MeOH-$CHCl_3$ fraction exhibited NO scavenging activity (Fig. 3). However, gradual decrease in activity (except fraction 7 and 10) was obtained at higher concentrations (data not shown).

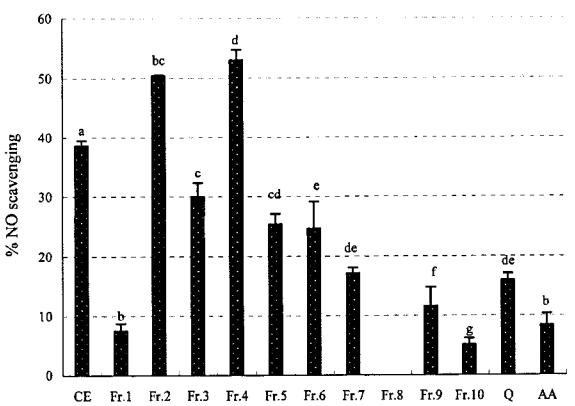

Fig. 3. NO scavenging activity of crude extract (CE) and fractions (1 - 10) of *Woodfordia fruticosa* at 25 μg/ml, each value is expressed as mean ± SD (n = 3), Values within same concentration bar with different letters differ significantly ($P < 0.05$), Q: Quercetin; AA: Ascorbic acid.

Metal chelating capacity

Crude extract and aqueous-methanol (9:1) fraction showed higher chelating activity among the tested samples. However, the activity was much lower than that of EDTA (Fig. 4). The standard compounds α-tocopherol and BHA did not exhibit any chelating activity at tested concentrations. Most of the fractions showed little chelating activity while aqueous-methanol (1:3), aqueous-methanol (1:9), methanol and hexane fraction did not exhibit any activity at tested concentrations (100 - 1,000 μg/ml).

Reducing power determination

In the present study, the reductive capabilities of ethanol extract and different solvent fractions of *W. fruticosa* were measured in terms of Fe^{3+} - Fe^{2+} transformation and compared with á-tocopherol, BHA and gallic acid. The extract and fractions have reduced iron (III) in a concentration-dependent manner. Crude extract and aqueous-methanol fractions exhibited significantly higher reducing

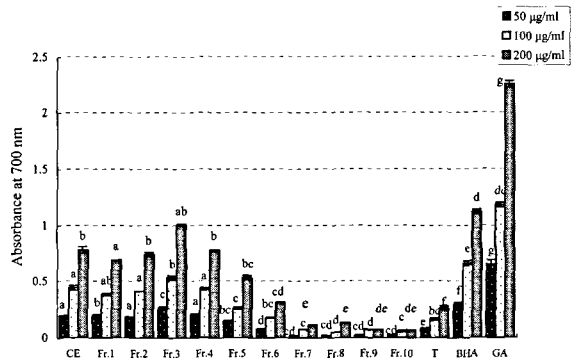

Fig. 5. Reducing power of crude extract (CE) and fractions (1 - 10) of *Woodfordia fruticosa*, each value is expressed as mean ± SD (n = 3), Values within same concentration bar with different letters differ significantly ($P < 0.05$), T: α-Tocopherol; GA: Gallic acid.

power than methanol and less polar fractions ($P < 0.05$). Their reducing activity was higher than α-tocopherol at all tested concentrations, but lower than that of BHA and gallic acid (Fig. 5). In particular, the reducing power of fraction 3 was comparable to BHA and was significantly higher than crude extract and fractions ($P < 0.05$). However, methanol and non-polar fractions showed poor reductive capability.

Total phenolic content

There was a wide range of phenol concentrations in the crude extract and fractions analyzed. As shown in Fig. 6, the crude extract and polar fractions of *W. fruticosa* contained very high level of total phenolics. The value varied from 14.59 ± 2.20 to 565.18 ± 3.09 mg GE/g of sample. The highest value of 565.18 ± 3.09 mg GE/g was detected in the crude extract, while the lowest value of 14.59 ± 2.20 in hexane fraction.

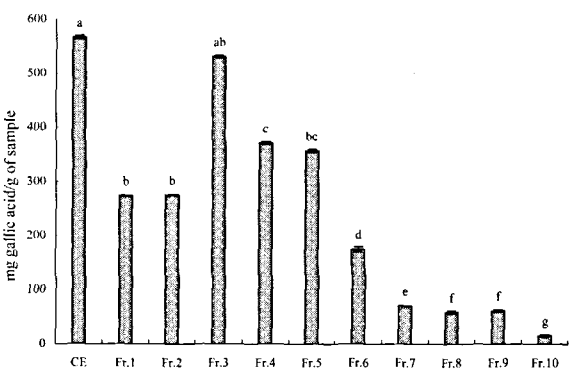

Fig. 6. Total phenolic content of crude extract (CE) and fractions (1 - 10) of *Woodfordia fruticosa*. Each value is expressed as mean ± SD (n = 3), standard deviations are too small to be seen, Values within each bar with different letters differ significantly ($P < 0.05$).

DISCUSSION

In the present study the antioxidant capacity of crude extracts and different polarity fractions of *W. fruticosa* were analysed. Radical scavenging activities are important due to the deleterious role of free radicals in foods and in biological systems. DPPH assay evaluates the ability of antioxidants to scavenge free radicals. The method is based on the reduction of alcoholic DPPH solution into non-radical form DPPH-H in the presence of a hydrogen-donating antioxidant. The plants have been reported to contain phenolic acids, flavanol glycosides, flavonol glycoside gallates and several tannins (Kadota *et al.*, 1990; Yoshida *et al.*, 1990; Yoshida *et al.*, 1991; Kuramochi-Motegi *et al.*, 1992; Dan and Dan, 1994). Many phenolic compounds have been reported to possess potent antioxidant activity, which vary according to the number and position of hydroxyl groups. Comparisons among the different classes of phenolic compounds showed that tannins were the most potential towards DPPH radical scavenging (Bouchet *et al.*, 1998; Yokozawa *et al.*, 1998; Cai *et. al.*, 2004). The strong scavenging capacity of polar fractions and crude extract might be due to the combined effect of tannins and other phenolic compounds that are reported to have ideal structural chemistry for free radical scavenging activities (Rice-Evans *et al.*, 1996).

The mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydro peroxides formed from linoleic acid. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated

β-carotene molecules. The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha *et al.*, 2001). In the present study most of the polar fractions and crude extract showed concentration dependent antioxidant activity. The results indicated that compounds with stronger β-carotene bleaching in this system were of high polarity. However, the activity was lower than that of BHA and α-tocopherol.

NO is an important bio-regulatory molecule, which has a number of physiological effects. Low concentration of NO are sufficient to affect these beneficial functions, however, during infections and inflammations, its formation is elevated and may bring about some deleterious effects (Maracocci *et al.*, 1994). The activity of most of the polar fractions and crude extract was higher than that of reference standards and were significantly different ($P < 0.05$). Fractions 2 and 4 scavenged NO radical most efficiently. The higher activity of polar fractions might be due to the presence of tannins, which have been reported to have strong NO scavenging activity (Nakagawa and Yokozama, 2002). Under physiological conditions nitric oxide can be generated or interconverted to different redox forms, which displays distinctive properties and reactivity (Maracocci *et al.*, 1994). Hence, the scavenging activity of samples might also be a consequence of the reaction of samples with other oxides of nitrogen.

The production of highly ROS, such as super oxide anion radicals, hydrogen peroxide, and hydroxyl radicals is also catalyzed by free iron through Haber-Weiss reaction. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red color of the complex. Extract and fractions of *W. fruticosa* showed poor iron chelating ability and the results suggested that the antioxidant mechanism of the plant was not due to iron chelating and is probably due to chain termination by radical-scavenging activity.

The reducing properties are generally associated with the presence of reductones (Pin-Der-Duh, 1998), which have been shown to exert antioxidative 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. A direct correlation between antioxidant activities and reducing power has been reported (Tanaka *et al.*, 1988). We also found a high correlation between reducing power and DPPH radical scavenging (r^2 = 0.982) at 10 μg/mL concentration and medium correlation between reducing power and inhibition of the bleaching of β-carotene at 400 μg/ml (r^2 = 0.661) among crude extract and different polarity fractions of *W. fruticosa*. Similarly positive correlation was found between phenolic content and reducing power (r^2 = 0.841 at 100 μg/ml).

Phenolic content of samples was evaluated using Folin-Ciocalteu method. The results suggest the possible influence of extracting and eluting solvent on total phenolic content estimation. Correlation between total phenolic content and antioxidant activity was determined. A high correlation between total phenolic content and DPPH scavenging activity (r^2 = 0.87) at 5 μg/ml concentration and reducing power (r^2 = 0.854) at 200 μg/ml was obtained. Similarly, the correlation was positive with inhibition of the bleaching of β-carotene (r^2 = 0.662) at 400 μg/ml, metal chelating activity (r^2 = 0.326) at 1 mg/ml and NO scavenging activity (r^2= 0.437). The results showed that, polyphenols present in *W. fruticosa* are multifunctional and can act as reducing agent, hydrogen donating antioxidants, NO scavengers and probably through other antioxidant mechanisms (Rice-Evans *et al.*, 1996).

CONCLUSION

In conclusion, the results indicated that crude

extract and aqueous-methanol fractions contained high phenolic content and possessed stronger antioxidant capacity. Their DPPH radical scavenging capacity and reducing power, has strongly proven their hydrogen donating capability. Similarly, they enabled inhibiting the bleaching of carotene in the β-carotene-linoleate assay system and efficiently scavenged NO radical. However, their iron chelating activity was poor, suggesting that the antioxidant mechanism of *W. fruticosa* might be due to the chain termination by radical-scavenging activity rather than iron chelation. The positive correlation between antioxidant capacity and phenolic content, suggested the contribution of phenolics towards antioxidant capacity. Further investigations should be done on isolation of active constituent(s) and their *in vivo* antioxidant activity and are necessary to determine the specific mechanisms.

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