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Pharmacological and Antioxidant Activities of Leaves of Ethanol Extract of *Diospyros blancoi*

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Abstract: The Ethanol extract of leaves of *Diospyros blancoi* (Ebenaceae) was tested for its pharmacological and antioxidant activities. Acetic acid induced writhing method, Brine shrimp lethality bioassay and disc diffusion method were applied for determining analgesic, cytotoxic and antibacterial activity, respectively. To investigate antioxidant activity, 2, 2-diphenyl-1-picryl-hydrazil (DPPH) free radical scavenging, total phenolic and flavonoid contents were applied. The extract produced dose-dependent analgesic activity in acetic acid-induced writhing test. Significant antibacterial and cytotoxic activity were also observed. In DPPH radical scavenging assay, the extract showed the IC_{50} value of 59 µg/mL (Ascorbic acid, 17 µg/mL). Total phenolic and flavonoids contents were 62.54 mg GAE and 9.63 mg QE/g of dry powder, respectively. In HPLC analysis, Catechin hydrate, Rutin hydrate, Vanillic acid, *p*-coumaric acid, and Quercetin were detected in the extract (39.51, 20.67, 1.68, 1.52, 1.08 mg/100 g of dry extract, respectively). Presence of polyphenolic compounds in the extract might have attributed towards the observed bio-activity.

Keywords: *Pharmacological activity; Antioxidant activity; Diospyros blancoi; Phenolic compounds; HPLC*

I. Introduction

Plants have been used as medicines since ancient civilization and are still playing a significant role in health care for human beings all around the world. Since Bangladesh is a country of low economic growth, scientific exploration and standardization of potential crude drugs is an urgent need to revolutionize our drug sector. Diverse bioactive metabolites like steroids, terpenoids, flavonoids, alkaloids, glycosides, etc. in plants have formed the therapeutic basis of herbal medication. Thus, emphasis is given on the biological screening of medicinal plants for further exploration of their active constituents. Extensive studies have been conducted on plant extracts based on traditional uses to find out therapeutically active constituents [1].

Diospyros blancoi, locally named as Bilati gub belongs to the family of Ebenaceae. Traditionally, *D. blancoi* is used as a good source of vitamins A, C, and minerals. Fruit is edible, the tannin content declining as it ripens. Barks and leaves are used as pain killer. These are also effective for snake bites and itchy skin ailments. Seeds oil and barks are used for diarrhea and dysentery. In Southeast Asia, juice of unripe fruit is used for wounds. [1]. Present study was conducted to evaluate selected pharmacological activity (Analgesic, cytotoxic and antibacterial activity) and rationalize the traditional uses of *D. blancoi*.

II. Experimental Section

II.1. Chemicals and reagents

Diclofenac-Na, 2, 2-diphenyl-1-picryl-hydrazil (DPPH), Ascorbic acid, Sodium dihydrogen phosphate, Disodium hydrogen phosphate, Potassium ferric cyanide, Trichloroacetic acid, Ferric chloride, Kanamycin discs and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetic acid, acetonitrile, methanol and ethanol were obtained from Merk (Darmstadt, Germany). Kanamycin and Vincristine sulfate were purchased from Incepta Pharmaceuticals Ltd., Bangladesh.

II.2. Microorganisms

Nine pathogenic bacterial strains (five Gram positive bacterial strains namely *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus pyogens*, *Streptococcus agalactiae*, *Enterococcus faecalis* and four Gram negative bacterial strains namely *Shigella flexneri*, *Shigella sonnei*, *Shigella dysenteriae*, *Escherichia coli*) were used in antibacterial activity test. The experiment was conducted in Microbiology Laboratory of Pharmacy Discipline, Khulna University.

II.3. Experimental animals

Swiss albino mice of both sexes (20-29 g body weight) were obtained from animal resources branch of the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and were used for the experiments. The animals were kept at animal house (Pharmacy Discipline, Khulna University, Khulna) for adaptation under standard laboratory conditions (relative humidity 55-65%, room temperature 25 ± 2 ^{0}C and 12 hour light: dark cycle) and fed with standard diets (International Centre for Diarrhoeal Disease Research, Bangladesh, ICDDR, B formulated) for period of 14 days prior to perform the experiments. The selected animals were treated under the ethical guidelines for animal experimentation authorized by Pharmacy Discipline, Khulna University.

II.4. Plant material and extraction

The leaves of *Diospyros blancoi* were collected from Magura, Bangladesh in August, 2013 and were identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka (Accession Number: DACB 4698). A voucher specimen has been submitted there for reference in future. The plants were washed by fresh water, cutting into small pieces with grinding machine, and shed dried for two weeks. The dried leaves were grounded into fine powdered form. The plant extract was made by cold extraction method by taking 200 mg powders in 700 mL ethanol (95.6%) in a glass container for 14 days [2]. The extract was separated from the plant debris by filtration using cotton plague and dried using rotary vacuum evaporator (Bibby RF200, Sterilin Ltd., UK) at 45ºC. The amount of yield was 7.31% and extract was stored in a refrigerator at 4ºC.

II.5. Antibacterial Activity

Antibacterial activity test was determined by disc diffusion method [3, 4]. Both gram positive and gram-negative bacterial strains were taken for the test. Nutrient agar media was used as culture medium and it was prepared by adding water to a dehydrated product that contains all the ingredients. Sample at 200, 250 and 500 µg/disc, Kanamycin at 30 µg/disc and ethanol at 10 µL/disc as negative control were applied. The growth media was prepared in aseptic condition and poured into petridishes. After proper incubation, the antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition in term of millimeter with calibrated digital slide calipers.

II.6. Brine Shrimp Lethality Bioassay (Cytotoxic activity)

Cytotoxic activity test was carried out according to the Meyer method [5]. For *Artemia salina* leach (brine shrimp eggs) was used as the test organism. The eggs of the brine shrimp were collected from

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an aquarium shop (Khulna, Bangladesh) and hatched in artificial seawater (3.8 % NaCl solution) for 48 hours to get mature shrimp called nauplii. Vincristine sulphate was used as positive control and the percent (%) of lethality of the brine shrimp nauplii was calculated for different concentrations of vincristine sulphate and LC_{50} was calculated.

II.7. Analgesic Activity

Analgesic activity of the sample was evaluated using the model of acetic acid induced writhing in swiss-albino mice [6]. The mice were randomly divided into groups each containing five mice. The first group, treated as control group, was administered orally with 1% (V/V) Tween-80 in distilled water at the dose of 10 mL/kg body weight. The second group was treated with standard Diclofenac sodium (25 mg/kg). Third and fourth groups were treated with the sample at the doses of 250 and 500 mg/kg body weight, respectively. Control, standard drug and samples were administered orally 30 minutes before intraperitoneal administration of 0.7% of acetic acid. At 5 minutes interval, the number of writhing was counted for a period of 15 minutes. The following formula was applied to calculate the percent inhibition of writhing of sample:

$$\% \text{ Inhibition of writhing} = (1\text{-}W_0/W_1) \times 100$$

Where, W_1 and W_0 represent the mean writhing of the control and standard or sample groups, respectively.

II.8. DPPH radical scavenging assay

The radical scavenging activity of the sample was quantitatively determined on the basis of its ability to scavenge the stable free radical 2, 2-diphenyl-1-picryl-hydrazil (DPPH) [7]. Ascorbic acid, a well-known natural antioxidant was taken as standard. The percentage of DPPH free radical scavenging activity of sample and standard drug was calculated by the following formula:

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

Where, A_0 and A are the absorbance of control solution containing all reagents except sample and standard or sample, respectively.

Finally, the concentration of sample required to scavenge 50% DPPH free radical (IC_{50}) was calculated from the plot of inhibition (%) against the concentration of sample.

II.9. Total phenolic content

The total phenolic content of the extract was determined by the modified Folin- Ciocalteu's method [8]. Ethanol solution of sample was mixed with 5 mL of 10% Folin- Ciocalteu reagent. Then an aliquot of 4 mL sodium carbonate (75 g/L) was added and kept it at 40ºC for 30 min. Absorbance of the reaction mixture was measured at 756 nm. Different concentrations (0.1-0.5 mg/mL) of gallic acid were taken for the preparation of standard calibration curve where total phenolic content was determined and expressed as mg gallic acid equivalent (GAE) per g of dry extract.

II.10. Total flavonoids content

The total flavonoids content of the extract was determined according to Aluminum trichloride colorimetric method [9]. In the sample solution, 4 mL distilled water was added followed by 0.3 mL 5% w/v aluminum chloride was added. At the sixth min, 2 mL of 1 M sodium hydroxide was added and the volume was adjusted up to 10 mL. Then absorbance was determined at 510 nm against blank solution. Quercetin at different concentrations (0.1-0.5 mg/mL) was used to prepare standard calibration curve and total flavonoids content of sample was expressed in terms of mg quercetin equivalent (QE) per g of dry extract.

II.11. HPLC profiling

HPLC analysis was carried out for the detection and quantification of phenolic compounds in the extract using DionexUltiMate 3000 system equipped with quaternary rapid separation pump (LPG-

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3400RS) and photodiode array detector (DAD-3000RS). Separation was done using C18 (5 µm) Dionex column (4.6 × 250 mm) at 30ºC with a flow rate of 1 mL/min and an injection volume of 20 µL. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30 min, 100%A. There was a 5 min post run at initial conditions for equilibration of the column. Standard stock solution was prepared in methanol containing gallic acid, (+)-catechin hydrate, vanillic acid, caffeic acid, (-)-epicatechin, *p*-coumaric acid, rutin hydrate, ellagic acid, myricetin, quercetin and kaempferol. A solution of sample was made in methanol having the concentration of 10 mg/mL. All the solutions were filtered through 0.2 µm syringe filter and degassed in an ultrasonic bath prior to HPLC analysis [10].

II.12. Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 5 statistical package (GraphPad Software, 2014, USA). The data were analyzed by one way analysis of variance (ANOVA). All the results were expressed as mean ± SE for triplicate determinations.

III. Results and Discussion

Plants, the molecular architect still offers a great potentiality for drug search. The plants contain several non-nutritive chemicals known as phytochemical constituents. Among these constituents phenolic compounds, flavonoids, tannins and alkaloids are the most valuable for therapeutic activity. So, identification of the nature of the compounds present in extracts is essential to evaluate the biological activity of the extract. It is already reported that the polyphenolic compounds like phenolic acids, flavonoids and tannins, commonly found in different plants and exert multiple biological response, including antioxidant, antibacterial, cytotoxic, analgesic activity [11, 12]. So, the result of the present study can be correlated with the previous study. Phenolic compounds, secondary plant metabolites abundantly found in both edible and non-edible plants possess biological properties of antioxidant, anti-apoptosis, anti-aging, anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, cardiovascular protection, improvement of the endothelial function, as well as inhibition of oxidative damage of DNA [13], angiogenesis and cell proliferation activity [14].

III.1. Antibacterial activity

Disc diffusion technique is widely acceptable, most popular, inexpensive and easy method for the preliminary screening of antibacterial activity. The extract showed accountable antibacterial activity against the bacterial strains *E. coli* and *S. dysenteriae* at the doses of 200, 250 and 500 µg per disc. Kanamycin (30 µg/ disc) antibiotic discs were used as positive control to ensure the activity of standard antibiotic against the test organisms (Table 1). Tannins, terpenoids, alkaloids and flavonoids are the phytochemical groups that showed the antibacterial properties [15, 16].

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Table 1. *In vitro Antibacterial Activity of D. blancoi extract*

Bacterial Strains	Diameter of Zone of Inhibition in mm ± S.E.			
	Extract (200 µg /disc)	Extract (250 µg /disc)	Extract (500 µg /disc)	Kanamycin (30 µg/disc)
Staphylococcus pyogens	5.1 ± 0.84	7.0 ± 0.92	11.2 ± 0.89	27.0 ± 0.12
Staphylococcus aureus	4.3 ± 0.76	6.6 ± 0.42	10.1 ± 0.91	28.0 ± 0.21
Staphylococcus epidermidis	5.3 ± 0.42	9.2 ± 0.42	13.0 ± 0.42	28.3 ± 0.12
Shigella dysenteriae	3.8 ± 0.78	9.6 ± 0.83	14.9 ± 0.88	25.6 ± 0.22
Shigella sonnei	3.1 ± 0.86	6.2 ± 0.78	9.4 ± 0.87	29.3 ± 0.19
Streptococcus agalactiae	4.6 ± 0.42	8.0 ± 0.62	13.2 ± 0.72	33.0 ± 0.14
Escherichia coli	5.6 ± 0.78	10.3 ± 0.85	15.3 ± 0.74	24.4 ± 0.17
Enterococcus faecalis	0	0	0	31.0 ± 0.18
Shigella flexineri	3.5 ± 0.72	6.1 ± 0.91	12.0 ± 0.84	32.3 ± 0.14

III.2. Cytotoxic activity

Brine shrimp lethality bioassay is a recent development for the assay of bioactive compounds and natural products which indicate cytotoxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, pesticidal, etc. The sample showed different mortality rate at different concentrations of the extract. The LC_{50} of brine shrimp nauplii was found to be 40 µg/mL which is comparable to standard vincristine sulphate (0.46 µg/mL) (Table 2, Fig. 1). The cytotoxic activity of plants is principally contributed by the presence of secondary metabolites like alkaloids, glycosides, steroids, tannins, terpenoids and flavonoids [17]. Phenolic compounds namely catechin hydrate, rutin hydrate, vanillic acid, *p*-coumaric acid, and quercetin may be responsible for cytotoxic potential of the sample. From the LC_{50} value, it can be said that the ethanol extract of *D. blancoi* leaves possesses significant cytotoxic activity. So, this result will help us for further research to isolate pure compound.

Table 2. *Results of brine shrimp lethality bioassay of standard drug (Vincristine sulfate)*

Concentration (µg/mL)	No. of alive shrimp (Standard)	% mortality	LC_{50} (µg/mL)
0.312	6	40	0.46
0.625	4	60	
1.25	2	80	
2.5	2	80	
5	0	100	

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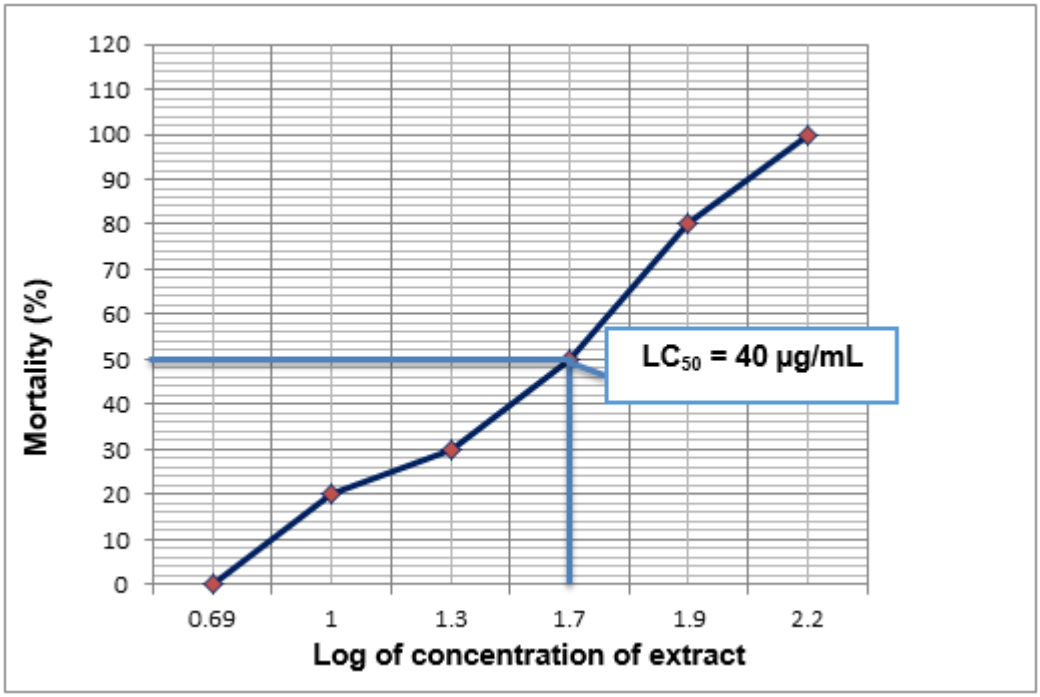

Figure 1. *Lethality test of ethanol extract of D. blancoi leaves*

III.3. Analgesic activity

Acetic acid-induced writhing model characterizes pain sensation by triggering localized inflammatory response. Acetic acid induces writhing by causing algesia by releasing endogenous substances, which then excite nerve endings [18]. Analgesic and antioxidant activities can be correlated to ameliorate pain sensation [19]. The sample showed significant ($P<0.005$) and dose dependent analgesic activity and revealed 27.28 and 51.29% writhing inhibition at the doses of 250 and 500 mg/kg body weight, respectively whereas standard Diclofenac sodium (25 mg/kg body weight) exhibited 75.76% writhing inhibition (Table 3). It is seen that ethanol extract of *D. blancoi* leaves showed promising analgesic activity. The active principles responsible for the analgesic activity of different plants are terpenoids, reducing sugar, gums, xanthoprotein, flavonoids and tannins [20, 21]. Phenolic compounds exert analgesic activity by inhibiting neutrophil degranulation and subsequently decreasing the release of local hormone arachidonic acid [22]. The sample might have exerted analgesic effect by interfering synthesis, release or antagonizing local hormones. Flavonoids as well as phenolic acids may be the active components of *D. blancoi* that narrates to its analgesic activity.

Table 3. *Effect of D. blancoi in acetic acid induced writhing in mice*

Treatment	Dose (mg/kg)	% Writhing	% Writhing inhibition
Control	-	100	0
Standard	25	24.24	75.76*
Extract	250	72.72	27.28*
Extract	500	48.71	51.29*

n = 5; *p<0.005 compared to control

III.4. DPPH radical scavenging assay

Antioxidants remove free radical intermediates from the body and responsible to protect us from some life-threatening diseases. DPPH free radical scavenging assay is a widely acceptable method to assess antioxidant properties of plant extracts [23]. In the DPPH radical scavenging assay, antioxidant activity was gradually increased with increasing concentration of the extract with the IC_{50} value of 59 µg/mL while that of ascorbic acid was 17 µg/mL (Fig. 2). DPPH based qualitative

antioxidant test indicates the presence of phytochemical compounds with free radical scavenging activity in ethanol extract of *D. blancoi* leaves.

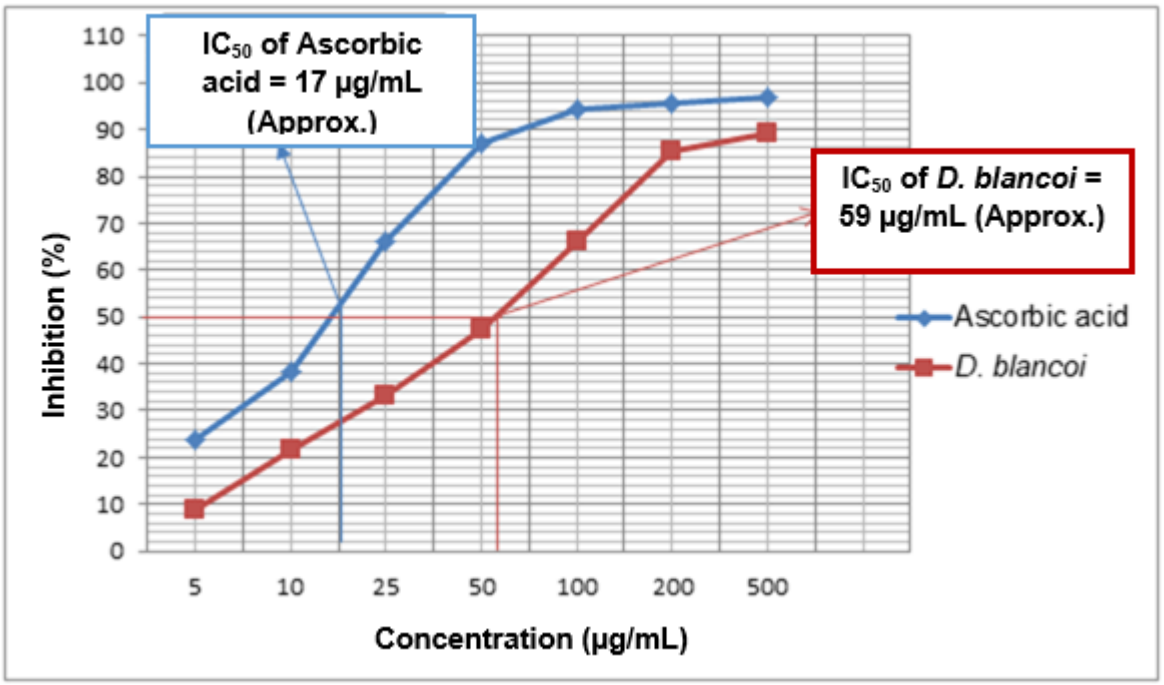

Figure 2. *DPPH scavenging assay of D. blancoi extract (% inhibition vs Conc.)*

III.5. Total phenolic and flavonoids content

In addition to DPPH test, we performed total phenolic and flavonoids content of *D. blancoi* extract. The absorbance values obtained at different concentrations of gallic acid were plotted against respective concentrations. A standard calibration curve was obtained with the equation y = 8.075 x + 0.2093 (R^2=0.9857). Total phenolic content of the extract was calculated using the equation and found to be 62.54 mg GAE/g of dry extract. Different concentrations of quercetin were used to obtain a standard calibration curve (y = 0.519 x+0.012; R^2= 0.988) from where total flavonoid content of the extract was calculated as 9.63 mg QE/g of dry extract.

III.6. HPLC analysis

The chromatographic separations of polyphenols in standard and ethanol extract are shown in Fig. 3 and 4, respectively. The content of phenolic compounds was calculated as shown in Table 4. The experimental results indicated that ethanol extract of *D. blancoi* contained a moderate concentration of catechin hydrate, and rutin hydrate (39.51 and 20.67 mg/100 g of dry extract, respectively). Vanillic acid, *p*-coumaric acid, and quercetin were also detected in low concentration (1.68, 1.52 and 1.08 mg/100 g of dry extract, respectively). The other polyphenolic compounds were not detected in the ethanol extract. The obtained phenolic compounds might be responsible for the aforementioned activities and also provide strong evidence with the traditional uses of this plant [24]. Pharmacological evaluation of the plant extract provides the strong evidence of the existence of analgesic, antibacterial and cytotoxic activities.

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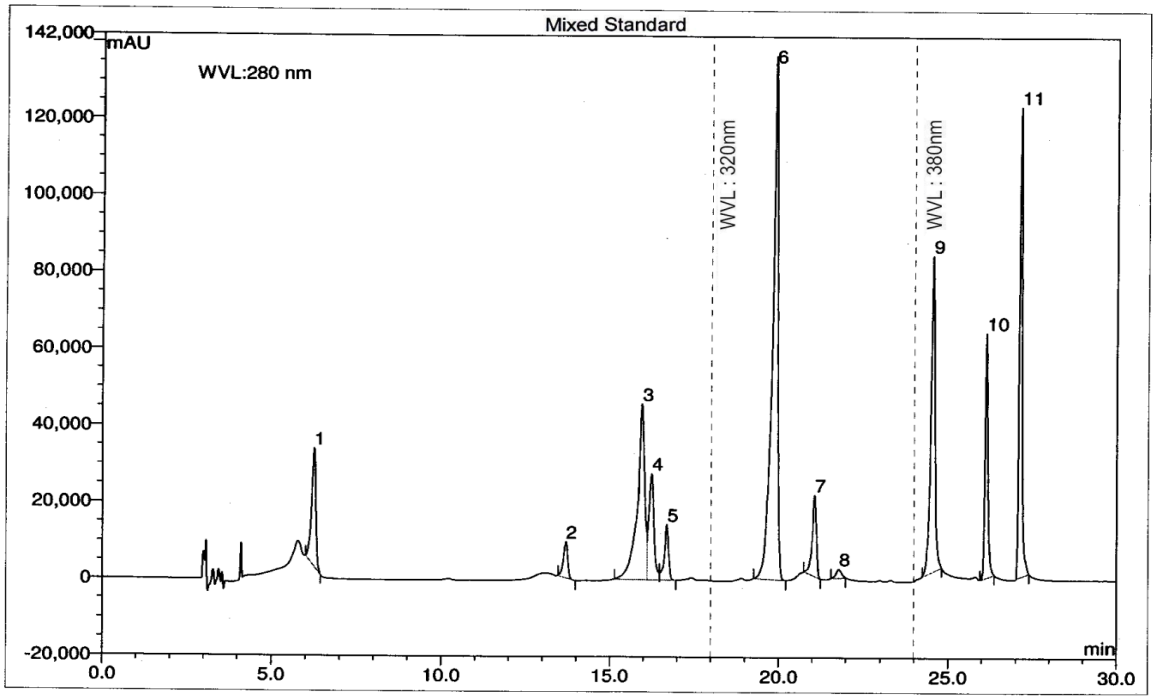

Figure 3. *HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, (+)-catechin hydrate; 3, vanillic acid; 4, caffeic acid; 5, (-)-epicatechin; 6, p-coumaric acid; 7, rutin hydrate; 8, ellagic acid; 9, myricetin; 10, quercetin; 11, kaempferol.*

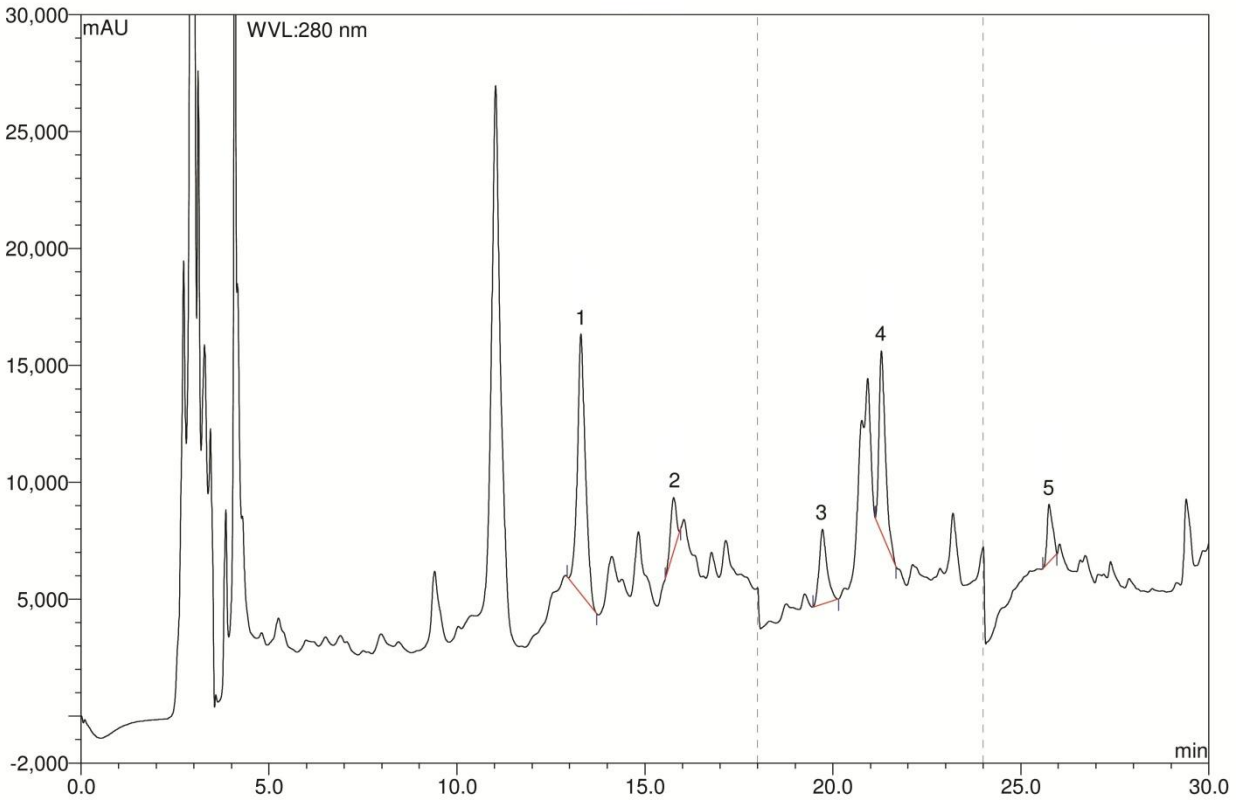

Figure 4. *HPLC chromatogram of ethanol extract of Diospyrus blancoi. Peaks: 1, (+)-catechin hydrate; 2, vanillic acid; 3, p-coumaric acid; 4, rutin hydrate; 5, quercetin.*

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Table 4. *Contents of phenolic compounds in D. blancoi extract (n=5)*

Polyphenolic compound	Ethanol extract of *Diospyrus blancoi*	
	Content (mg/100 g of dry extract)	% Relative Standard Deviation (RSD)
Catechin Hydrate (CH)	39.51	0.36
Vanillic Acid (VA)	1.68	0.05
p-Coumaric Acid (PCA)	1.52	1.04
Rutin Hydrate (RH)	10.67	0.16
Quercetin (QU)	1.08	0.02

IV. Conclusions

The present study confirmed us that the ethanol extract of *Diospyros blancoi* possesses some selected pharmacological and antioxidant activity. The antioxidant activity exhibited by the extract provides the justification for the therapeutic use of this plant in folkloric medicine due to the phytochemical constituents. The present study suggests that this extract could be of great importance for the treatment of radical related diseases and age associated diseases. Hence, further pharmacological investigation and bioactivity guided studies are suggested for isolation and purification of active principle(s) responsible for these activities. *In-vivo* studies of its medicinal active components should be carried out in order to determine their exact mechanism of action and to improve nutritional profile and health benefits as well as to prepare natural pharmaceutical products of high value.

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