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NATURAL DRUGS

ANTIOXIDANT, ANTIHEMOLYTIC AND NEPHROPROTECTIVE ACTIVITY OF AQUEOUS EXTRACT OF *DIOSPYROS LOTUS* SEEDS

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Abstract: This study was conducted to quantitatively evaluate the antioxidant, antihemolytic and nephroprotective effects of *Diospyros lotus* seeds extract in experimental *in vitro* and *in vivo* models. Antioxidant potential of *Diospyros lotus* seeds extract was examined by employing seven *in vitro* models i.e., DPPH, nitric oxide and hydrogen peroxide radicals scavenging activity, iron ion chelating, reducing power and lipid peroxidation through linoleic acid. Antihemolytic activity of extract was examined against hydrogen peroxide-induced erythrocytes hemolysis. Also, nephroprotective effect of extract against gentamicin (GM)-induced renal injury was evaluated. Renal injury was achieved by injecting 100 mg/kg, intraperitoneally (*i.p.*) of GM in normal saline. Extracts were administrated *i.p.* in doses 200 and 400 mg/kg. Blood samples were examined for serum creatinine and blood urea nitrogen after 10 consecutive days of treatment. Results show that extract showed different level of antioxidant and antihemolytic activity in the studied models. Also, results show that GM-induced nephrotoxic animal model was successfully constructed. Extract attenuated the gentamicin-induced increase in level of serum creatinine and blood urea nitrogen. The present study shows that the extract offered significant biological action compared with standard compound.

Keywords: *Diospyros lotus*, antioxidant, antihemolytic, nephroprotective, gentamicin

Renal failure is of global concern, when selecting new drug candidates during the early stage of drug development (1). Kidney is an important target of the toxicity of drugs, xenobiotics, oxidative stress and toxic materials (1). In addition, reactive oxygen species (ROS) derived from chemical materials that are exposed to renal cells appear to mediate renal necrosis, although the mechanisms of free radical toxicity are not well understood (2). Therefore, it is important to understand the role played by antioxidants e.g., phenolic compounds, such as flavonoids, phenolic acids and tannins during drug-mediated toxicity to determine if they can show protective effect against oxidative stress induced by reactive intermediates produced by various chemical materials (3). The fruits of *Diospyros lotus* L. (*Ebenaceae*) are febrifuge and used to promote secretions (4). The seed is regarded in China as a sedative (4). Chemical constituents of *D. lotus* have been published. Fatty acid compositional changes and changes in phenolic acid contents during fruit development of this plant were studied (4). To the best of our knowledge there is no report on antioxidant, antihemolytic and nephroprotective effects of *Diospyros lotus* seeds extract. In this study, these activities were examined employing various *in vitro*

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assay systems in order to understand the usefulness of this medicinal plant.

EXPERIMENTAL

Chemicals

Ferrozine, linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide and hydrogen peroxide were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Gallic acid, quercetin, butylated hydroxyanisole (BHA), vitamin C, sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck (Darmstadt, Germany). Gentamicin was purchased from Daru-pakhsh Co. (Iran). All other chemicals were of analytical grade or purer.

Sample preparation

Diospyros lotus seeds were collected from Panbeh chuleh, near the Caspian Sea, Sari, Mazandaran, Iran and identified by Department of Biology, University of Mazandaran, Iran.

Preparation of extract

The materials were oven dried at 38°C, for 5 days. Dried materials were coarsely ground (2–3 mm) before extraction. Materials were extracted by percolation method using distilled water for 24 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No. 1 filter paper, repeated three times. The resultant extracts were concentrated in a rotary evaporator until a crude solid extracts were obtained, which were then freeze-dried for complete solvent removal.

Antioxidant activity

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the samples (5). Different concentrations of sample were added, at an equal volume, to ethanolic solution of DPPH (100 mM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Assay of nitric oxide-scavenging activity

For the experiment, sodium nitroprusside (10 mM) in phosphate-buffered saline, was mixed with different concentrations of sample dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract, but with an equivalent amount of water, served as control. After the incubation period, 0.5 mL of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (5).

Metal chelating activity

The chelating of ferrous ions by the extract was estimated by the method of our recently published paper (6). Briefly, the sample (0.2–3.2 mg/mL) was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/standard. Na_2EDTA was used as positive control.

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Sample (0.1–1 mg/mL) in distilled water was added to a hydrogen peroxide solution (0.6 mL, 40 mM). The absorbance of sample at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extract and standard was calculated as follows:

$$\% \text{ scavenged } [H_2O_2] = [(A_o - A_1)/A_o] \times 100$$

where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard (6).

Determination of antioxidant activity by the FTC method

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are targets of lipid peroxidation. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. The inhibitory capacity of *Diospyros lotus* extract against oxidation of

linoleic acid by FTC method was tested. This method was adopted from Nabavi et al. (4). Twenty mg/mL of samples dissolved in 4 mL of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL), and distilled water (3.9 mL) and kept in screw-cap containers at 40°C in the dark. To 0.1 mL of this solution was then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely, 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured, and it was measured again every 24 h until the day when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as:
(%) inhibition = 100 – [(absorbance increase of the sample/absorbance increase of the control) × 100].

All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged. Vitamin C and BHA were used as positive controls.

Antihemolytic activity of extract
Preparation of rat erythrocytes

All the animal experiments were carried out with the approval of institutional ethical committee. Male rats in the body weight range of 180–220 g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with standard diet. The animals were sacrificed under anesthesia and blood was collected in heparinized tubes by heart puncture. Erythrocytes were isolated and stored according to the method described by Ebrahimzadeh et al. (6). Briefly, blood samples collected were centrifuged (1500 × g, 10 min) at 4°C, erythrocytes were separated from the plasma and were washed three times by centrifugation (1500 × g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4, PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes were stored at 4°C and used for further studies.

Antihemolytic activity of extract against H_2O_2-induced hemolysis

Antihemolytic activity of the extract was assessed as described by Alinezhad et al. (5). Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. One gram of samples/mL of saline buffer was added to 2 mL of erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation, the reaction mixture was centrifuged at 1500 rpm for 10 min and the extend of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

Animals

The study was performed on male NMRI mice of approximately the same age-group and body weight (2–3 weeks; 20–25 g), housed in ventilated animal rooms at a temperature of 24 ± 2°C with a 12 h light/dark cycle and 60 ± 5% humidity. They were fed with standard laboratory animal feed, manufactured by pasture institute, Tehran, Iran. Water was provided *ad libitum*. Experiments were performed between 10:00 and 14:00. All experiments were performed according to the norms of the ethical committee of University of Mazandaran, Babolsar, which are in accordance with the national guidelines for animal care and use.

Experimental protocol

Animals were randomly divided into three groups of 10 animals each. Group I was kept as normal control receiving isotonic saline (0.5 mL, *i.p.*) for 8 consecutive days, and animals of groups II were administered gentamicin, manufactured by Darupakhsh, Iran (100 mg/kg/day, *i.p.*) for 8 consecutive days, which is well known to produce significant nephrotoxicity in mice (7). Injections of gentamicin were made daily at 08:00 h to minimize the circadian variation in nephrotoxicity (8). Animals of Group III received extract (250 mg/kg/day, *i.p.*) for 8 consecutive days. After the last application, animals were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) given *i.p.* Blood samples were collected *via* retro-orbital puncture in plain plastic tubes, left to stand at 48°C for 1 h and centrifuged (900 × g for 15 min at 5°C) to separate serum. The serum obtained was stored at –5°C until analysis.

Biochemical analysis

Blood urea nitrogen (BUN) and creatinine (Cr) concentrations were assessed as markers of nephrotoxicity. BUN and Cr were determined spectrophotometrically (UV–Visible EZ201, Perkin Elmer spectrophotometer, USA) from serum samples using commercially available kits (Sigma).

Statistical analysis

The values are presented as the means ± SEM. Differences between group means were estimated using a one-way ANOVA followed by Duncan's multiple range test. Results were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron-donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (9). IC_{50} for DPPH radical-scavenging activity was 13.30 ± 0.50 mg/mL. The IC_{50} values for ascorbic acid, quercetin and BHA were 5.05 ± 0.10, 5.28 ± 0.22 and 53.96 ± 3.13 mg/mL, respectively. Phytochemical contents of this plant may be the reason of its good DPPH-scavenging activity (5). Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as thalassemia major (10). Common consequence of β-thalassemia is excessive iron deposition in the liver, which can cause further complications such as fibrosis (10). The usage of chelators can ameliorate the symptoms of iron overload and improve the quality of life and overall survival rate for sufferers. So many of researches focused on some natural product especially flavonoids that possess direct influence on iron(III) ions level within tissues (10). Foods are often contaminated with transition metal ions which may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions *via* Fenton reaction (5). An excess of body iron ions causes that an increased free iron ion induces free radical oxygen species as in Fenton's reaction (5):

$$Fe\ (II) + H_2O_2 \rightarrow Fe\ (III) + {\bullet}OH + OH^-$$

So, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated according to our recently published papers (5, 10). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. *Diospyros lotus* seeds extract showed potent chelating activity (IC_{50} = 42 ± 2.54 µg/mL). EDTA showed better activity (IC_{50} = 18 µg/mL). It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (6). According to results, it has been supposed that iron ion chelating activity is one of the most important mechanisms in renoprotective activity of the extract. The nitric oxide (NO) assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent (5). Scavengers of NO compete with oxygen, leading to reduced production of nitrite ions. The % inhibition was increased with increasing concentration of the extracts. *Diospyros lotus* extract showed potent NO scavenging activity (IC_{50} = 160.25 ± 6.61 µg/mL *vs*. quercetin 17.01 ± 0.03 µg/mL). Although quercetin showed very potent NO radical scavenging, but its carcinogenic activity has been reported (6). In addition to reactive oxygen species, NO is also implicated in inflammation, cancer and other pathological conditions (11). The plant/plant products may have the property to counteract the effect of NO formation and in turn, may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. Scavenging of H_2O_2 by *Diospyros lotus* extract may be attributed to their phenolics and other active components, which can donate electrons to H_2O_2, thus neutralizing it to water molecule (5). The extract was capable of scavenging H_2O_2 in a concentration dependent manner. *Diospyros lotus* showed good activity (IC_{50} was 923.51 ± 28.81 mg/mL). The IC_{50} values for ascorbic acid and BHA were 21.40 ± 1.10 and 52.00 ± 2.60 mg/mL, respectively. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are targets of lipid peroxidation (11). The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and

hydroxyl radical (10). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Figure 1 shows the time-course plots for the antioxidative activity of *Diospyros lotus* seeds using the FTC method. The peroxidation inhibition (antioxidant activity) of extract exhibited values of 92.3 (at 24th h) and of 96.1 (at 96th h). There were significant differences among antioxidative activity of extracts and two controls (vitamin C and BHA) at different incubation times ($p < 0.01$). The effect of *D. lotus* extract showed moderate antihemolytic activity (IC_{50} = 953.00 ± 27.54 µg/mL *vs.* vitamin C 235.00 ± 8.21 µg/mL). Antihemolytic activity of quercetin (15) and the relation between iron ion chelating activity and protective activity against oxidative damage to erythrocyte membrane by the flavonoids was previously reported (11).

The extract shows nephroprotective effect in the studied model. The results are shown in Table 1. Gentamicin (100 mg/kg) when injected for 8 consecutive days caused significant ($p < 0.001$) increases in serum creatinine (45.03%) and blood urea nitrogen (100.3%). The extract-treated mice (250 mg/kg/day) differed from normal control mice by an elevated concentration of serum creatinine ($p > 0.05$) and blood urea nitrogen ($p > 0.05$). The extract at 250 mg/kg/day decreased blood urea nitrogen and creatinine levels more than in normal group ($p > 0.05$). Gentamicin, aminoglycoside antibiotic, was isolated from *Micromonospora purpurea* in 1963 and, being active against *P. aeruginosa* and *Serratia marcescens*, is widely used in the treatment of life threatening infections (7). Nephrotoxicity is a major complication of the gentamicin administration. Thus, reduction of nephrotoxicity would enhance its clinical use (7). Some antioxidant agents that have been used for recovery of gentamicin-induced nephrotoxicity in rats include deferoxamine, methimazole, vitamin E, vitamin C, diethyldithiocarbamate, L-histidinol and thymoquinone (12). But none of these compounds have proved to be clinically efficient to provide complete protection in patients. Recently, interest has considerably increased in finding natural antioxidants that are able to recover cisplatin- and gentamicin-induced nephrotoxicities, to replace synthetic antioxidants, which were restricted due to their side effects such as carcinogenesis (9). In this study, we have explored the possible recovery effect of *Diospyros lotus* seeds extract on gentamicin-induced renal injury (Tab. 1). The results of the present study indicate that gentamicin at a dose of 100 mg/kg/day for 8 days administration brought about a significant increase in BUN and serum creatinine and extract in 250 mg/kg/day has therapeutic effect (Tab. 1). The effects induced by gentamicin were significantly reversed by the extract, adding further evidence that this plant has the potential to be used to recovery of gentamicin-induced nephrotoxicity. Reactive oxygen species, including hydroxyl radical, have been implicated in the etiology of gentamicin-induced nephrotoxicity (12, 13). Fenton's reaction caused oxidative stress

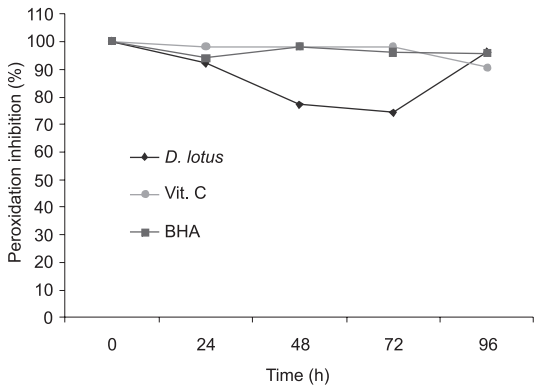

Figure 1. Antioxidant activity of aqueous extract of *Diospyros lotus* seeds in FTC method at different incubation times. *Diospyros lotus* (0.4 mg/mL), vitamin C and BHA (0.2 mg/mL).

Table 1. Effect of extract (250 mg/kg/day) on serum creatinine and blood urea nitrogen levels in gentamicin-induced nephrotoxic mice.

Groups	Serum creatinine µmol L^{-1}	Blood urea nitrogen mg dL^{-1}
Gentamicin control (100 mg/kg, *i.p.*)	34.88 ± 8.54 **	52.5 ± 5.5 **
Normal	24.05 ± 6.24	38.5 ± 3.5
Extract-treated (250 mg/kg, *i.p.*)	17.8 ± 0.88 *	25.5 ± 4.2 *

Values are the mean ± SD (n = 10). Data for normal animals are considered as base-line data; there was no significant base-line difference between the groups. * $p > 0.05$ and ** $p < 0.05$ *versus* control group.

and the formation of aminoglycoside–iron complexes that have been proposed to be the major mechanisms in the development of GM-induced acute renal failure (14). Walker and Shah (15) showed that gentamicin *in vitro* enhances the generation of hydrogen peroxide by renal cortical mitochondria and that iron chelators and hydroxyl-radical scavengers protect against gentamicin-mediated renal damage (16). In addition, previous study emphasize the important role of concomitant oxidative and nitrosative stress and the role of peroxynitrite and NO in the ensuing renal dysfunction (17), especially in GM-induced acute renal failure. Previous study showed that a scavenger of NO and peroxynitrite might be more effective than iNOS inhibitors as a therapeutic intervention (18). On the other hand, in this study we showed that the extract has good antioxidant and chelating activity (4). So, the possible mechanism of protective action of the extract may be attributed to its iron ion chelating and free radical-scavenging properties that may result from the presence of phytochemical compounds such as phenols and flavonoids. The exact mechanism by which the extract reduces gentamicin-induced nephrotoxicity remains to be elucidated.

CONCLUSION

The present study showed that the extract has antioxidant activity similar to the standard compound. Also it shows good nephroprotective activity. Further investigation of individual compounds and characterization of bioactive compounds responsible for the observed significant efficacy is needed.

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