

In vitro anti-oxidant activity of the leaves of *Dillenia indica*

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SUMMARY

The methanol extract of *Dillenia indica* was tested for antioxidant activity as determined by free radical scavenging of DPPH radical scavenging assay, reducing power, total antioxidant capacity measured by phosphomolybdenum method, total phenolic content and total flavonoids content determination assays. The extract showed significant activities in all antioxidant assays compared to the standard antioxidant in a dose dependent manner. In DPPH radical scavenging assay the IC₅₀ value of the extract was found to be 100.53 µg/ml while ascorbic acid has the IC₅₀ value 58.92 µg/ml. *Dillenia indica* extract showed strong reducing power and total antioxidant capacity. Moreover, methanol extracts also possess high amount of phenolics and flavonoids and expressed as gallic acid and rutin equivalent respectively. The remarkable activities exhibited in reactive oxygen species scavenging may attributed to the high amount of hydrophilic phenolics present in *Dillenia indica*.

Key words: *Dillenia indica*; DPPH; Total antioxidant capacity; Reactive oxygen species

INTRODUCTION

Endogenous free radicals such as superoxide, nitric oxide and hydroxyl free radicals are produced in the human body, everyday. In recent years, oxygen-derived free radicals have been reported to be closely involved in many biological symptoms such as inflammation, cancer, atherosclerosis, and coronary heart disease (Kourounakis *et al.*, 1999; Gulcin *et al.*, 2002). To prevent or delay the oxidation process, addition of antioxidants to foods is the most extensively used method. Although synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertbutylhydroquinone (TBHQ) have been commonly

used as antioxidants in foods for years, their safety has long been questioned (Branen, 1975; Ito *et al.*, 1983). However BHT and BHA have been suspected of being responsible for liver damage and carcinogenesis (Grice, 1986; Wichi, 1988; Hettiarachchy *et al.*, 1996). This has led to an increased interest in natural antioxidants (Braca *et al.*, 2002; Gyamfi and Aniya, 2002; Kayano *et al.*, 2002; Lim *et al.*, 2002). The plant under investigation is a tree *Dillenia indica* belonging to the family Dilleniaceae. The fruit of *Dillenia indica* is said to possess tonic and laxative properties and is used of abdominal pain. The bark and leaves are astringent (Ghani, 2003). Alcoholic extract of the leaves of *Dillenia indica* had been reported to possess CNS depressant activities in mice (Ghani, 2003). Seeds of *Dillenia indica* showed antimicrobial activity (Ghani, 2003). Fruits of *Dillenia indica* also possessed antioxidant activity

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(Abdille *et al.*, 2005). New pentacyclic triterpene lactone, 3 β -hydroxy lupane-13 β ,28-lactone with betulinaldehyde, butilin, lupeol, β -sitosterol, betulinic acid and myricetine has been isolated from the stem barks of *Dillenia indica* (Ghani, 2003). Two flavone, 3,4',5,7-tetrahydroxy-3'-methoxy flavone (dihydroisorhametin) and 3,5,7-trihydroxy-3'-4'-dimethoxy flavone (dillenetin) has also been reported to be present in the plant (Pavanasivam *et al.*, 1975).

As part of investigation into natural antioxidants from local medicinal plants of Bangladesh (Alam *et al.*, 2008a, b), in this paper, we reported a study of the antioxidant activity of the leaves of *Dillenia indica*. The evaluation of antioxidant power was performed in vitro by the DPPH radical scavenging, reducing power, total antioxidant capacity, total phenolics and total flavonoids assays.

MATERIALS AND METHODS

Chemicals

DPPH (1, 1-diphenyl, 2-picryl hydrazyl), TCA (Trichloro acetic acid), Ammonium molybdate, Folin-Ciocalteu, Aluminum trichloride and Ferric chloride were obtained from Sigma Chemical Co. USA. Ascorbic acid was obtained from SD Fine Chem. Ltd., Biosar, India. Sodium Nitroprusside was obtained from Ranbaxy Lab., Mohali, India. Potassium ferricyanide was obtained from May and Backer, Dagenham, UK.

Plant material

Leaves of *Dillenia indica* were collected from Gazipur, Bangladesh in June 2007, and identified by Professor Abdul Ghani (Stamford University, Dhaka, Bangladesh); a voucher specimen (SU-MAA-2007-5) for this collection has been retained in the Pharmacognosy Laboratory, Stamford University, Dhaka, Bangladesh.

Extraction

The shade-dried leaves were coarsely powdered and extracted with mixture of methanol: water (7:3

ratios) by a Soxhlet apparatus. The solvent was completely removed by rotary evaporator and obtained greenish gummy exudates. This crude extract was used for further investigation for potential antioxidant properties.

Phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed according to the procedure described earlier (Ghani, 2003).

Antioxidant activity test

The free radical scavenging capacity of the extracts was determined using DPPH (Hasan *et al.*, 2006; Alam *et al.*, 2008). A methanol DPPH solution (0.004% w/v) was mixed with serial dilutions (1 to 500 μ g) of *Dillenia indica* extracts and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV - visible spectrophotometer). Ascorbic acid was used as a standard. The inhibition curve was plotted and IC₅₀ values obtained by probit analysis (Viturro *et al.*, 1999).

Reducing power

The reducing power of *Dillenia indica* was determined according to the method previously described by Oyaizu (Oyaizu, 1986). Different concentrations of *Dillenia indica* extract (100 - 1,000 μ g) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (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 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a standard.

Determination of total antioxidant capacity

The antioxidant activity of the extracts of *Dillenia indica* was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al.* (1999). The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (Shimadzu, UV-150-02) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Determination of total phenolic content

The total phenolic content of extracts was determined using to the Folin-Ciocalteu method (Singelton *et al.*, 1999). The extracts were oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm after 60 min. Using galic acid as standard total phenolic content (standard curve was prepared using concentrations 2.5 - 50 mg/l) was expressed as mg GA equivalent/l of extract. Data reported of two replications.

Determination of total flavonoids content

The flavonoids content was determined using a method previously described by Kumaran and Karunakaran (2007) using rutin as a reference compound. One millilitre of plant extract in methanol (50 - 250 µg/ml) was mixed with 1ml aluminium

trichloride in ethanol (20 mg/ml) and a drop of acetic acid, and then diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 ml of plant extract and a drop of acetic acid, and then diluted to 25 ml with ethanol. The absorption of standard rutin solution (0.5 mg/ml) in ethanol was measured under the same conditions. All determinations were carried out in duplicates. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the following formula: $X = (A \times m0) / (A0 \times m)$, where X is the flavonoid content, mg/mg plant extract in RE, A is the absorption of plant extract solution, A0 is the absorption of standard rutin solution, m is the weight of plant extract in mg and m0 is the weight of rutin in the solution in mg.

RESULTS

Phytochemical screening

Phytochemical analyses of the crude extract revealed the presence of alkaloid, tannin, gum and flavonoid (Table 1). Phytochemical analysis revealed the presence of alkaloids, gums and flavonoids copound in the methanolic extract of *Dillenia indica*.

DPPH radical scavenging activity

Comparison of the antioxidant activity of the extract and ascorbic acid was shown in Table 2. The

Table 2. Scavenging of free radical by crude hydroethanolic extract of *Dillenia indica* and ascorbic acid in DPPH method

Sample	DPPH method IC ₅₀ (µg/ml)
Crude Hydroethanolic extract of <i>Dillenia indica</i>	100.53
Ascorbic acid	58.92

Table 1. Result of chemical group test of the methanol extract of *Dillenia indica* leaf

Extract	Steroid	Alkaloid	Reducing sugar	Tannin	Gum	Flavonoid	Saponin
ME of <i>D. indica</i>	-	+	-	++	+	++	-

ME: Methanol extract; (+): Present; (-): Absent

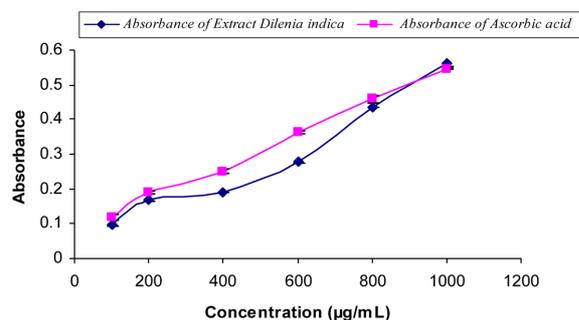


Fig. 1. Reducing power of the crude plant extract *Dillenia indica* and ascorbic acid. Increase in absorbance is a sign of increasing reducing power in ferric/thiocyanate system. Values represents duplicate experiments and expressed as mean \pm standard deviation.

hydro-alcoholic extract of *Dillenia indica* exhibited a significant dose dependent inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of IC_{50} 100.53 μ g/ml. The IC_{50} value of the extract was found to be greater than the standard, Ascorbic acid (IC_{50} 58.92 μ g/ml).

Reducing power

Fig. 1 shows the reductive capabilities of the plant extract compared to Ascorbic acid determined using the potassium ferricyanide reduction method. The reducing power of extract of *Dillenia indica* was very potent and the power of the extract was increased with quantity of sample. The plant extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of Ascorbic acid.

Table 4. Total amount of plant phenolic compounds and flavonoids of the methanol extract of *Dillenia indica*

Concentration (μ g/ml)	Total Phenols mg/g plant extract (GAE)	Total flavonoids mg/g plant extract (RE)
50	5.537 \pm 0.633	10.433 \pm 0.442
100	13.000 \pm 0.633	21.750 \pm 1.390
150	32.179 \pm 3.061	30.962 \pm 2.641
200	32.478 \pm 5.805	39.152 \pm 4.264
250	43.075 \pm 3.272	54.651 \pm 5.683
Average	25.25 \pm 2.681	31.3896 \pm 2.884

The result of total phenol was obtained by using calibration curve but that of total favonoids was obtained using the equation mentioned in the text.

Table 3. Total antioxidant capacity of extract of *Dillenia indica*. Values are given for two consecutive experiments and expressed as mean \pm standard deviation

Concentration (μ g/mg)	Equivalent of ascorbic acid
100	0.859 \pm 0.041
200	1.095 \pm 0.458
400	1.519 \pm 0.116
600	1.965 \pm 0.073
800	2.407 \pm 0.521

Total antioxidant activity

The yield of total antioxidant capacity of the methanol extract of *Dillenia indica* is given in Table 3. Total antioxidant capacity of *Dillenia indica* are expressed as the number of equivalents of ascorbic acid.

Total phenolics and flavonoid content

The total phenolics and flavonoids content of *Dillenia indica* extract was expressed in gallic acid and rutin equivalents respectively and are presented in Table 4. The amount of phenolic content was determined from regression equation of calibration curve ($y = 0.0067x + 0.1609$). There is positive correlation of total phenolic content as it was increased due to the increment of the concentration of extract.

Likewise phenolic content total flavonoids were also increased due to the increment of the concentration of extract. Total favonoids was obtained using the equation mentioned in the text and expressed in rutin equivalents.

DISCUSSION

Antioxidants which are found naturally in many foods and beverages provide health benefits in preventing diseases such as heart disease and cancer by fighting cellular damage caused by free radicals in the body (Svilaas *et al.*, 2004). A possible mechanism mediating the protective effect is related to bioactive compounds in fruits and vegetables that reduce oxidative stress, a condition that arises when the formation of reactive oxidants (most importantly reactive oxygen species, reactive nitrogen species, and reactive iron species) outstrips the antioxidant defense, and oxidative damage occurs (Svilaas *et al.*, 2004). Antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from their phenolic hydroxyl groups, thereby forming a stable end-product, which does not initiate or propagate further oxidation of lipids (Sherwin, 1978). Fruits and vegetables contain at least several hundred different types of antioxidants (i.e., electron- or hydrogen-donating reductants) that may react directly with such reactive oxidants, forming products with much lower reactivity and therefore potentially protecting against oxidative damage (Halliwell, 1996; Gutteridge and Halliwell, 2000). Another mechanism involves activation of genes encoding proteins in the antioxidant defense system and/or silencing of genes that may contribute to the oxidative stress (Wild and Mulcahy, 2000). Owing to the complexity of the oxidation-antioxidation process, no single testing method is capable of providing a comprehensive view of the antioxidative profile of a sample (Parejo *et al.*, 2002). Therefore, a multi-method approach is necessary to assess antioxidative activity. In present study, the total antioxidant effect of methanol extracts of *Dillenia indica* that are traditionally used in Bangladesh, were measured and compared to ascorbic acid. Three commonly accepted assays, DPPH free radical scavenging assay, reducing power and total antioxidant capacity by phosphomolibdenum method were employed to evaluate the

total antioxidant effects of tested plant.

Scavenging activity for free radicals of 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources. The DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants (Alam *et al.*, 2008). The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple colour. The essence of DPPH method is that the antioxidants react with the stable free radical i.e., α,α -diphenyl- β -picrylhydrazyl (deep violet colour) and convert it to α,α -diphenyl- β -picrylhydrazine with discolouration. The degree of discolouration indicates the scavenging potentials of the sample antioxidant. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins, and aromatic amines (p-phenylene diamine, p-aminophenol, etc.) reduce and decolourise DPPH by their hydrogen donating ability (Yokozawa *et al.*, 1998). Phenolic compounds of the *Dillenia indica* extracts are probably involved in their antiradical activity.

The reducing ability of a compound generally depends on the presence of reductones (Duh, 1998), which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom (Gordon, 1990). The presence of reductants (i.e. antioxidants) in the fractions *Dillenia indica* extract causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Our data on the reducing power of the tested extracts suggest that it is likely to contribute significantly towards the observed antioxidant effect. However, the antioxidant activity of antioxidants has been attributed by various mechanisms, among which

some of them are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997). Since phenolic compounds present in the extracts are a good source of electron donors, they show reducing power.

The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts (Prieto *et al.*, 1999). Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract.

Phenolic compounds, as secondary metabolite, are ubiquitous in vegetables, fruits and cereal grain. They are one kind of natural antioxidants and have multiple biological effects, in particular, play an important role in the defence against cardiovascular disease, aging and cancer. In our present study, antioxidant activity of *Dillenia indica* extracts assayed through the three methods DPPH radical scavenging, reducing power and total antioxidant capacity was found to follow the increasing order with increasing of concentration. This is similar to the extent of antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract. In the present study it is found that the methanol extract of *Dillenia indica* contains substantial amount of phenolics and flavonoids and it is the extent of phenolics present in this extract is responsible for its marked antioxidant activity as assayed through various *in vitro* models. Several reports have conclusively shown close relationship between total phenolic content and antioxidative activity of the fruits and vegetables (Vinson *et al.*,

1998; Deighton *et al.*, 2000). It has been reported that phenolic compounds with ortho- and para-dihydroxylation or a hydroxy and a methoxy group are more effective than simple phenolics (Frankel *et al.*, 1995). Since the chemical composition and structures of active components are important factors governing the efficacy of natural antioxidants the extract of *Dillenia indica* needs their characterization.

CONCLUSION

The extract found to have different levels of antioxidant activity in all the systems tested. In view of the discussion, it can be concluded that the methanolic extract possesses the antioxidant substances which may be a potential mechanism using this plants extract in folkloric remedies. Further studies are warranted for the isolation and identification of individual phenolic compounds and also *in vivo* studies are needed for understanding their mechanism of action as an antioxidant.

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