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Antioxidant and Antidiarrhoeal Potentiality of *Diospyros blancoi*

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Abstract: Phytochemical analysis of the MeOH extract of leaves of *Diospyros blancoi* (Ebenaceae) indicated the presence of Reducing Sugar, Gum, Tannin, Alkaloid and Flavonoid types of compounds. The pharmacological interest of these compounds prompted us to check its antidiarrhoeal and anti-oxidant activities. Antioxidant potential was evaluated by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay. The extract showed significant ($p < 0.001$) antioxidant activity in a dose-dependent manner with the IC_{50} value of $15.06 \pm 0.49 \mu\text{g mL}^{-1}$, compared to the IC_{50} value of the reference standard ascorbic acid which was $8.11 \pm 0.21 \mu\text{g mL}^{-1}$ suggested its potent anti-oxidant activity. The anti-diarrhoeal activity was investigated on the castor oil-induced mice models. The doses displayed significant reduction in faecal output of about 24.49 and 44.89%, respectively at 250 and 500 mg kg^{-1} , compared to the reference standard loperamide which was 59.18% $\mu\text{g mL}^{-1}$. The obtained results provide a support for the use of this plant in traditional medicine as an antidiarrhoeal remedy.

Key words: *Diospyros blancoi*, anti-oxidant, antidiarrhoeal, DPPH, castor oil

INTRODUCTION

In human, cancer and atherosclerosis are caused by free radical, extremely unstable species; ROS (reactive oxygen species) includes OH^{\cdot} (hydroxyl radical), $\text{O}_2^{\cdot-}$ (superoxide radical), NO^{\cdot} (nitric oxide radical), LOO^{\cdot} (lipid peroxyl radical) (Bagchi and Puri, 1998). There are several endogenous reactions and some external reactions caused by enzyme as well as non-enzyme generate free radicals. These reactions are respiratory chain reaction, the phagocytosis, prostaglandin synthesis, cytochrome P450 system and oxidative phosphorylation (i.e., aerobic respiration) in the mitochondria (Tiwari, 2004; Halliwell, 2007; Pacher *et al.*, 2007). Though ROS damages the cells it plays some important functions in the body as it is the normal cellular metabolite (Valko *et al.*, 2004). Oxidative stresses are built up for the deficiency of antioxidant to reduce the level of free radical in the body subsequently cause chronic health damage. So equilibrium must be maintained between free radicals and antioxidants in the body for healthiness (Bagchi and Puri, 1998). Antioxidants defense is, the transfer electrons to extremely unstable, free radical. Free radical accepts the electron from antioxidant and they do not attack the cell

and the chain reaction of oxidation is inhibited. Thus cell damage is prevented (Dekkers *et al.*, 1996). Phenolic compounds, flavonoids and triterpenoids containing foods and beverages with antioxidant activity have been reported (Brown and Rice-Evans, 1998; Krings and Berger, 2001; Cipak *et al.*, 2006; Somova *et al.*, 2003). Very recent, health risks and toxicity have been reported using synthetic antioxidants restricted (Buxiang and Fukuhara, 1997).

Some plant species containing antioxidants (rosemary and sage) are reported as commercial antioxidant preparation as well as food and nutritional supplements (Rahman *et al.*, 2011).

In few years, natural antioxidants are very much interesting bioactive compounds for the scientists. Among them probing of antioxidants from plant origin has markedly augmented (Jayaprakasha and Rao, 2000).

A common health problem in the developing countries like Bangladesh is Diarrhoea (Syder and Merson, 1982). Diarrhoea is one of the most threatening disease in developing countries as about 7.1 million death in 1998 estimated by World Health Organization (Park, 2000). The most dangerous symptoms of GI problems is secretory diarrhea associated with excessive faecal

output, loose stool (Aranda-Michel and Giannella, 1999). Oral saline (rehydrate therapy) and chemotherapy are the clinical management for this diarrhoea (Irfan *et al.*, 2001).

Diospyros blancoi (Ebenaceae), commonly known as Gub, is a small to large evergreen tree found all over the different parts of Bangladesh, Pakistan and India, juice of bark and leave used for snakebites, diarrhea and dysentery (Ghani, 2003). *Diospyros* species possesses various biological activities e.g., antibacterial, antidiarrhoeal, antifungal, antiprotozoal, molluscicidal, anti-inflammatory and cytotoxicity activity (Maridass, 2008). *D. blancoi* is reported to 24 components (Collins and Halim, 1976).

However, there is no scientific proof justifying the traditional use of *Diospyros blancoi* leaf in the treatment of diarrhoea. Hence, the present study was undertaken to evaluate its potential antidiarrhoeal efficacy in experimental models of diarrhoea in mice. As a part of ongoing investigations on local medicinal plants of Bangladesh, in this study, we reported antioxidant and antidiarrhoeal potentiality of the leaves of *Diospyros blancoi*.

MATERIALS AND METHODS

Plant materials: Leaves of *Diospyros blancoi* was collected from district of Barisal, Bangladesh in February 2010 and was identified by the experts at Bangladesh National Herbarium, Dhaka (Accession No. 34970) and a voucher specimen was also deposited there.

Chemicals: 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA), Loperamide was a gift sample from Square Pharmaceuticals Ltd., Bangladesh and other chemicals were of analytical grade.

Preparation of plant extract: The plant material was shade dried with occasional shifting and then powdered with a mechanical grinder and stored in a tight container. The dried powder (1.5 kg) was refluxed with MeOH for three hours. The total filtrate was concentrated to dryness, in vacuo at 40°C to render the MeOH extract for investigation.

Animal: For the experiment, Twenty Swiss albino mice of either sex, weighing between 20-25 g, were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDRDB). Animals were maintained under standard environmental conditions (temperature: $24.0 \pm 1.0^\circ\text{C}$, relative humidity: 55-65% and 12 h light/dark cycle) and had free access to feed and water *ad libitum*. The animals were acclimatized

to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

Phytochemical screening: The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, by using the following reagents and chemicals, for example, alkaloids were identified by the Dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions and steroids with Liebermann-Burchard reagent. Reducing sugars with Benedict's reagent (Ghani, 2003).

Chemical group tests of the extract: Testing different chemical groups present in the extract are performed the preliminary phytochemical studies. The chemical group test that was performed along with the results obtained is as follows (Trease and Evans, 1989). In each test 10% (w/v) solution of extract was taken unless otherwise mentioned in individual test.

Reagents used for the different chemical group test: The following reagents were used for the different chemical group test (Ghani, 1998; Harborne, 1984).

Mayer's reagent: The 1.36 g mercuric iodide in 60 mL of water was mixed with a solution containing 5 g of potassium iodide in 20 mL of water.

Dragendorff's reagent: The 1.7 g basic bismuth nitrate and 20 g tartaric acid were dissolved in 80 mL water. This solution was mixed with a solution containing 16 g potassium iodide and 40 mL water.

Fehling's solution A: The 34.64 g copper sulphate was dissolved in a mixture of 0.50 mL of sulphuric acid and sufficient water to produce 500 mL.

Fehling's solution B: One hundred seventy six gram of sodium potassium tartrate and 77 g of sodium hydroxide were dissolved in sufficient water to produce 500 mL. Equal volume of above solution were mixed at the time of use.

Benedict's reagent: The 1.73 g cupric sulphate, 1.73 g sodium citrate and 10 g anhydrous sodium carbonate were dissolved in water and the volume was made up to 100 mL with water.

Molisch reagent: The 2.5 g of pure α -naphthol was dissolved in 25 mL of ethanol.

Liebermann-Burchard reagent: Five milliliter acetic anhydride was carefully mixed under cooling with 5 mL concentrated sulphuric acid. This mixture was added cautiously to 50 mL absolute ethanol with cooling.

Tests performed for identifying different chemical groups: The following tests were performed for identifying different chemical groups.

Tests for tannins

Ferric chloride test: Five milliliter solution of the extract was taken in a test tube. Then 1 mL of 5% Ferric chloride solution was added.

Potassium dichromate test: Five milliliter solution of the extract was taken in a test tube. Then 1 mL of 10% potassium dichromate solution was added.

Lead acetate test: One milliliter of 10% lead acetate solution was added to 5 mL of extract solution.

Test for flavonoids: A few drops of concentrated hydrochloric acid were added to a small amount of extract of the plant material.

Test for saponins: One milliliter solution of the extract was diluted with distilled water to 20 mL and shaken in a graduated cylinder for 15 min.

Test for gums: Five milliliter solution of the extract was taken and then Molisch reagent and sulphuric acid were added.

Tests for steroids

Liebermann-Burchard test: One milliliter solution of chloroform extract was taken and then added 2 mL Liebermann-Burchard reagent.

Sulphuric acid test: One milliliter solution of chloroform extract was taken and then 1 mL sulphuric acid was added.

Tests for alkaloids

Mayer's test: Two milliliter solution of the extract and 0.2 mL of dilute hydrochloric acid were taken in a test tube. Then 1 mL of Mayer's reagent was added.

Dragendorff's test: Two milliliter solution of the extract and 0.2 mL of dilute hydrochloric acid were taken in a test tube. Then 1 mL of Dragendorff's reagent was added.

Wagner's test: Two milliliter solution of the extract and 0.2 mL of dilute hydrochloric acid were taken in a test tube. Then 1 mL of iodine solution (Wagner's reagent) was added.

Hager's test: Two milliliter solution of the extract and 0.2 mL of dilute hydrochloric acid were taken in a test tube. Then 1 mL of picric acid solution (Hager's reagent) was added.

Tests for carbohydrates

Benedict's test (test for reducing sugar): The 0.5 mL of aqueous extract of the plant material was taken in a test tube. Five milliliter of benedict's solution was added to the test tube, boiled for 5 min and allowed to cool spontaneously.

Fehling's test (standard test for reducing sugar): Two milliliter of aqueous extract of the plant material was added to 1 mL of a mixture of equal volumes of Fehling's solutions A and B, boiled for few minutes.

Combined reducing sugar test: One milliliter of aqueous extract of plant material was boiled with 2 mL of dilute HCl acid for 5 min. The mixture was cooled and neutralized with NaOH solution and performed the Fehling's test as described above.

In vitro tests for antioxidant activity

Free radical scavenging activity by DPPH method: Quantitative assay was performed on the basis of the modified method of Gupta *et al.* (2003). Stock solutions (10 mg mL⁻¹) of the plant extracts were prepared in ethanol from which serial dilutions were carried out to obtain concentrations of 1, 5, 10, 50, 100 and 500 mg mL⁻¹. Diluted solutions (2 mL) were added to 2 mL of a 0.004% ethanol solution of DPPH, mixed and allowed to stand for 30 min for reaction to occur. The absorbance was determined at 517 nm and from these values corresponding percentage of inhibitions were calculated. Then %inhibitions were plotted against log concentration and from the graph IC₅₀ was calculated. The experiment was performed in duplicate and average absorption was noted for each concentration. Ascorbic acid was used as positive control.

DPPH free radical scavenging activity was determined by the method described by Choi *et al.* (2007) and Desmarchelier *et al.* (1997). Plant extract (0.1 mL) was added to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition was calculated from:

$$\frac{A_0 - A_1}{A_0} \times 100$$

where A₀ is the absorbance of the control and A₁ is the absorbance of the extract/standard. IC₅₀ value was calculated from the equation of line obtained by plotting a graph of concentration (µg mL⁻¹) versus % inhibition.

Antidiarrhoeal activity: Antidiarrhoeal activity of the MeOH extract of *D. blancoi* was tested using the model by castor oil induced diarrhoea in mice (Uddin *et al.*, 2005). The mice were all screened initially by giving 0.5 mL of castor oil and only those showing diarrhoea were selected for the final experiment. The test animals were randomly chosen and divided into two groups having five mice in each. Group-I was kept as control and received 1% Tween-80 at the dose of 10 mL kg⁻¹ b.wt.; group-II received loperamide at 3 mg kg⁻¹; group-III and IV were 'test groups' and were treated with extract of *D. blancoi* at 250 and 500 mg kg⁻¹. Control vehicle and the extract were administered orally, 1/2 h prior to the oral administration of 0.5 mL castor oil. Individual animals of each group were placed in separate cages having adsorbent paper beneath and examined for the presence of diarrhoea every hour in four hours study after the castor oil administration. Number of stools or any fluid material that stained the adsorbent paper was counted at each successive hour during the experiment (4 h). The latent period of each mouse was also counted. At the beginning of each hour new papers were placed for the old ones.

Finally percent reduction of faecal output was calculated (Mohammed *et al.*, 2009).

Statistical analysis: Student's t-test was used to determine a significant difference between the control group and experimental groups.

RESULTS

Phytochemical screening: Phytochemical analyses of the crude extract revealed the presence of Flavonoid, alkaloid and tannin, Gum and sugar (Table 1).

In vitro antioxidant activity

DPPH free radical scavenging activity: MeOH extract of *D. blancoi* showed potential antioxidant activity where the IC₅₀ was 15±0.49 µg mL⁻¹ (p<0.001), as compared to that of ascorbic acid (IC₅₀ 8±0.21 µg mL⁻¹) (p<0.001) which is a well known antioxidant (Table 2). The extract caused an increase in DPPH free radical scavenging activity (% inhibition) as increasing dose (Table 3).

Antidiarrhoeal activity test: Antidiarrhoeal activity of the methanolic extract of *D. blancoi* was tested by castor oil induced diarrhoea in mice. The extract caused an increase in latent period (2.81 and 3.290 h)% (p<0.001) i.e., delayed the onset of diarrhoeal episode at 250 and 500 mg kg⁻¹, respectively (Table 4). The extract also decreased the frequency of defecation at the same dose where the mean

Table 1: Test result for chemical groups of *D. blancoi*

Sample	Flavonoid	Alkaloid	Saponin	Tannin	Steroid	Sugar	Gum
ME of <i>D. blancoi</i>	+	+	-	+	-	+	+

ME: Methanolic extract, +: Present, -: Absent

Table 2: DPPH free radical scavenging activity of *D. blancoi*

Sample	IC ₅₀ (µg mL ⁻¹)
ME of <i>D. blancoi</i>	15.06±0.49*
Ascorbic Acid	08.11±0.21*

*p<0.001 vs. control

Table 3: DPPH radical scavenging activity of *D. blancoi*

Concentration (µg mL ⁻¹)	Inhibition (±SEM) (%)	
	ME extract of <i>D. blancoi</i>	Ascorbic acid
1	3.36±0.21	9.68±0.92
5	16.60±0.11	30.79±1.20
10	58.99±0.88	82.87±0.82
25	84.44±1.12	86.85±1.05
50	84.94±0.45	87.19±1.35
100	85.64±0.85	87.54±1.40
200	86.50±0.25	87.71±1.04
500	86.50±0.30	91.00±0.52

Values represent the Mean±SEM, No. of readings in each group = 3

Table 4: Effect of the MeOH extract of *D. blancoi* on castor oil induced diarrhoea in mice

Treatment	Mean latent period (h)	Total No. of faecal matter passed in 4 h	Reduction of faecal output (%)
Test group-I Distilled water, 10 mL kg ⁻¹	1.52±0.22	9.8±0.66	-
Test group-II Loperamide, 3 mg kg ⁻¹	3.89±0.14	4±0.32	59.18
Test group-II ME, 250 mg kg ⁻¹	2.81±0.32	7.4±0.76	24.49
Test group-III ME, 500 mg kg ⁻¹	3.29±0.10	5.02±0.50	44.89

Values represent the Mean±SEM, No. of readings in each group = 3

numbers of stool at the 4th h of study were 7.4±0.76 and 5.4±0.50, respectively and % reduction of faecal output were 24.49 and 44.89% (p<0.001), respectively (Table 4).

DISCUSSION

Preliminary phytochemical screening showed the presence of Flavonoid, tannin, alkaloid in the plant extract. Multiple biological effects, including antioxidant activity commonly found in plants containing polyphenolic compounds, like flavonoids, tannins and phenolic acids (Brown and Rice-Evans, 1998; Vinson *et al.*, 1995; Gil *et al.*, 1999; Kahkonen *et al.*, 1999). Tannic acid present in the plant extract, as evident from phytochemical screening, may be responsible for the antioxidant action (Fig. 1, 2). It was shown that the percentage (%) scavenging of DPPH radical was increased significantly with increasing dose, p<0.001. However, bioactive triterpenes were obtained from *Diospyros blancoi*. Isoarborinol methyl ether, a mixture of alpha-amyrin palmitate, alpha-amyrin palmitoleate, beta-amyrin palmitate

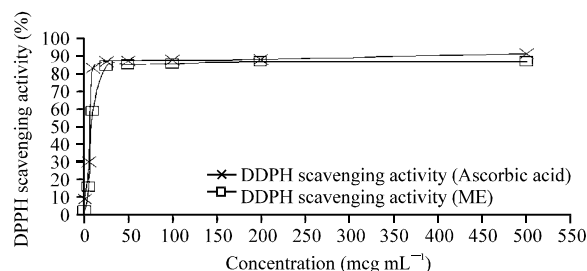


Fig. 1: Percent inhibition of crude extract of *D. blancoi* and with ascorbic acid (Standard), N = 3, Error bars not shown to ensure clarity

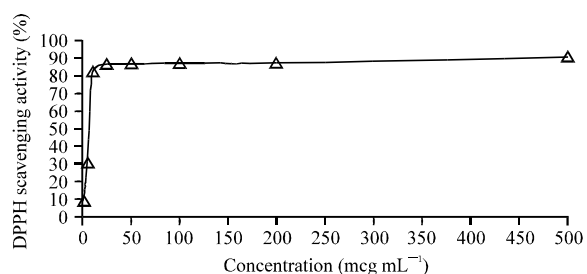


Fig. 2: DPPH radical scavenging activity of *D. blancoi*, N = 3, Error bars not shown to ensure clarity

and beta-amyrin palmitoleate, in a 13:4:3:1 ratio and squalene (Ragasa *et al.*, 2009). Many plants conveniently available are used in traditional folklore medicine for the treatment of diarrhoea. Of the indigenous plants used *Andrographis paniculata*, *Asparagus racemosus*, *Butea monosperma*, *Cassia auriculata* and others are mentioned (Chopra *et al.*, 1956). Some plant extracts have been reported to a protective effect on the intestinal tract (Rani *et al.*, 1999; Majumdar *et al.*, 2000; Kumar *et al.*, 2001). In the present study, ethanol extracts of *Diospyros blancoi* that have not been studied so far, was evaluated for its antidiarrhoeal potential against castor oil induced diarrhea in mice.

The MeOH extract of *Diospyros blancoi* exhibited significant anti-diarrhoeal activity against castor oil induced diarrhoea in mice. The extracts had a similar activity as loperamide, when tested at 250 and 500 mg kg⁻¹ and statistically significant reduction in the frequency of defecation (p<0.001) and the wetness of the faecal droppings when compared to untreated control mice. Thus it can be suggested that the MeOH extract of *D. blancoi* might possess antidiarrhoeal activity.

Castor oil or its active component ricinoleic acid induces permeability changes in mucosal fluid and electrolyte transport that results in a hypersecretory response and diarrhoea (Ammon *et al.*, 1974; Gaginella *et al.*, 1975). Diarrhoea results from an imbalance

between the absorptive and secretory mechanisms in the intestinal tract, accompanied by hurry, resulting in an excess loss of fluid in the faeces. In some diarrheas, the secretory component predominates, while other diarrhoeas are characterized by hypermotility. The use of castor oil induced diarrhoea model in our study is logical because the autacoids and prostaglandins are involved these have been implicated in the causation of diarrhoea in man (Horton *et al.*, 1968; Greenbargena *et al.*, 1978). The liberation of ricinoleic acid from castor oil results in irritation and inflammation of the intestinal mucosa, leading to release of prostaglandins which stimulate motility and secretion (Pierce *et al.*, 1971). These observations tend to suggest that those extracts at a dose of 500 mg kg⁻¹ reduced diarrhoea by inhibiting castor oil induced intestinal accumulation of fluid. These results are recommended for previous report on ripen fruits extract of *Rhus javanica* (Tangpu and Yadav, 2004).

The antidiarrhoeal activity of tannin has been reported (Mukherjee *et al.*, 1998), flavonoids (Galvez *et al.*, 1993), alkaloids (Shoba and Thomas, 2001), saponins, reducing sugars and sterols and/or terpenes (Otshudi *et al.*, 2000) containing plant extracts. The phytochemical analysis of the extract showed the presence of alkaloids, saponins, flavonoids, sterols and/or terpenes and sugars. These constituents may be responsible for the antidiarrhoeal activity of *Diospyros blancoi*.

CONCLUSION

The present study reveals that the MeOH extract of *Diospyros blancoi* possesses significant free radical scavenging activity. The data suggests that the extract contains compounds that may be effectively utilized as a wide spectrum of antioxidant agent. The antidiarrhoeal activity was dose dependent. Further analysis including additional purification of extract and chemical characterization of isolated compounds, along with further testing should be required for identification of compounds and possible mechanism of action.

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