Diospyros lotus L. fruit extract protects G6PD-deficient erythrocytes from hemolytic injury in vitro and in vivo: prevention of favism disorder

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Abstract. – *Objective:* The aim of this study was to evaluate the protective effect of *Diospyros lotus* L. fruit extract against the hemolytic damage induced by *Vicia faba* beans extract in both G6PD enzyme-deficient human and rat erythrocyte *in vitro* and *in vivo*.

Materials and Methods: In the former model, venous blood samples were obtained from five subjects with known G6PD deficiency and erythrocyte hemolysis induced by *Vicia faba* L. bean extract was asessed spectrophotometrically in the presence and absence of *Diospyros lotus* L. fruits extract. In the *in vivo* model, G6PD-deficient rats (induced by intraperitoneal injection of dehydroepiandrosterone for 35 days) pre-treated with different doses of *Diospyros lotus* L. (500, 750, 1000, and 1500 mg/kg, p.o for 7 days) were challanged with *Vicia faba* beans extract and the protective effect of the fruit extract against hemolysis was evaluated as above.

Results and Conclusions: The results have shown that *Diospyros lotus* L. fruits extract has antioxidant activity that may protect against hemolytic damage induced by *Vicia faba* bean extract in both G6PD-deficient human and rat erythrocytes. The study gives a scientific basis for the efficacy of the fruit extract as used in Iran. The fact that this was shown in human erythrocytes *in vitro* is significant and provides a rationale for further testing *in vivo* in G6PD-deficient human populations.

Key Words:

G6PD-deficient erythrocytes, Favism disorder, *Diospyros lotus* L, Antioxidant activity, Hemolytic damage.

Introduction

Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is the most common human enzymopathy in the world; a conservative estimate is that more than 400 million people are affected worldwide[1]. It is most common in people of Mediterranean descent,[2] but is also found in people in many other parts of the world[3-5]. G-6-PD deficiency is an X-linked disorder, which is characterized by susceptibility to hemolytic crisis following the ingestion of *Vicia faba L.* beans or antimalarial drugs (primaquine, quinine), antipyretics, and analgesics such as aspirin and quinones[6]. As a consequence of enzymatic alterations that take place during the hemolytic crises of favism[7], glucose-6-phosphate dehydrogenase is essential for the production of reduced nicotinamide adenine dinucleotide (NADPH), which provides a protective effects against oxidative cell damage[8]. People with G6PD deficiency show hemolytic anemia in response to oxidative stress, while newborn infants have prolonged jaundice, both of which may be potentially lethal. Even if the neonatal jaundice is not fatal, it is often serious enough to produce a permanent neurological damage in the newborn infant[9]. Many scientists have shown that G6PD-deficient erythrocytes have a higher sensitivity toward lipid peroxidation[10], and the hemolytic damage in this cell is considerably higher when they are exposed to oxidizing agents[11,12]. Because of the increased peroxidative damage to the erythrocyte membrane, several antioxidant agents such as seleni-

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um, and vitamin C and E have been tested to prevent the hemolytic crises of the G-6-PD-deficient subjects[13-15]. Among these, vitamin E (Vit E), which has strong antioxidant characteristics, and has been studied extensively both *in vivo* and *in vitro*. However, the results are controversial[16-18].

Diospyros lotus L. is indigenous to the temperate Asian forests, China and also seen in north of Iran from parts of the coast of Caspian Sea up to 1100 meter from sea level in Astara to Ramian, Gorgan in north of Iran. Juice can be obtained from the fruits; its leaf blade elliptic to ovate-oblong, 5-13 × 2.5-6 cm. The surface of the leaves is dark green, the reverse side is light, the flowers are small, and the fruits are globe, and are as big as a hazel nut. When ripe in Fall, the fruit turns brown, and it has 2-3 clear and big seeds[19]. *Diospyros lotus* L., similar to other species of Diospyros, has a high amount of naphthoquinones especially 7-methyljuglone. Many studies have shown that it has numerous biological and pharmacological properties include: including its use as an antifebrile agent, secretions, as a sedative, and for controlling cough[20]. It is commonly known as "Khormendi and Fermoni" in the Mazandaran province, north of Iran. A study conducted in Sari city, northern Iran, showed that the prevalence of G6PD deficiency was high[21]. Moreover, because northern Iran is an agricultural region, and there are vast expanses of *Vicia faba* bean fields, there is an increased risk of favism disorder. Antioxidant and other supportive therapies protect red blood cells (RBC) against oxidant damage[22,23]. A recent study in 2009 investigated the antioxidant activity of *Diospyros lotus* L. fruits extract employing six different *in vitro* assay systems. It was found that the *Diospyros lotus* L. fruits extract with a high amount of total phenolic and flavonoid contents had an antioxidant activity and a free radical scavenging[24]. The fruits of Khormendi are used traditionally to treat favism in this region though it is not scientifically proven.

The present study investigates the protective effects of *Diospyros lotus* L. fruits extract on hemolytic injury-induced fava bean G6PD deficiency in both human and rat erythrocytes.

Materials and Methods

Plant Material

Fresh matured fruits of *Diospyros lotus L.* were collected from the mountains of the Mazan-

daran province, Iran, in October-November 2007. Green and fresh *Vicia faba L.* beans were harvested from the fields in Sari City, Northern Iran. A voucher specimen of the Herbarium has been deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran.

Preparation of *Diospyros lotus L.* fruits extract

The design of the method was based on the traditional extraction that is practiced in the Mazandaran province, north of Iran. In this method, the pip was separated from the ripe fruits. 500 g of the crushed fresh fruit was extracted with water as solvent heated to boiling temperature for 3 h. Concentrated extract was put into multiple plates so that a thin layer of aqueous extract was produced. The plates containing the aqueous extract were put in an oven in order to evaporate its aqueous phase at 45°C. After drying, 36.5 g of dried powder was obtained. The dried powder was then removed from the plates and placed in a light-proof container and in a cool and dry place.

Preparation of *Vicia Faba* **L. Beans Extract**

After the collection of the *Vicia faba* L. beans from Mazandaran province, Iran, the outer crust of the beans was separated. The green beans were ground, put into a glass container, placed in an oven, and dried at 45°C. After drying, the beans were powdered. The powder was extracted by method of maceration. 250 g of powdered beans was extracted with 2500 ml of aqueous methanol (70%) while stirred for one hour. The mixture was then kept at room temperature for 48h. This process was done in triplicate. After filtration, solvent was evaporated under reduced pressure at 40°C by rotary evaporator apparatus. In this manner, 26.5 g of the powdered extract was obtained.

Chemicals

Dehydroepiandrosterone (DHEA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were purchased from Sigma Chemical Co. (Buchs, Switzerland) and Fluka Chemical Co. (Buchs, Switzerland), respectively. All other chemicals were of analytical grade and obtained from either Sigma or Merck (Darmstadt, Germany).

Measurement of Free Radical-scavenging Activity

The free radical-scavenging capacity of the Diospyros *lotus* L. fruit extract was determined as the bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH)[25,26]. Different concentrations of the Diospyros *lotus* L. fruits (0.5 to 8 mg/ml) were added, at an equal volume, to a methanol solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. IC50 values denote the concentration of the sample that is required to scavenge 50% of DPPH free radicals.

Animals

Male Wistar albino rats (n=40), 5-7 months old with a weight of 150-200 g was purchased from the Pasteur Institute of Iran (North Branch, Amol, Iran). They were well kept at the University's animal section and were given standard food pellets and water *ad libitum*. All the animals were maintained under controlled conditions of light (12h/24h) and temperature (23±1°C). Their use and the experimental protocol were approved by the Research Committee of the Mazandaran University of Medical Sciences,

Experiment Design

Human Experimental (in Vitro Study)

The experiments were conducted at the Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran. The study protocol was approved by the Research Committee of the University. This method is similar to Sharma's study with minor modifications[29]. After obtaining written informed consent, 10-ml blood samples were collected in heparinized tubes from five male subjects with known G6PD deficiency, which was confirmed using a standard assay kit purchased from Saba Lab (Tehran, Iran). The rest of the blood was centrifuged at 1500 g for 15 min at 4°C using a swinging-bucket cold centrifuge. The plasma (with Buffy coat) was carefully removed and its volume accurately determined. The sedimented cells were washed three times with a buffer and suspended in 154 mM NaCl (in 10 mM sodium phosphate buffer), pH 7.0. Initially, the volume of the added buffer was equal to the volume of plasma removed, but later more buffers were added to prepare an erythrocyte suspension. For estimation of which concentration of the *Vicia faba* bean extract could produce approximately 50% hemolysis of the G6PD-deficient erythrocytes, aliquots of this suspension were incubated with varying amounts of the *Vicia faba L.* beans extract (20, 40, 80, 160, 320, and 640 μg/ml), which is soluble in an aqueous environment, at 37°C for 2 hours. Aliquots of the suspensions were also incubated in the buffer to give control values and with distilled water to provide values for complete hemolysis. At the end of the incubation period, the samples were centrifuged at 1500 g for 15 min at 4°C and the content of hemoglobin in the supernatant estimated by a spectrophotometer (UV-VIS Spectrophotometer Shimadzu UV-160A, Shimadzu Scientific Instruments, Kyoto, Japan) by recording its optical density[27-29] at 540 nm. The percentage of hemolysis was calculated by dividing the absorbance reading from the experimental tubes by the mean value of complete hemolysis induced by distilled water multiplied[29] by 100. The background absorbance, if any, was subtracted from the absorbency values of the induced hemolysis and freshly prepared solutions were used in the all the experiments. This screening test was used to estimate which concentration of the *Vicia faba L.* bean extract produced approximately 50% hemolysis of the G6PD-deficient erythrocytes. After obtaining the concentration of the inducer 50% hemolysis for the *Vicia faba L.* beans extract, the main protocol was started. The protocol was similar to the aforementioned protocol, except for the preparation of an erythrocyte suspension. Subsequently, aliquots of this suspension were incubated with varying amounts of the *Diospyros lotus* L.fruit extract (40, 80, 160, 320, and 640 μg/ml), which is soluble in an aqueous environmental, at 37°C for 30 min and were challenged with the *Vicia faba L.* bean extract (280 μg/ml; the concentration induced approximately 50% hemolysis of the G6PD-deficient erythrocytes) at the same temperature for 2 hours. Also, in one group, Vitamin E (100 μM), which is soluble in distilled water plus one drop of tween 80 as a surfactant, was added as a positive control and another group was given only the *Vicia faba L.* bean extract (280 μg/ml). In this step, a small amount of the sample from each group was taken for the evaluation of the hematocrit through the microhematocrit method for the measurement of the volume of red blood cells. Subsequently, aliquots of suspensions were also incubated in the buffer to give control values and with distilled water for complete hemolysis. Later, different groups were used for the evaluation

of their hemoglobin content in the supernatant through a Shimadzu spectrophotometer (UV) by recording the optical density at 540 nm. In the optical density method similar to the aforementioned protocol, the percentage of hemolysis was calculated by dividing the absorbance reading from experimental tubes by the mean value of complete hemolysis induced by distilled water multiplied by 100.

Animal Models (*In vivo* Experiment)

Induction of G6PD Deficiency in Rat Erythrocyte

The G6PD deficiency was induced by an intraperitoneal (i.p.) injection dose of DHEA at dose 100 mg/kg body weight of DHEA dissolved in its solvent, which was made up of 1 vol of 95% ethanol and 9 vols of 16% Tween 80 in 0.9% NaCl for 35 consecutive days (5 weeks). After the last DHEA injection, the blood of rats fasting overnight was drawn from their tail artery, and the accuracy of the G6PD enzyme deficiency was evaluated by a standard assay kit purchased from Saba Lab (Tehran, Iran). The rats with known G6PD enzyme deficiency were selected for the next experiment.

Experimental Protocol

In the experiment, after the induction of the G6PD enzyme deficiency, a total of 40 rats were used. The rats were randomly divided into the 8 groups (groups 1-8), with 5 animals involved to the each groups:

Group 1: Normal control; received distilled water (10 ml/kg b.w.) by gavage for 7 consecutive days.
Group 2: Control G6PD deficiency; received distilled water (10 ml/kg b.w.) by gavage for 7 consecutive days after the induction of G6PD deficiency.
Group 3: Control Faba; received distilled water (10 ml/kg b.w.) by gavage for 7 consecutive days after the induction of G6PD deficiency.
Group 4: Treated with *Diospyros lotus L.* extract (500 mg/kg b.w.) in distilled water (10 ml/kg b.w.) by gavage for 7 consecutive days after the induction of G6PD deficiency.
Group 5: Treated with *Diospyros lotus L.* extract (1000 mg/kg b.w.) in distilled water (10 ml/kg b.w.) by gavage for 7 consecutive days after the induction of G6PD deficiency.

Group 6: Treated with *Diospyros lotus L.* extract (1500 mg/kg b.w.) in distilled water (10 ml/kg b.w.) by gavage for 7 consecutive days after the induction of G6PD deficiency.
Group 7: Control extract; treated with *Diospyros lotus L.* extract (1500 mg/kg b.w.) in distilled water (10 ml/kg b.w.) by gavage for 7 consecutive after the induction of G6PD deficiency.
Group 8: Treated with Vitamin E (15 iu/kg b.w., PO) for 7 consecutive days after the induction of G6PD deficiency.

Twenty-four hours from the completion of 7 days, the *Vicia faba* bean extract (40 mg/kg b.w.) in distilled water (10 ml/kg b.w.) was given orally (by gavage) to the all groups except group 1 as it was a normal control group, group 2 as it was a control G6PD deficiency group, and group 7 as it was a control extract. One hour after the *Vicia faba L.* bean extract treatment, the animals were anesthetized with petroleum ether. Blood was then removed by a cardiac puncture and hematocrit and total hemoglobin values were measured by standard methods.

Measurement of Blood Hemoglobin

Blood was collected in heparinized tubes through the cardiac puncture of the animals and assayed within 4 h. The hemoglobin level was measured using a total hemoglobin assay kit (ZistChem Diagnostics, Tehran, Iran) by following the manufacturer's protocol.

Measurement of the Hematocrit (HCT)

The standard method for measuring hematocrit involves collecting a blood sample in a capillary tube (also known as a microhematocrit tube) and centrifuging the tube at 10,000 RPM for 5 min to separate the red blood cells from the plasma. By measuring the height of the resulting layer of red blood cells in the capillary and referencing it to the total blood volume, the volume percentage of red blood cells can be quantified.

Statistical Analysis

The data are presented as means ± SD. The GraphPad InStat software was used for the statistical analysis. One-way analysis of variance (ANOVA) and Tukey's post test were used for multiple comparisons of data. IC50 values were calculated from linear regression analyses. The differences were considered significant, when $p < 0.05$.

Results

DPPH Radical-Scavenging Activity

The scavenging effect of *Diospyros lotus* fruit extract was enhanced with increasing concentration. The maximum inhibitory effects were obtained in 86.43±1.27% at a concentration of 8 mg/ml for it. The IC50 value of the extract for DPPH radical-scavenging activity was 1.7 mg/ml (Figure 1).

In Vitro **Study**

The results in Figure 2 show that the G6PD-deficient human erythrocytes undergo hemolysis when exposed to the *Vicia faba* bean extract. The degree of hemolytic injury is shown in relation to 100% hemolysis produced by distilled water and is dependent on the concentration of the hemolytic agent present in the incubating medium. The concentration that produces approximately 50% hemolysis of the G6PD-deficient erythrocytes is 280 μg/ml for the *Vicia faba L.* bean extract. This extract concentration was selected for the induction of hemolysis in subsequent studies in which G6PD-deficient erythrocytes were incubated at 37°C with or without the *Diospyros lotus L.* fruit extract. The results in Figure 3 show that erythrocytes incubated with the *Vicia faba L.* bean extract release 49.68±4.56% hemoglobin in the supernatant, which is reduced in a concentration-dependent manner in the presence of the *Diospyros lotus L.* fruit extract. A significant difference in the *Vicia faba L.* bean extract group values is observed when the *Diospyros lotus* L. fruit extract concentration in the incubating medium is increased to 320 μg/ml. The *Diospyros lotus* L. fruit extract at doses of 320 and 640 μg/ml reduced the release of hemoglobin in the supernatant at 40.34±2.81% and 33.5±3.1, respectively, when compared to the *Vicia faba L.* bean extract group ($p < 0.001$), The *Diospyros lotus L.* fruit extract at higher doses did not have any side effects on the red blood cells in the group that used this extract only.

The results in Figure 4 show that the *Vicia faba L.* bean extract group reduced the values of hematocrit from 45.4±2.43% to 25.3±2.43% ($p < 0.001$) of control group. There is a significant difference in the *Vicia faba* bean extract group values of hematocrit when the *Diospyros lotus L.* fruit extract concentration in the incubating medium is increased to 320 μg/ml. The *Diospyros lotus L.* fruit extract at doses 320 and 640 μg/ml recovered this reduction of hematocrit to 34.1±2.16% and 37.6±1.78, respectively, to the Vitamin E group ($p < 0.001$).

In Vivo **Experiment**

For the in vivo study, G6PD deficiency was induced by an intraperitoneal (IP) injection of DHEA (100 mg/kg), a specific G6PD enzyme inhibitor, dissolved in a suitable solution, into rats for 35 consecutive days. After 35 days, the accuracy of the induction of G6PD deficiency by DHEA was evaluated using a standard assay kit. All animals showed a DHEA-induced G6PD de-
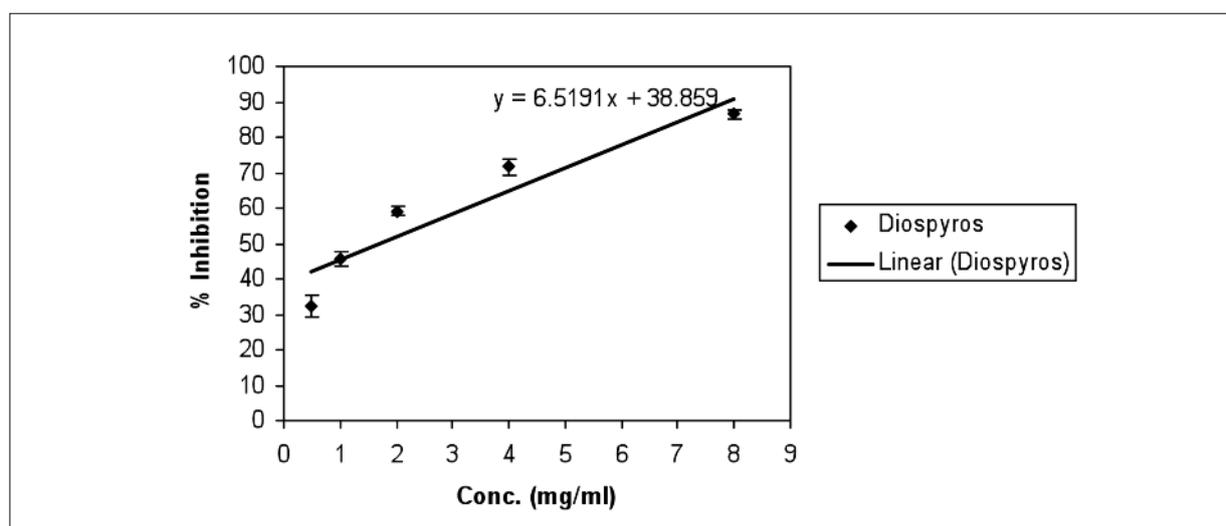

Figure 1. Scavenging effect of different concentrations of the Diospyros lotus L. fruit extract on the 1,1-diphenyl-2- picrylhydrazyl free radical at 517 nm.
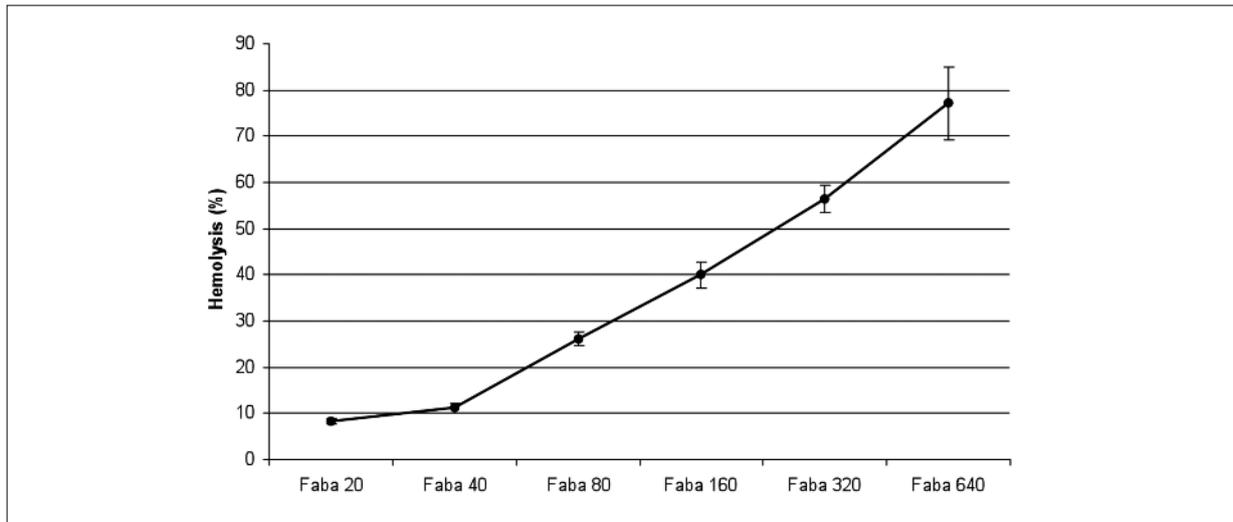
Figure 2. Hemolysis of G6PD deficient erythrocytes after 2 h incubation at 37°C with different concentrations (µg/ml) of *Vicia faba* L. beans extract. Water induced hemolysis as 100%. The data represent the mean ± S.D. of five human G6PD deficient volunteers.

ficiency in their erythrocytes, which was tested by qualitative methods. The results in Figure 5 show that the oral administration of the *Vicia faba L.* bean extract at a dose of 40 mg/kg into the G6PD enzyme-deficient rats significantly reduced the value of their hemoglobin erythrocyte compared to the normal group ($p < 0.01$). The values of the hemoglobin for the normal group and G6PD enzyme- deficient group that induced

oxidative damage by the *Vicia faba L.* bean extract were 14.3±0.96 and 9.54±0.44, respectively. As shown in Figure 5, there was a dose dependency in the recovery of the hemoglobin reduction by the *Vicia faba L.* bean extract in the G6PD enzyme-deficient rats up to a dose of 1000 mg/kg of *Diospyros lotus L.* fruit extract. But higher doses up to 1500 mg/kg did not show any dose-dependent effect and maximum protection
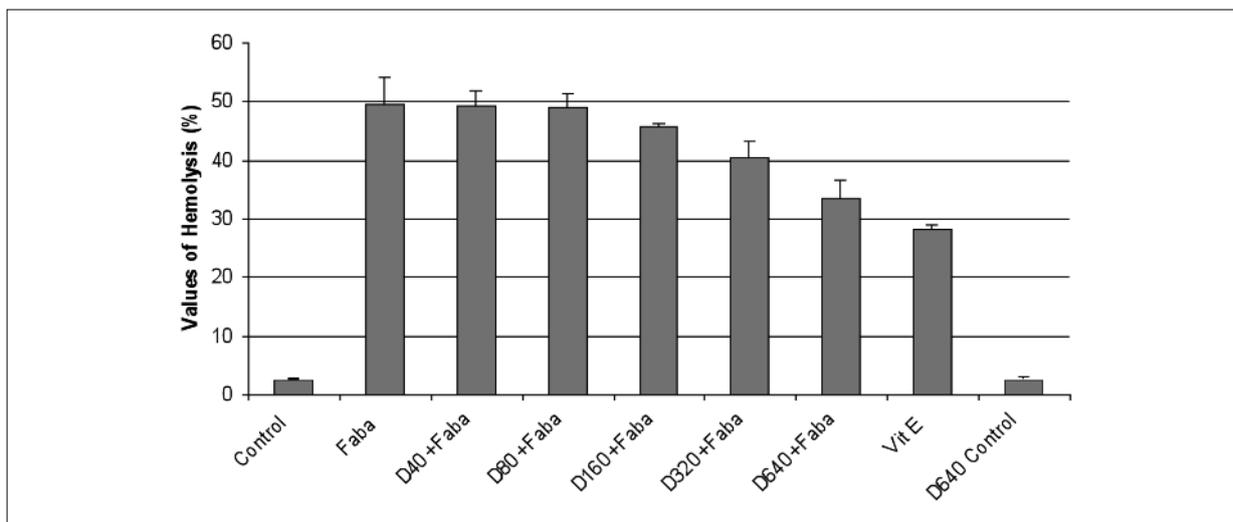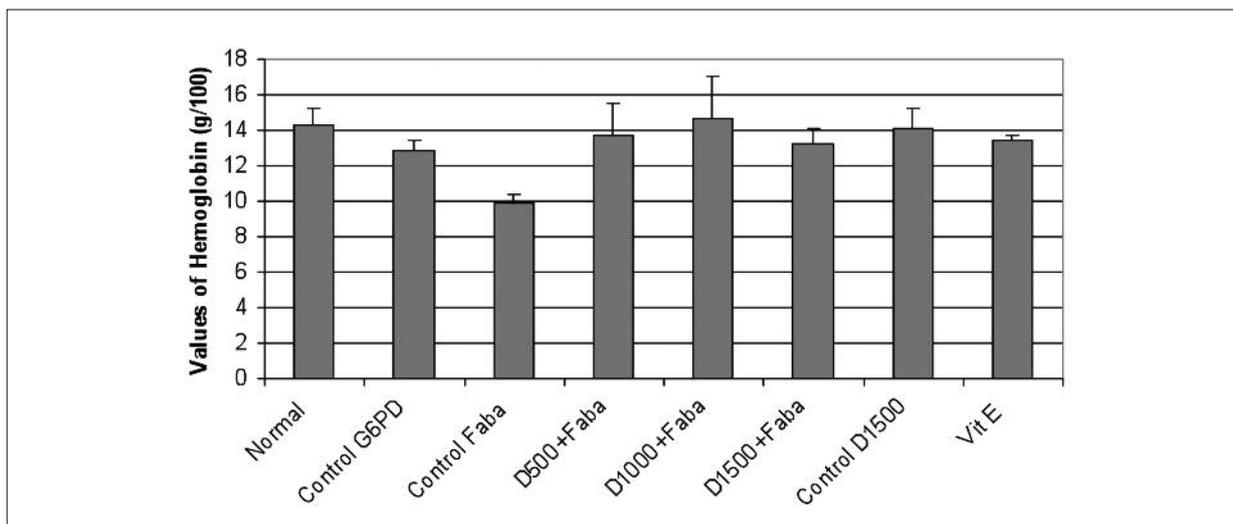

Figure 3. *In vitro* protection by *Diospyros lotus* fruits extract (D) at different concentrations (µg/ml) and Vit E (100 µg/ml) against induction of hemolysis induced by *Vicia faba* L. beans extract (Faba) at (280 µg/ml) in G6PD deficiency human erythrocyte. The data represent the mean ± S.D. of five human volunteers. *P* < 0.001: Control samples compared with similarly damaged RBC from the blood samples treated with Faba. Also *p* < 0.001: Faba samples compared to D320+Faba, D640+Faba, Vit E+Faba and D640 Control samples. *P* > 0.05: Faba samples compared to D40+Faba, D80+Faba and D160+Faba samples.
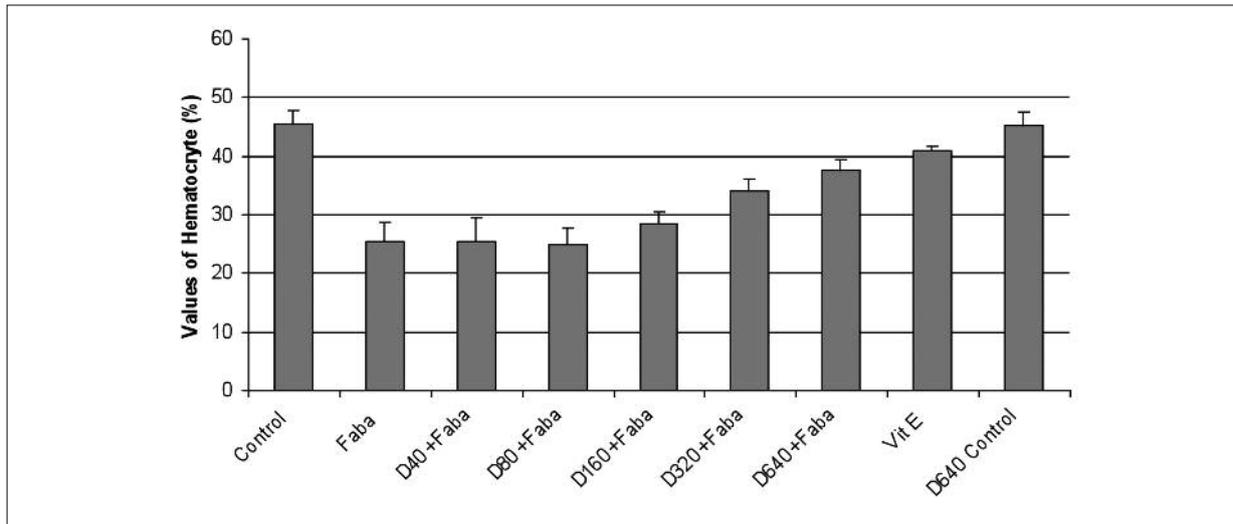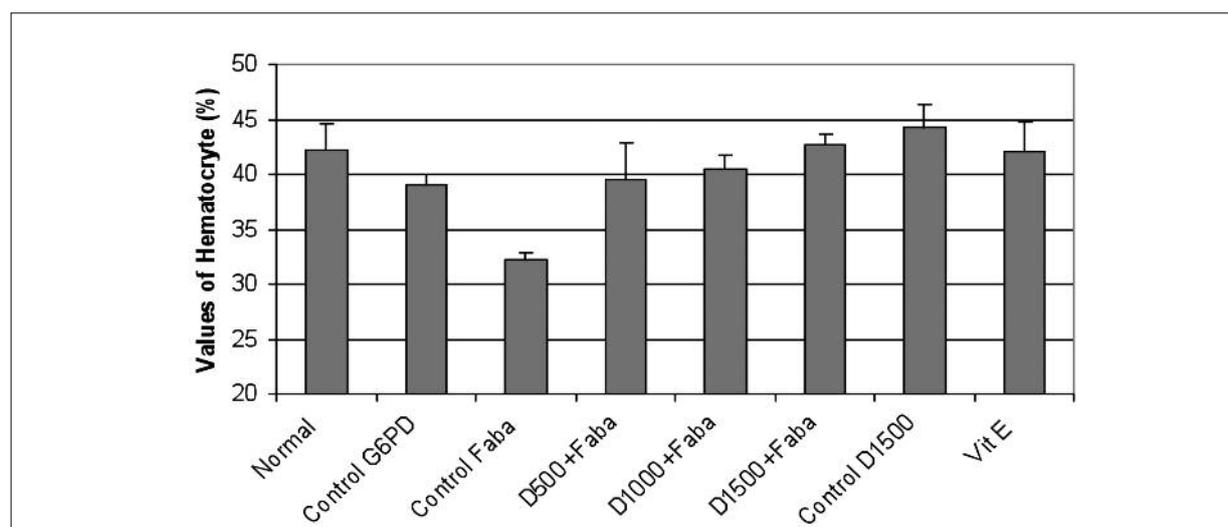
Figure 4. *In vitro* protection by *Diospyros lotus* fruits extract (D) at different concentrations (μg/ml) and Vit E (100 μg/ml) against reduction of hematocrit induced by *Vicia faba* L. beans extract (Faba) at (280 μg/ml) in G6PD deficiency human erythrocyte. The data represent the mean ± S.D. of five human volunteers. $P < 0.001$: Control samples compared with similarly damaged RBC from the blood samples treated with Faba. Also $p < 0.001$: Faba samples compared to D320+Faba, D640+Faba, Vit E+Faba and D640 Control samples. $P > 0.05$: Faba samples compared to D40+Faba, D80+Faba and D160+Faba samples.

was observed at a dose of 1000 mg/kg of *Diospyros lotus L.* that significantly raised the value of hemoglobin from 9.54±0.44 for the *Vicia faba L.* bean extract to 14.67±2.37 ($p < 0.01$). *Diospyros lotus L.* at a dose of 1000 mg/kg had a better effect than Vit E for the protection of the G6PD en- zyme-deficient rat erythrocytes against any injury induced by the *Vicia faba L.* bean extract. The results in Figure 6 also show that the oral administration of the *Vicia faba L.* bean extract at a dose of 40 mg/kg into the G6PD enzyme-deficient rats significantly reduced the value of their

Figure 5. *In vivo* protection by *Diospyros lotus* L. fruits extract (D) at different concentrations (mg/kg) and Vit E (15 iu/kg) against reduction of values of hemoglobin induced by *Vicia faba* L. beans extract (Faba) at (40 mg/kg) in G6PD deficiency rat's erythrocyte. The data represent the mean ± S.D. of five animals each group. $P < 0.01$: Normal samples compared with similarly damaged RBC from the blood samples treated with Faba. Also $p < 0.01$: Faba samples compared to D1000+Faba and Control D1500 samples. $P < 0.05$: Faba samples compared to D500+Faba and Vit E+Faba samples. $P > 0.05$: Faba samples compared to D1500+Faba samples. Also $p > 0.05$: Normal samples compared to Control G6PD samples.

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Figure 6. *In vivo* protection by *Diospyros lotus* L. fruits extract (D) at different concentrations (mg/ml) and Vit E (15 iu/kg) against reduction of values of hematocrit induced by *Vicia faba* L. beans extract (Faba) at (40 mg/ml) in G6PD deficiency rat's erythrocyte. The data represent the mean ± S.D. of five animals each group. $P < 0.001$: Normal samples compared with similarly damaged RBC from the blood samples treated with Faba. $P < 0.001$: Faba samples compared to D1000+Faba, D1500+Faba, Vit E +Faba and Control D1500 samples. $P < 0.01$: Faba samples compared to D500+Faba sample. $P > 0.05$: Faba samples compared to D1500+Faba samples. Also $p > 0.05$: Normal samples compared to Control G6PD samples.

hematocrit erythrocyte compared to the normal group ($p < 0.001$). The value of the hematocrit for the normal group and G6PD enzyme-deficient group that induced oxidative damage by the *Vicia faba* L. bean extract was 42.3±2.33% and 32.16±0.76%, respectively. As shown in Figure 6, there was a dose dependency in the recovery of the hematocrit reduction by the *Vicia faba* L. bean extract in the G6PD enzyme-deficient rats up to a dose of 1500 mg/kg of *Diospyros lotus* L. fruit extract. Maximum protection was observed at a dose of 1500 mg/kg of *Diospyros lotus* L. that significantly recovered the value of hematocrit from 32.16±0.76% for the *Vicia faba* L. bean extract to 42.7±0.97% ($p < 0.001$) to the vitamin E group with a value of 42±2.78%. The *Diospyros lotus* L. fruit extract at higher doses did not have any toxic effect on the G6PD enzyme-deficient rats' erythrocytes by oral administration.

Discussion

The results of the present study have shown that the *Diospyros lotus* L. fruit extract can effectively protect the G6PD-deficient erythrocytes against hemolytic injury induced by the *Vicia faba L.* bean extract both *in vitro* and *in vivo*. The

ability of the *Diospyros lotus L.* fruit extract for the inhibition of the DPPH radical was examined. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical-scavenging ability of various samples[35]. The 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 that are able to perform this reaction can be considered as antioxidants and, therefore, as radical scavengers[36]. It was found that the radical-scavenging activity of the *Diospyros lotus L.* fruit extract increased with increasing concentration. A 2009 study, proved that *Diospyros lotus L.* fruit extract with a high amount of total phenol and flavonoid contents and IC50 for DPPH radical-scavenging activity of 1.54±0.03 mg/ml, had a potent antioxidant activity and free radical scavenging[24]. The value of IC50 in this study was similar to the IC50 obtained in our study. Another study on the phytochemical investigation of the *Diospyros lotus L.* plant revealed the presence of some fatty acids and nonvolatile acids in the fruit[37], total phenol and flavonoid content[38], terpenes[39] and naphthoquinones[40]. In a study conducted by Said et al[41], eight phenolic compounds were isolated and identified as gallic acid, methylgallate, ellagic acid, kempferol, quercetin, myricetin, myricetin 3-O-β-glucuronide and

myricetin 3-O-α-rhamnoside. The phenolic compounds from the *Diospyros lotus* in this study were isolated for the first time from the genus *Diospyros*. It seems phenol and flavonoid, especially quercetin, myricetin, and also naphthoquinones, particularly 7-methyljuglone, are responsible for antioxidant activity; moreover, methyl phenol rings existed in the chemical structure of 7-methyljuglone may be a good hydrogen donor to the DPPH radical.

This work showed that the *Diospyros lotus L.* fruit extract with potent antioxidant activity and radical scavenging could protect erythrocytes against the damage-inducing *Vicia faba L.* bean extracts, both in the G6PD-deficient human erythrocyte and the G6PD-deficient rat erythrocyte. Many scientists have shown that the G6PD-deficient erythrocytes are unable to maintain sufficient NAD (P) H levels to convert oxidized glutathione (GSSG) into its reduced form (GSH) to detoxify hydrogen peroxide and organic peroxides in the erythrocyte[42]. In the current study, G6PD deficiency in the animals was induced by dehydroepiandrosterone as a specific G6PD enzyme inhibitor. DHEA is known to inhibit the activity of glucose 6-phosphate dehydrogenase (G6PD) *in vitro*[30], though *in vivo* studies of the DHEA treatment on the hepatic G6PD have produced mixed results[31]. For example, Mc Inntosh et al[32] showed that injection of rats with 100 mg DHEA/kg for 5 weeks significantly decreased the hepatic G6PD activity in rats. In contrast, Finan and Cleary[33] showed that rats fed with powdered chow plus 0.6% DHEA for 1 week had a significantly increased G6PD activity as compared with control rats. Nevertheless, the method of the induction of the G6PD deficiency in vivo used in this study was similar to another (*Hee Poh*) study with minor modifications[34].

Favism as a life-threatening hemolytic crisis can result from the ingestion of fava beans (*Vicia faba L.*) by susceptible individuals who have low-activity variants of erythrocytic glucose 6-phosphate dehydrogenase (G6PD). As G6PD regulates the production of NADPH in the red cell by the hexose monophosphate (HMP) shunt, G6PD-deficient individuals have a decreased capacity to maintain sufficient levels of NADPH in response to an oxidative stress[1] Early studies identified two components of fava beans, divicine and isouramil, as the probable causative agents based on their ability to deplete reduced glutathione (GSH) in isolated suspensions of human G6PD-deficient red cells[43]. The mechanism

underlying the onset of favism is not yet understood. However, it has been postulated that both pyrimidine aglycones, liberated upon digestion of their parent glucosides[44], are absorbed into the blood and induce oxidative damage within erythrocytes as a consequence of their redox activity[45]. Studies of red blood cells withdrawn from patients during the early and the late stages of the favic crises have indicated that GSH depletion and HMP shunt stimulation are key events that precede red cell loss[46]. As the *Diospyros lotus L.* fruit extract is a potent scavenger of free radicals, it could have provided a defense against hemolytic injury by the *Vicia faba L.* bean extract-related fall in reducing glutathione in the G6PD-deficient erythrocytes. Erythrocytes have a high content of polyunsaturated lipids that, along with their rich oxygen supply and transition metals, make them more susceptible to lipid peroxidation[47,48]. Any process that increases the peroxidation of unsaturated bonds in membrane lipids also causes an increase in erythrocyte fragility and their susceptibility toward hemolysis[49]. Although a variety of free-radical species are formed by the interaction of xenobiotics with erythrocytes, there are several membrane systems that protect them from free radical damage. Among these are superoxide dismutase, glutathione peroxidase, catalase, and a number of free radical scavengers that include ascorbic acid, uric acid, and α-tocopherol[50]. Whether the *Diospyros lotus L.* fruit extract has the ability to affect any of the membrane protective mechanisms is not yet clear, but it could act by quenching the depletion of these components to enhance their membrane-preserving effect in the G6PD-deficient erythrocytes. Studies involving plasma have indicated that flavonoids have the ability to delay the consumption of some endogenously present antioxidants in the human body[51]. Also as mentioned earlier, the *Diospyros lotus* L. fruit extract with a high amount of total phenol and flavonoids could protect some endogenously present antioxidants. Therefore, in the G6PD-deficient erythrocytes, the *Diospyros lotus* L. fruit extract could act as a supportive agent against the high consumption of endogenous antioxidants in the human body following the induction of oxidative stress by the *Vicia faba L.* bean extract. Free-radical-induced structural changes in the G6PD-deficient erythrocyte membrane are not fully understood[52], but despite its complex nature, the process of hemolysis falls under two categories: one that involves cell damage and the

other that results from permeability changes in the cell membrane[53]. While some xenobiotics have the ability to produce hemolytic changes of both types,[54] it seems that the *Vicia faba L.* bean extract has the potential of affecting cell membrane permeability, in addition to their ability of causing oxidative cell damage. Therefore, the *Diospyros lotus* L. fruit extract probably has a membrane-stabilizing property, and this was a contributing factor for its antihemolytic effect in the present study. Many studies showed the biological activity of *Diospyros* genus in vitro. One of these studies showed that *Diospyros gaultheriifolia L.* has an antioxidant activity and a low toxicity *in vitro*[55]. It is possible that the radical scavenging of this species may be related to the presence of naphthoquinones. In the another investigation, Maiga et al[56] showed that *Diospyros abyssinica L.* with a high content of naphthoquinones has a radical-scavenging activity and 15-lipoxygenase inhibition. Also, Ganapaty et al[57] showed that antiprotozoal and cytotoxic effect of *Diospyros assimilis L.* to be related to the presence of naphthalene derivatives and naphthoquinones.

In conclusion, the present study showes that fruit extracts of *Diospyros lotus L.* possess antioxidative properties, which suggest the presence of biologically active components such as flavonoids and naphthoquinones that may be worthy of further investigation and elucidation. The exact mechanism is not clear, but it seems *Diospyros lotus L.* with a high amount of naphthoquinones, especially 7-methyljuglone, has a potent antioxidant activity that could protect membrane erythrocytes against oxidative damage induced by the *Vicia faba* bean extract. Moreover, it could be suggested that flavonoids existing on the *Diospyros lotus L.* fruit extract can be a supportive agent against the high consumption of endogenous body antioxidants during oxidative injury. These results suggest that the product of *Diospyros lotus L.* may provide a new therapeutic avenue to the prevention of favism disorder.

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