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Comparative investigation of the free radical scavenging potential and anticancer property of *Diospyros blancoi* (Ebenaceae)

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ABSTRACT

Objective: To investigate the comparative effects of *Diospyros blancoi* (Ebenaceae) leaves (DBL), root bark (DBRB) and stem bark (DBSB) on free radicals and cancer.
Methods: The polyphenol contents, antioxidant and free radical scavenging properties were determined using standard spectrophotometric methods. Cytotoxicity and anticancer activities were performed on brine shrimp nauplii and Ehrlich ascite carcinoma cells, respectively.
Results: Among the extracts, DBSB showed the highest total antioxidant capacity and reducing capacity on ferrous ion. Based on 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radical scavenging activities, DBSB showed (95.760 ± 0.343)% and (67.460 ± 2.641)% scavenging with IC_{50} of (3.10 ± 0.17) and (50.00 ± 3.11) μg/mL, respectively. The IC_{50} values of standard butylated hydroxytoluene and catechin (CA) for 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radicals were (8.50 ± 0.25) and (75.00 ± 0.14) μg/mL, respectively suggesting that DBSB had a significant ($P < 0.05$) radical scavenging activity than standards. In lipid peroxidation inhibition assay, the inhibitory activity of the extracts and the standard was in the following order: DBSB > DBRB > CA > DBL. Also, the phenolic [(139.91 ± 3.924) mg gallic acid equivalent/g] and flavonoid contents [(412.00 ± 16.70) mg catechin equivalent/g)] of DBSB were higher than that of other extracts. In addition, the DBSB showed the moderate cytotoxic and anticancer properties.
Conclusions: Our results indicate that *Diospyros blancoi* stem bark had the significant highest antioxidant and free radical scavenging properties as well as moderate anticancer activity. Hence, we assume that the anticancer activity of this plant can be, at least in part, attributed to its content in phenolic compounds as well as its significant free radical scavenging properties.

1. Introduction

Recent research community and their accumulating evidences suggest that most of the dangerous pathological manifestations, such as cancer, diabetes, cardiovascular and neurodegenerative disorders, are associated with the accumulation of free radicals [1,2]. These free radicals have the tendency to become stable through electron pairing with biological macromolecules, such as proteins, lipids, and DNA in healthy human cells and cause protein and DNA damage, leading to cancer. This oxidative mediated cellular damage can become widespread because of the weakened cellular antioxidant defense systems. All biological systems have antioxidant

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defense mechanisms that protect oxidative damages and help to remove damaged molecules [1]. However, this inherent antioxidant defense mechanism can be inefficient; hence dietary intake of antioxidants is important to provide effective protection to ameliorate various disease states, including cancer.

Antioxidants are substances that combat free radicals and prevent damage to cells and protect them either by scavenging the reactive oxygen species (ROS) or by protecting the antioxidant defense mechanism [3]. Antioxidants also turn free radicals into waste by products, and they eventually get eliminated from the body. However, consumption of fruits and vegetables is known to lower the risk of several diseases caused by free radicals or oxidative stress [4], and such health benefits are mainly attributed to the presence of various phytochemicals such as polyphenols, carotenoids, vitamins E and C available in the plant [1].

Scientific research suggests that phenolic compound possesses antioxidant activity and is widely distributed in both edible and non edible herbs, cereals, fruits, vegetables, oils, spices and other plant materials [5,6]. However, antioxidant properties of endemic plants, limited to certain regions and known only by local populations, are still scarce. Even if in this modern era, many people are still depending on the traditional medicine for their primary health care. Therefore, the evaluation of such properties remains an interesting and useful task, particularly to find new promising sources of natural antioxidants for functional foods and/or nutraceuticals [7].

Diospyros blancoi (*D. blancoi*), which belongs to Ebenaceae family, grows well in areas with a monsoon climate and on almost any soil. In Bangladesh, *D. blancoi* is known as "bilati gab" and widely distributed all over the country including the region in Rajshahi District. The genus *Diospyros* consists of more than 240 species, which are reported to be economically important [8]. Folkloric usage of *D. blancoi* suggests that the juice of unripe fruits is used as a natural treatment for diarrhea and first aid treatment for wounds; while other parts such as bark, leaves, and roots are used to treat respiratory diseases and skin ailments including eczema [9–11]. In Southeast Asia, traditionally the juice of unripe fruit is used for wounds; oil from seeds is used for diarrhea and dysentery; infusion of fruit is used as gargle for stomatitis [12].

Evidences of antioxidant and free radical scavenging properties of *D. blancoi* leaves are found in different spectrophotometric assay systems [13]. Although antioxidant activity of leaves of *D. blancoi* has been reported previously, scientific data particularly on free radical scavenging, cytotoxicity and anticancer property of *D. blancoi* leaves (DBL), root bark (DBRB) and stem bark (DBSB) are very rare. Therefore, in this study, we evaluated comparative polyphenolic contents, antioxidant activity, free radical scavenging potential, cytotoxic and anticancer property from leaves, root bark and stem bark of *D. blancoi*.

2. Materials and methods

2.1. Plant collection

Leaves, stem and root barks of *D. blancoi* were collected from Rajshahi University Campus, Rajshahi, Bangladesh, in February, 2014 and were identified by an expert taxonomist at the Department of Botany, University of Rajshahi. A voucher specimen (access number 1330/Pharm) is deposited to the herbarium in the Department of Botany, University of Rajshahi. Plant materials were then washed separately with fresh water to remove dirty materials and were shade dried for 7 days with occasional sun drying (at around 30 °C for 2 h, twice a day). The dried materials were ground into coarse powder by grinding machine and the materials were stored at room temperature (RT) for future use.

2.2. Extract preparation

The extraction was performed according to Alam *et al.* [14]. About 500 g of each powdered plant materials were taken in three amber colored extraction bottles and the materials were soaked with 1.5 L of ethanol. The sealed bottles were kept for 15 days with occasional shaking and stirring. The final extracts were filtered separately through cotton and then Whatman No. 1 filter papers and were concentrated with a rotary evaporator (Bibby Sterilin Ltd., UK) under reduced pressure at 50 °C to afford 30, 45 and 40 g extract of leaves, stem and root barks, respectively.

2.3. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, catechin (CA), ferrous ammonium sulfate, butylated hydroxyl ltoluene (BHT), gallic acid (GA), ascorbic acid (AA), $AlCl_3$, trichloro acetic acid (TCA), sodium phosphate, sodium nitrate, ammonium molybdate, 2-Deoxy-D-ribose, sodium hydroxide, ethylene diamine tetraacetic acid and $FeCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA); potassium acetate, phosphate buffer, thiobarbituric acid (TBA), HCl, H_2SO_4, H_2O_2 were purchased from Sigma–Aldrich, vinblastine sulphate (VBS) from Cipla India, folin-ciocalteu's phenol reagent and sodium carbonate were obtained from Merck (Dam-stadt, Germany).

2.4. Determination of antioxidant and free radical scavenging activity

2.4.1. Determination of total phenolics

Total phenolic contents of the extracts were determined by folin-ciocalteu method described by Wolfe *et al.* [15]. An aliquot of the extracts was mixed with 2 mL folin-ciocalteu reagent (previously diluted with water 1:10 v/v) and 2 mL (75 g/L) of sodium carbonate. The tubes were vortexed thoroughly for 15 s and allowed to stand for 20 min at 25 °C for color development. Absorbance was then measured at 760 nm UV-spectrophotometer (Shimadzu, USA). Samples of extract were evaluated at a final concentration of 0.1 mg/mL. Total phenolic contents were expressed in terms of gallic acid equivalent (GAE) (standard curve equation: $y = 0.073x + 0.17$, $R^2 = 0.999$), mg of GAE/g of dry extract.

2.4.2. Determination of total flavonoids

Total flavonoid contents were estimated using aluminum chloride colorimetric assay method described by Kiranmai *et al.* [16]. To 0.5 mL of samples/standard, 150 μL of 5% sodium nitrate and 2.5 mL of distilled water were added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At 6 min, 1 mL of 0.001 mol/L NaOH and 0.55 mL distilled water were added to the mixture and left at RT for 15 min. Absorbance of the mixtures was measured at 510 nm. Total flavonoid contents

were expressed in terms of catechin equivalent (CAE) (standard curve equation: $y = 0.003x + 0.001$, $R^2 = 0.998$), mg of CAE/g of dry extract.

2.4.3. Determination of total antioxidant capacity (TAC)

TAC of the extracts was determined according to Costa *et al.* [17]. Samples/standard (0.5 mL) at different concentrations was mixed with 3 mL of reaction mixture containing 0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 1% ammonium molybdate into the test tubes. The test tubes were incubated at 95 °C for 10 min to complete the reaction. The absorbance was measured at 695 nm using a spectrophotometer against blank after cooling at RT. CA was used as standard. A typical blank solution contained 3 mL of reaction mixture and the appropriate volume of the same solvent used for the samples/standard were incubated at 95 °C for 10 min and the absorbance was measured at 695 nm. Increased absorbance of the reaction mixture indicates increased TAC.

2.4.4. Ferrous reducing antioxidant capacity assay

The ferrous reducing antioxidant capacity of the extracts was evaluated by the method of Jayanthi *et al.* [18]. A volume of 0.25 mL samples/standard of solution at different concentrations, 0.625 mL of potassium buffer (0.2 mol/L) and 0.625 mL of 1% potassium ferricyanide, $K_3[Fe(CN)_6]$ solution were added into the test tubes. The reaction mixtures were incubated for 20 min at 50 °C to complete the reaction. Then 0.625 mL of 10% TCA solution was added into the test tubes. The total mixture was centrifuged at 3 000 r/min for 10 min. After which, 1.8 mL supernatant was withdrawn from the test tubes and was mixed with 1.8 mL of distilled water and 0.36 mL of 0.1% $FeCl_3$ solution. The absorbance of the solution was measured at 700 nm using a spectrophotometer against blank. A typical blank solution contained the same solution mixture without plant extracts/standard was incubated under the same conditions and the absorbance of the blank solution was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing capacity.

2.4.5. DPPH radical scavenging assay

Free radical scavenging ability of the extracts was tested by DPPH radical scavenging assay according to Shen *et al.* [19]. A solution of 0.1 mmol/L DPPH in methanol was prepared and 2.4 mL of this solution was mixed with 1.6 mL of extract in methanol at different concentrations. The reaction mixture was vortexed thoroughly and left in the dark at RT for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. BHT was used as reference. Percentage DPPH radical scavenging activity was calculated by the following equation:

$$\% \text{ DPPH radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extracts/standard. Then percentage of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

2.4.6. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extracts was determined by the method of Nagai *et al.* [20]. The reaction mixtures contained 0.8 mL of phosphate buffer solution (50 mmol/L, pH 7.4), 0.2 mL of extracts/standard at different concentrations, 0.2 mL of ethylene diamine tetraacetic acid (1.04 mmol/L), 0.2 mL of $FeCl_3$ (1 mmol/L) and 0.2 mL of 2-Deoxy-D-ribose (28 mmol/L) were taken in the test tubes. The mixtures were kept in a water bath at 37 °C and the reaction was started by adding 0.2 mL of AA (2 mmol/L) and 0.2 mL of H_2O_2 (10 mmol/L). After incubation at 37 °C for 1 h, 1.5 mL of cold TBA (10 g/L) was added to the reaction mixture followed by 1.5 mL of HCl (25%). The mixture was heated at 100 °C for 15 min and then cooled down with water. The absorbance of the solution was measured at 532 nm with a spectrophotometer. The hydroxyl radical scavenging activity was evaluated with the inhibition of percentage of 2-Deoxy-D-ribose oxidation on hydroxyl radicals. The percentage of hydroxyl radical scavenging activity was calculated according to the following formula:

$$\% \text{ Hydroxyl radical scavenging activity} = [A_0 - (A_1 - A_2)] \times 100/A_0$$

where, A_0 is the absorbance of the control without a sample, A_1 is the absorbance after adding the sample and 2-Deoxy-D-ribose, A_2 is the absorbance of the sample without 2-Deoxy-D-ribose. Then percentage of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

2.4.7. Lipid peroxidation inhibition assay

The lipid peroxidation inhibition assay was determined according to the method described by Rahman *et al.* [21]. Excised rat liver was homogenized in buffer and then centrifuged to obtain liposome. Firstly, 0.5 mL of supernatant, 1 mL of 0.15 mol/L KCl and 0.3 mL of extracts/standard at different concentrations were mixed. Peroxidation was initiated by the addition of 300 μL of 0.5 mmol/L $FeCl_3$ [21]. The mixture was incubated at 37 °C for 30 min and the reaction was stopped by adding 2 mL of ice-cold TBA-TCA-HCl-BHT solution. The TBA-TCA-HCl solution was prepared by dissolving 1.68 g TCA and 41.60 mg TBA in 10 mL of 0.125 mol/L HCl. A volume of 1 mL BHT solution (1.5 mg/mL ethanol) was added to 10 mL TBA-TCA-HCl solution. The reaction mixture was heated for 60 min at 90 °C and then cooled on ice and centrifuged at 3 000 r/min for 5 min. The supernatants were removed and absorbance was measured on a spectrophotometer at 532 nm. A control experiment was performed in the presence of distilled water without the extract. The percentage of lipid peroxidation inhibition in the samples was calculated using the following formula:

$$\% \text{ Lipid peroxidation inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 is the absorbance of the control (300 μL of distilled water), and A_1 is the absorbance of extracts/standard. Then percentage of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

2.5. Determination of cytotoxicity and anticancer activity

2.5.1. Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is a general bioassay, which is indicative of cytotoxicity, various pharmacological actions and pesticidal effects. Extracts with $ED_{50} \leq 30$ μg/mL are

considered to be cytotoxic [22]. All extracts (12.5–150.0 μg/mL) and control VBS were diluted with hatching medium for the brine shrimp lethality bioassay in a 50-well plate (n = 10 live shrimps/well). The live healthy nauplii with constant motion were counted after 24 h. The percentage of viability of the nauplii was calculated at each concentration by the following formula:

$$\% \text{ Nauplii viability} = N_t/N_0 \times 100$$

where, N_t is the number of viable nauplii after 24 h of incubation, N_0 is the number of total nauplii transferred, *i.e.* 10.

2.5.2. Tumor growth inhibition

Protocol used in this study for the use of mice as animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh. *In vivo* tumor cell growth inhibition was carried out by the method previously described by Sur and Ganguly [23]. For this study, 5 groups of mice (6 in each group) were used. For therapeutic evaluation, 1×10^7 Ehrlich ascite carcinoma (EAC) cells/mouse was inoculated into each group of mice on the first day. Treatment was started after 24 h of EAC inoculation and continued for 5 days. Groups 1–3 received the test compounds (DBL, DBSB, and DBRB) at the doses of 25 mg/kg, respectively per day per mouse. In each case, the volume of the test solutions injected (*i.p.*) was 0.1 mL/day per mouse. Group 4 received standard anticancer agent, bleomycin (0.3 mg/kg, *i.p.*) and was considered as positive control. Finally, the group 5 mice were treated with the vehicle (normal saline) and were considered as untreated control. The mice were sacrificed on the 6th day after transplantation and tumor cells were collected by repeated *i.p.* wash with 0.9% saline. Viable tumor cells per mouse of the treated group were compared with those of control.

The tumor growth inhibition was calculated using the following formula:

$$\% \text{ of cell growth inhibition} = \left(1 - \frac{Tw}{Cw}\right) \times 100$$

where, Tw is the mean of number of tumor cells of the treated group of mice and Cw is the mean of number of tumor cells of the control group of mice.

2.6. Statistical analysis

All analyses were carried out in triplicates. Data were presented as mean ± SD. Free R-software version 2.15.1 (http://www.r-project.org/) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

3. Results

3.1. Determination of TAC and ferrous reducing antioxidant capacity

The TAC and ferrous reducing antioxidant capacity of ethanolic extracts of different parts (DBL, DBRB, DBSB) of *D. blancoi* are shown in Table 1. From the table it was shown that at a concentration of 25 μg/mL, the absorbance of DBL, DBRB, DBSB and CA was in the range of (0.204 ± 0.007)–(0.383 ± 0.006); whereas at 50 μg/mL, the absorbance of DBL, DBRB, DBSB and CA was in the range of (0.396 ± 0.012)–(0.719 ± 0.010). Hence, the extracts showed significant ($P < 0.01$) antioxidant activity when compared with standard CA. The extracts were found to increase the total antioxidant capacity with the increasing concentration of the extracts.

Table 1
Absorbance of TAC and ferrous reducing antioxidant capacity of different parts (DBL, BDRB and DBSB) of *D. blancoi* at two different concentrations.

Extract	TAC		Ferrous reducing antioxidant capacity	
	25 μg/mL	50 μg/mL	25 μg/mL	50 μg/mL
DBL	0.204 ± 0.007	0.396 ± 0.012**	1.051 ± 0.010	2.233 ± 0.032*
DBRB	0.214 ± 0.008	0.422 ± 0.018**	1.132 ± 0.027	2.286 ± 0.082**
DBSB	0.229 ± 0.012	0.468 ± 0.026**	1.834 ± 0.033	3.376 ± 0.032**
CA	0.383 ± 0.006	0.719 ± 0.010	–	–
AA	–	–	1.580 ± 0.060	1.820 ± 0.060

Data are represented as mean ± SD (n = 3). *: $P < 0.05$ and **: $P < 0.01$ when compared with standards.

The extracts of *D. blancoi* showed significant ($P < 0.05$) ferrous reducing antioxidant capacity when compared to standard AA. At 25 μg/mL, the absorbance of DBL, DBRB, DBSB and AA was in the range of (1.051 ± 0.010)–(1.834 ± 0.033); in contrast at 50 μg/mL, the absorbance of DBL, DBRB, DBSB

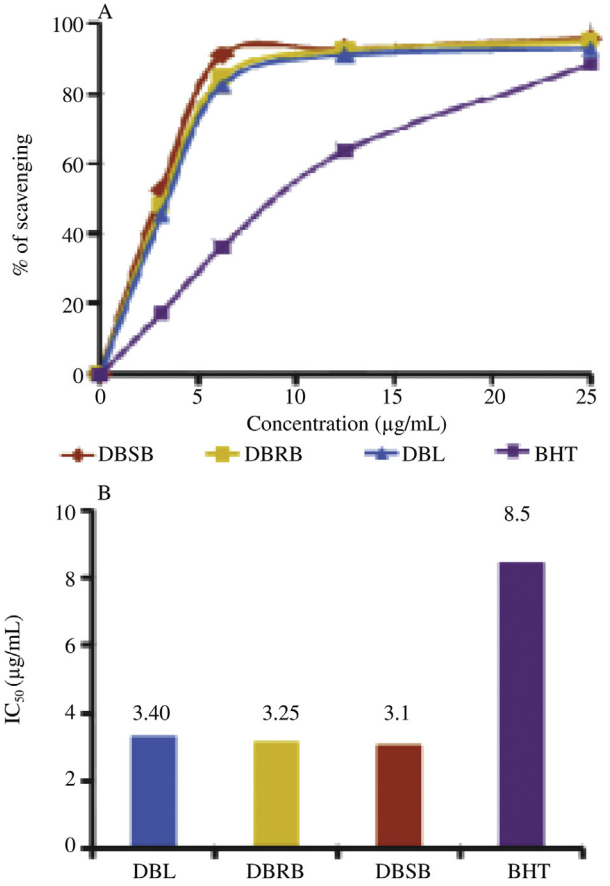

Figure 1. Determination of DPPH radical scavenging activity and IC_{50} of ethanolic extracts (DBL, DBRB and DBSB) of *D. blancoi*.
A: DPPH radical scavenging activity; B: IC_{50} values. Data are expressed as mean ± SD (n = 3) for all tested dosages.

and AA was in the range of (1.820 ± 0.060)–(3.376 ± 0.032). A higher absorbance indicates a higher reducing power. These results demonstrated that the ethanolic extracts of DBSB were found to possess the highest TAC and ferrous reducing antioxidant capacity among the extracts even though the DBSB showed higher ferrous reducing antioxidant capacity than that of standard AA (Table 1).

3.2. DPPH radical scavenging activity

Figure 1A shows the free radical scavenging activity of DBL, DBRB and DBSB and standard BHT. Among the extracts, the DBSB was found to possess the highest activity. At a concentration of 25 µg/mL, the scavenging activity of DBL, DBRB and DBSB was (93.050 ± 1.003)%, (95.430 ± 0.020)% and (95.760 ± 0.343)%, respectively; whereas at the same concentration, the standard BHT was (88.510 ± 3.277)% (Figure 1A). The IC_{50} of DBL, DBRB and DBSB was found to be (3.40 ± 0.08), (3.25 ± 0.35) and (3.10 ± 0.06) µg/mL, respectively; in contrast the IC_{50} of BHT (standard) was (8.50 ± 0.25) µg/mL (Figure 1B). Higher the IC_{50} means lower the radical scavenging activity. The free radical scavenging activity of different extracts and BHT were in the following order: DBSB > DBRB > DBL > BHT.

3.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of DBL, DBRB and DBSB was dose dependent. Among the extracts, DBSB had higher activity than that of the other extracts. At a concentration of 100 µg/mL, the scavenging activity of DBL, DBRB and DBSB was found to be (58.930 ± 1.394)%, (63.120 ± 1.818)% and (67.460 ± 2.641)%, respectively; whereas at the same concentration, the standard CA was (63.590 ± 0.231)% (Figure 2A). The hydroxyl radical scavenging activity of DBSB was higher than not only the other extracts but also the standard CA. The IC_{50} of DBL, DBRB, DBSB and CA was (74.00 ± 2.39), (65.80 ± 1.86), (50.00 ± 1.17) and (75.00 ± 0.14) µg/mL, respectively, demonstrating that the inhibitory activity of DBSB was higher than DBL, DBRB and standard CA (Figure 2B).

3.4. Lipid peroxidation inhibition assay

The lipid peroxidation inhibition activity of DBL, DBRB and DBSB was compared with CA. At a concentration of 100 µg/mL, the inhibitory activity of DBL, DBRB and DBSB was (73.24 ± 2.65)%, (60.33 ± 1.85)% and (75.59 ± 1.24)%, respectively; whereas that of the CA was (80.64 ± 2.44)% (Figure 3A). The IC_{50} of DBL, DBRB and DBSB and CA was (63.80 ± 0.19), (46.00 ± 0.77), (36.00 ± 0.41) and (54.00 ± 0.37) µg/mL, respectively (Figure 3B). The DBSB had the highest inhibitory activity among the extracts as well as standard CA.

3.5. Brine shrimp lethality bioassay

Figure 4B showed the effect of DBL, DBRB and DBSB of *D. blancoi* at different concentrations (12.5–150 µg/mL) on the

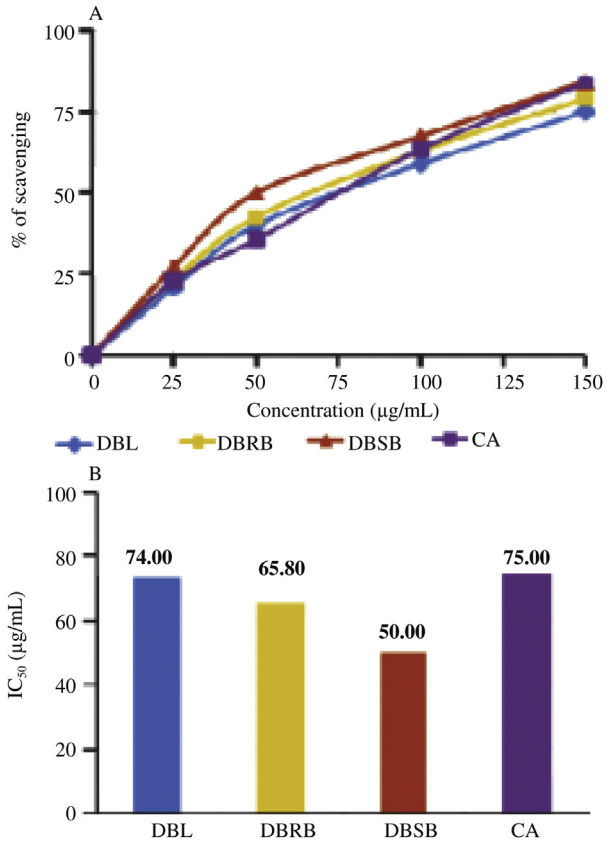

Figure 2. Determination of hydroxyl radical scavenging activity and IC_{50} of ethanolic extracts (DBL, DBRB and DBSB) of *D. blancoi*.
A: Hydroxyl radical scavenging activity; B: IC_{50} values. Data are expressed as mean ± SD (n = 3) for all tested dosages.

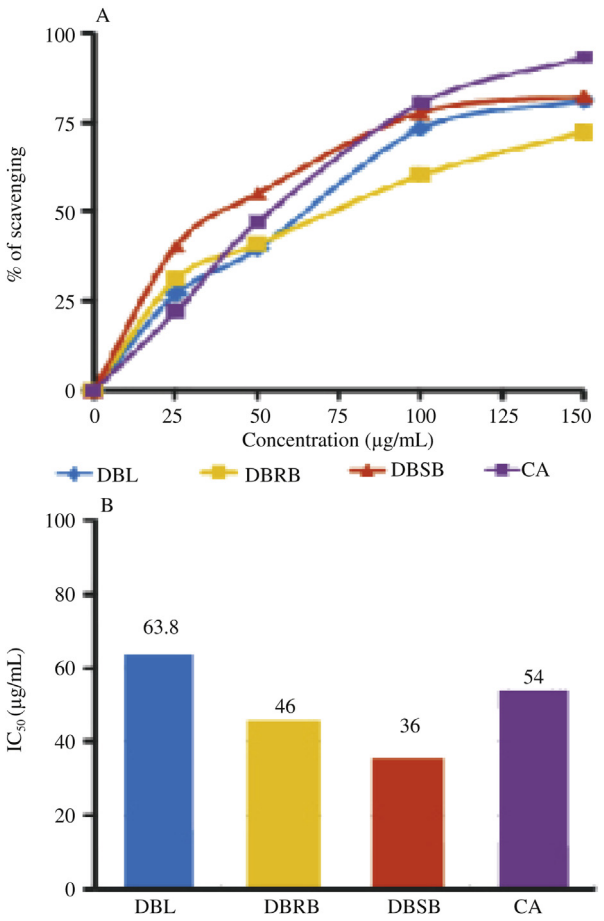

Figure 3. Determination of lipid peroxidation inhibition activity and IC_{50} of ethanolic extracts (DBL, DBRB and DBSB) of *D. blancoi*.
A: Lipid peroxidation inhibition activity; B: IC_{50} values. Data expressed as mean ± SD (n = 3) for all tested dosages.

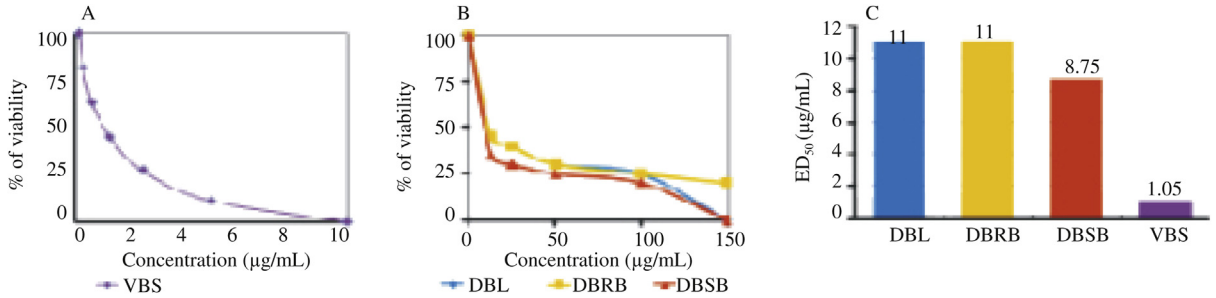

Figure 4. Determination of cytotoxic effect and ED_{50} of VBS (standard) and ethanolic extracts of DBL, DBRB and DBSB of *D. blancoi* on the viability of brine shrimp nauplii.
A: Cytotoxic effect of VBS (standard); B: Cytotoxic effect of ethanolic extracts of DBL, DBRB and DBSB of *D. blancoi*; C: ED_{50} values. Data expressed as mean ± SD (n = 3) for all tested dosages.

brine shrimp nauplii. The ED_{50} of the tested samples was calculated using the concentration versus % of nauplii viability curve of the samples (Figure 4C). The lower ED_{50} means higher toxicity. Among the extracts, the DBSB showed most potent activity with ED_{50} value of (8.75 ± 0.70) µg/mL. The ED_{50} of DBL and DBRB was equally similar to (11.00 ± 0.67) µg/mL; whereas the control VBS showed viability of nauplii when the concentration was lowered gradually from 10 µg/mL (zero viability) to 0.16 µg/mL (100% viability) (Figure 4A). The ED_{50} of VBS was found to be 1.05 µg/mL. Our results demonstrated that all the extracts of *D. blancoi* had significant cytotoxic activity.

3.6. Total phenolic and flavonoid contents

Table 2 shows the total polyphenolic contents of DBL, DBRB and DBSB expressed as GAE and CAE. The DBSB showed the highest phenolic (139.910 ± 3.924 mg GAE/g) and flavonoid (412.000 ± 16.700 mg CAE/g) contents among the extracts.

Table 2
Polyphenol contents of the ethanolic extracts of DBL, DBRB and DBSB.

Extracts	Phenolics (GAE/g of dry extract)	Flavonoids (CAE/g of dry extract)
DBL	135.850 ± 1.503	268.000 ± 6.557
DBRB	132.700 ± 3.784	382.000 ± 12.097
DBSB	139.910 ± 3.924*	412.000 ± 16.700*

Data are represented as mean ± SD (n = 3). *: $P < 0.05$ when phenolic and flavonoid contents of DBSB compared with DBL and DBRB.

3.7. Effect of antioxidant on EAC-induced tumor cells

The anticancer activity of the extracts (DBL, DBRB and DBSB) was performed on EAC cells-induced tumor bearing mice and was compared with standard anticancer agent, bleomycin. The significant tumor cell growth inhibition was observed after treatment with the extracts at the dose of 25.0 mg/kg (*i.p.*) on day six of tumor inoculation. The tumor growth inhibition was (63.70 ± 0.47)%, (82.41 ± 0.66)%, (81.04 ± 0.27)% for DBL, DBSB and DBRB, respectively. On the other hand, standard bleomycin showed the tumor growth inhibition by (82.78 ± 0.75)% at the dose of 0.3 mg/kg *i.p.* (Table 3).

Table 3
Effect of DBL, DBRB and DBSB on EAC tumor growth inhibition in mice, *in vivo*.

Group	Dose (mg/kg/day, *i.p.*)	No. of EAC cells in mice on Day 6 after tumor cell inoculation	% Of tumor growth inhibition
Control (EAC cell bearing mice)	–	(3.60 ± 0.100) × 10^7	–
Bleomycin (standard)	0.3	(0.62 ± 0.050) × 10^{7***}	82.78 ± 0.75
DBL	25	(1.31 ± 0.210) × 10^{7*}	63.70 ± 0.47
DBRB	25	(1.525 ± 0.250) × 10^{7**}	81.04 ± 0.27
DBSB	25	(1.415 ± 0.050) × 10^{7**}	82.41 ± 0.66

Number of mice in each case was six. Results were shown as mean ± SD. *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$ when compared with EAC bearing control mice.

4. Discussion

Polyphenols are the most abundant as well as important class of compounds in the plant kingdom due to their antioxidant nature and various diseases curing ability [24]. The plant, *D. blancoi*, contains significant amount of polyphenols (Table 2). Different techniques were used in order to perform rapid screening of *in vitro* antioxidant activity of *D. blancoi*. The antioxidant capacity of *D. blancoi* was estimated from the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acidic pH medium. The total antioxidant ability of the extracts was in the range of (0.396 ± 0.012)–(0.468 ± 0.026) µm green phosphate/Mo (V) (Table 1). Antioxidant activity was increased proportionally with the increase of polyphenolic contents. According to recent reports, a highly positive relationship appears between total phenolic contents and antioxidant activity in many plant species [1,21].

Recent research shows that disruption of Fe^{2+} regulation can induce the production of oxyradicals, lipid peroxidation and can also be responsible for neurological disorder [25]. The iron reducing capacities were estimated from their ability to reduce the Fe^{3+} – ferricyanide complex to the ferrous due to the presence of reductants in the solution that causes donating an electron. The reducing ability of the extracts was in the range of (2.233 ± 0.032)–(3.376 ± 0.032) µm Fe (II)/g (Table 1).

Increasing absorbance indicates an increasing of reducing activity of the plant extracts. In this study, ferrous reducing antioxidant capacity was increased with the increase of phenolic contents. Our results are consistent with the data published previously [6,21,26].

The DPPH antioxidant assay is based on the decolorizing of DPPH solution by addition of free radical species or antioxidants. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability [27]. The degree of color change is proportional to the concentration and potency of the antioxidants. Radical scavenging activities are very important to prevent the deleterious role of free radical in different diseases including cancer. Our results demonstrated that the ethanolic extract of DBSB had the higher free radical scavenging activity when compared with other extracts as well as standard BHT (Figure 1). This result indicates the proton-donating ability of the extracts which serve as free radical inhibitors or scavengers and also serve as effective antioxidants. It has been reported that total phenolic and flavonoid contents are highly correlated with radical scavenging and antioxidant activity of plant parts [26]. Interestingly the DBSB had the highest phenolic and flavonoid contents, which are mainly due to presence of the highest antioxidant capacity as well as the radical scavenging activity of DBSB of *D. blancoi* [28].

Hydroxyl radicals are one of the major ROS generated by various biochemical reactions, where superoxide dismutase catalyzes and subsequently produces highly reactive hydroxyl radicals in the presence of divalent metal ions, such as iron and copper through Fenton reaction [25]. Then they attack every non-selective biopolymer molecule leading to cell or tissue injury associated with degenerative disease and contributing to carcinogenesis and mutagenesis. The mutagenic capacity is due to the direct interactions of hydroxyl radicals with DNA and therefore playing an important role in cancer formation [29]. The potential radical scavenging ability of phenolic substances might be due to the active hydrogen donor ability. The DBL, DBRB and DBSB had significant hydroxyl radical scavenging activity when compared with standard CA (Figure 2) and could be served as anticancer agent.

Over production of ROS induces membrane damage by a chain reaction known as lipid peroxidation that leads to functional abnormalities of cells [30]. The ability of the extracts to quench hydroxyl radicals might be directly related to the prevention of lipid peroxidation. In this study, rat liver homogenates was induced by ferric ion plus potassium chloride. Antioxidants can chelate and deactivate transition metals from participating in the initiation of lipid peroxidation and oxidative stress through metal catalysis reaction [31]. Significant lipid peroxidation inhibition activity of different extracts was observed and suggested that *D. blancoi* plant was effective free radical neutralizer as well as lipid peroxidation inhibitor.

Cytotoxic effect represents a wide range of pharmacological activities of the bioactive natural products. The cytotoxic property might be due to the presence of phytochemicals such as saponins, triterpenes, tannins and polyphenolic compounds in the extracts [32], and these phytochemicals in plants exhibited anti-tumorigenic effects via multiple anticancer pathways such as by interaction with key enzymes in cellular signaling pathways, cell cycle arrest, apoptosis and metastasis [33]. Gao *et al.* [34] reported that the polyphenolic compounds have a correlation between antioxidative and cytotoxic activities. All the extracts of *D. blancoi* possess cytotoxic property; especially DBSB showed (Figure 4B, C) the remarkable cytotoxic property than that of other extracts. This result suggests that *D. blancoi* shows potent cytotoxic activity due to the presence of bioactive compounds that might have anti-tumor effect.

Indian and Chinese medicinal plants are well known to the researchers due to their traditional healing activities including cancer [35]. In humans, the most common form of free radicals is oxygen. When an oxygen molecule (O_2) becomes electrically charged or radicalized, it tries to steal electrons from other molecules causing damage to the DNA, lipid, protein and other molecules. Over time such damage may become irreversible and leads to diseases including cancer. Antioxidants are often described as “mopping up” free radicals. They neutralize the electrical charge and prevent the free radical from taking electrons from other molecules thereby prevent cancer. Several laboratory evidences from chemical, cell culture and animal studies indicate that antioxidants may slow or possibly prevent the development of cancer [36]. The anticancer activity of antioxidant rich fractions especially DBSB on EAC cells-induced tumor bearing mice showed significant tumor growth inhibition which was closely resemble to that of standard anticancer agent, bleomycin (Table 3); hence DBSB might be a good source for isolating anticancer agent.

In conclusion, our results indicate that *D. blancoi* stem bark had the highest antioxidant and significant free radical scavenging properties. The plant also showed moderate anticancer activity. Here, the finding from the research indicated that the potential content of phenolic compounds might be responsible for significant scavenging properties of free radicals as well as anticancer activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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