Effects of *Pistacia atlantica* (*subsp. Mutica*) oil extracts on antioxidant activities during experimentally induced cutaneous wound healing in rats

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Abstract

The fruits of *Pistacia atlantica* (*subsp. mutica*) have been used traditionally for the treatment of peptic ulcer, as a mouth freshener and have recently been introduced as a source of antioxidant vegetable oils. The aim of this study was to investigate the antioxidant activity of the gel forms, from *P. atlantica* (*subsp. mutica*) oil extraction on enzymatic antioxidants in experimental wound created in rat. A square-shaped skin defect (2×2 cm) was created aseptically by surgical excision at the first thoracic vertebrae. Then animals were randomly allocated in four groups (I, untreated controls; II, topically treated base gel; III, topically treated 5% gel; IV, topically treated 10% gel). Blood sampling was accomplished at 3, 7, 10, 14 and 21 days post-injury. Samples were collected for measuring antioxidant enzymes activities (superoxide dismutase, catalase and glutathione peroxidase activity in red cells) and lipid peroxidation (plasma malondialdehyde). The data analysis generally evidenced that the activities of the main antioxidant enzymes began to decrease significantly at 7 days after the wound was created in control and base gel groups. This remarkable decline became more evident in the period between 10 to 21 days post injury but increased progressively in *P. atlantica* (*subsp. mutica*) treatment groups, especially in gel 10% treatment group during wound healing. The results of this study suggest that excision of the wound leads to oxidative stress and topical administration of *P. atlantica* (*subsp. mutica*) gels causes remarkable changes in antioxidant parameter during wound closure (especially gel 10%) via pro-oxidative, and antioxidant activity can improve oxidative stress.

Introduction

Cutaneous wound repair is a complex and intricate process which initiates in response to injury that restores the function and integrity of damaged tissue.[1,2] The skin is a biological interface with the environment, and is frequently and directly exposed to prooxidative stimuli including chemical oxidants, ultraviolet and visible irradiation, which are known to promote the generation of reactive oxygen species (ROSs) and lipid peroxides.[3,4] Particularly large amounts of ROS are produced in wounded and inflamed tissue by NADPH oxidase, an enzyme complex expressed at specifically high levels by inflammatory cells.[5,6] ROSs are produced in response to cutaneous injury, which further impede the healing process by causing damage to cellular membranes, DNA, proteins and lipids as well.[7] Further, elevated lipid peroxide levels have also been demonstrated in certain inflammatory skin lesions such as traumatic wounds and radiation dermatitis.[8]

P. atlantica (*subsp. mutica*) trees grow in large populations and cover an area more than 1,200,000 ha mainly in the western, central and eastern parts of Iran. The fruits of *P. atlantica* (*subsp. mutica*), which have been used traditionally for the treatment of peptic ulcer and as a mouth freshener,[9] are called *Bene* in Iran and are used by the natives as food after grinding and mixing with other ingredients.[10] Bene hull oil (BHO) as a new source of highly stable and antioxidative vegetable oils has recently been introduced to the world.[10] They are round to oval, somewhat flat, and 0.5-0.7 cm in diameter. Their wooden hard shell is covered with a rather dry hull which could be easily removed by pressing between the fingers. This soft hull is dark green in color, comprises 24% of the whole fruit (25% kernel and 51% hard shell) and yields up to 30% oil.[10-12]

P. atlantica (*subsp. mutica*) oil contains saturated fatty acids [mainly palmitic acid in BHO (26.45%) and bene kernel oil (BKO) (13.15%)], mono unsaturated fatty acid [palmitoleic acid in BHO (13.38%) and BKO (1.78%); and oleic acid ranges from 52 to 61%], and the lowest percentage of polyunsaturated fatty acids (mainly linoleic acid in BHO (6.60%) and BKO (33.00%)]. The BHO contains about 6.5% unsaponifiable matter (USM) which is considered to be the highest content amongst all common vegetable oils.[13] The tocopherols and tocotrienols are the major constituents (48%) of the USM.[13] They display antioxidant properties and are active as vitamin E, which makes them particularly important for human health.[13]

The second major constituents of the USM with a total yield of about 37% are the triterpenic compounds (sterolic types and dialcoholic ones).[13] These steroidal phytochemicals contained in vegetable oils are hypocholesterolemic and may also be potent antioxidants. The sterol composition of the BHO consists of seven compounds with b-sitosterol as the major constituent (746.1 mg/kg).

Bene kernel oil contains 817.92 mg/kg of total tocopherol and 213.47 mg/kg of the total phenolic content respectively.[14] Regarding the fatty acid composition and total tocopherols and phenolic compounds determined, the oil extracted from the kernel of *P. atlantica* (*subsp. mutica*) is considered as one of the most oxidatively stable vegetable oils in the world.[14] Both tocopherols and phenolic compounds are very important natural antioxidants for the stabilization of unsaturated fatty acids and provide an effective protection against oxidative stress in the human body.[15,16] Tocopherols have antioxidant properties and they are active as vitamin E, which makes them particularly important for human health.[10] Antioxidants have been shown to promote wound healing.[7,17,18] Their levels have been shown to be depleted in the healing of cutaneous wounds in normal subjects, which either partially or completely recover following healing.[19] Therefore, the aim of our work was to evaluate the effect of the *P. atlantica's* gels extraction on

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enzymatic antioxidants in experimental wound created in rat.

Materials and Methods

Animals

One hundred Sprague dawley male rats, weighing 200±20 g were used in the experiment. The animals were kept in standard individual cages, at constant temperature (22-24°C) in animal lab of Shiraz University, with 12-hour light and dark cycles. They were fed a standard rat chow, and had free access to food and water (*ad libitium*).

Injury induction

The animals were anaesthetized by intramuscular injection of 10 mg/kg xylazine HCl (Xylazine 2%; Alfasan) as premedication, and 90 mg/kg ketamine HCl (Ketamine 10%; Alfasan) for anesthesia. A square-shaped skin defect (2×2 cm) was created aseptically by surgical incision at the first thoracic vertebrae and 1cm proximal to the first lumbar vertebrae. This method of wound induction has been used previously to investigate cutaneous wound healing in rats.[20]

Oil extraction

Fresh *P atlantica* (*subsp. mutica*) fruit were purchased from a retail food store (Chehel giah store, Ghasre Dasht Street, Shiraz, Iran) in March 2012. After drying in the shade, *P atlantica* (*subsp. mutica*) fruit were ground to powder in a grinder. The powders were extracted with n- hexane (1: 4 wt/vol) by agitation in a dark place at ambient temperature for 48 h. The solvent was evaporated *in vacuo* at 40°C to dryness.[21]

Preparation of the gel

To prepare the gel 1 g carbopol (polymers of high molecular weight of acrylic acid cross linked with a polyalkenyl polyether and flexibility to develop products with a wide range of flow and rheological properties) was added to 95 cc distilled water (5% gel), and 1 g to 90 cc distilled water (10% gel); after 4 or 5 h, all carbopol powder was dissolved and sodium hydroxide was added to make the gel base. Finally, 5 g (5% gel) and 10 g (10% gel) oil extract were added to the gel base and used for all experiments.

Experimental design

After wound creation, the animals were divided into four groups of twenty-five rats and each group was divided in five subgroups and, at this time, treatment was initiated with specific gels.

Group I, untreated controls; Group II, topically treated base gel; Group III, topically treated 5% gel; Group IV, topically treated 10% gel. At this time rats were treated with these gels.

Animal ethics

This experiment was performed under the approval of the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of the European Council Directive (86/609/EC) of November 24, 1986, regarding the protection of animals used for experimental purposes were considered.

Blood sample preparation

Blood sampling was accomplished at 3, 7, 10, 14 and 21 days post-injury. Samples were collected from the heart into tubes containing heparin for measuring antioxidant enzymes activities [superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activity in red cells] and lipid peroxidation [plasma malondialdehyde (MDA)] within 5-6 h after blood sampling.

Hemolysate preparation

Whole blood (0.5 mL) was centrifuged at 700 g for 15 min. The plasma was aspirated off, and the erythrocyte pellet was washed three times with normal saline solution, then, distilled water was slowly added up to 2 mL. The biochemical tests were performed immediately after preparing the hemolysate.

Biochemical assays

The activities of superoxide dismutase and glutathione peroxidase

Superoxide dismutase activity was measured with a commercial kit (RANSOD kit, Randox Com, UK). According to the manufacturer's manual, 0.5 mL of whole blood was centrifuged for 10 minutes at 700 g and the plasma was aspirated off. The erythrocytes were washed four times with 3 mL of 0.9% NaCl solution and were centrifuged each time to separate the supernatant. The washed erythrocytes were diluted to 2 mL with cold redistilled water. After thorough mixing the lysate was diluted with 0.01 mol/L phosphate buffer (pH=7) with a final dilution factor of 200. The reagents were then added to the diluted samples. In this method, xanthine and xanthine oxidase are employed to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity in hemolysate was determined by the degree of inhibition of this reaction as one unit of SOD corresponded to 50% inhibition of INT reduction under assay condition. Finally, the enzyme activity was expressed as units/g of hemoglobin.

GPx activity was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia and Valentine.[22] According to the manual, 0.05 mL of whole blood was diluted and incubated with 3 ml of a diluting agent to form the hemolysate. The GPx present in the hemolysate catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The absorbance was measured at 340 nm and the enzyme activity was expressed as units/g of hemoglobin.

Catalase assay

Tissue catalase activity was assayed spectrophotometrically by monitoring the decomposition of H_2O_2 using the procedure of Aebi.[23] Briefly, 0.5 mL of 30 mmol/L H_2O_2 solution in 50 mmol/L phosphate buffer (pH=7.0), 1 mL of 1: 10 diluted erythrocyte lysates was added and the consumption of H_2O_2 was followed spectrophotometrically at 240 nm for 2 min at 25°C. The molar extinction coefficient was 43.6 L/mol per cm for H_2O_2. Catalase activity was expressed as the unit that is defined as µmol H_2O_2 consumed/min per gram hemoglobin.

Measurement of malondialdehyde

To evaluate lipid peroxidation in erythrocytes, a modified HPLC method based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colored MDA-TBA adduct was used.[24] Erythrocytes were washed three times with phosphate-buffered saline. Then, 40 µL of sample was diluted with 100 µL of H_2O and mixed with 20 µL of 2.8 mmol/L butylated hydroxytoluene (BHT) in ethanol, 40 µL of 81 g/L sodium dodecyl sulfate and 600 µL of TBA reagent (8 g/L TBA diluted 1:1 with 200 ml/L acetic acid adjusted to pH 3.5 with NaOH). The mixture was immediately heated (60 min at 95°C) and cooled with running water; 200 µL of H_2O and 1000 µL of butanol-pyridine (15:1, v/v) were then added. After vigorous mixing, the organic layer was separated by centrifugation (3 min at 16,000 *g*). The supernatant was analyzed on a UV-visible spectrophotometer fitted with an 80 µL flow cell. The absorbance was measured at 532 nm (the mobile phase consisted of 300 mL/L methanol in 50 mmol/L potassium dihydrogen phosphate buffer, pH 7.0). 1, 1, 3, 3-tetraethoxypropane was used as a standard, and MDA-TBA reactive substances values were expressed as MDA nano-moles per grams of hemoglobin (nmol/g Hb). The HPLC system was comprised of a solvent delivery pump (JASCO 980-PU, Tokyo, Japan), a reversed-phase column (Luna C18, 250×4.6 mm, Phenomenex, CA, USA), and a UV-Vis detector (Jasco, UV-975, Tokyo, Japan) operated at 532 nm.

Statistical analysis

The differences between means of measured parameters between groups in different times of blood sampling were analyzed by Multivariate analysis of variance (ANOVA). Differences between means were estimated using Tukey test. The values are expressed as mean±standard error of mean (SEM). All data were analyzed with SPSS/17 software. $P<0.05$ was considered significant.

Results

Our data revealed remarkable changes in antioxidant-oxidant related parameters in experimental wound during a period of 21 days after topical *P. atlantica* (*subsp. mutica*) gels administration. These changes are presented in Tables 1-4.

As shown, the data analysis generally evidenced that the activities of the main erythrocyte antioxidant enzymes began to decrease significantly at 7 days after wound creation in control and base gel groups. This remarkable decline became more evident in a period between 10 and 21days post injury but increased progressively in *P. atlantica* (*subsp. mutica*) treatment groups, especially in 10% gel treatment group during wound healing.

Table 1, shows the mean±SE of SOD activity in red cells of control, base gel, *P. atlantica* (*subsp. mutica*) gel 5% and *P atlantica* (*subsp. mutica*) 10% gel treated rats. There was no significant difference in the SOD activities of control and base gel treated rats, which remained relatively constant throughout the healing period. 10% gel treated group showed significant increase in SOD levels 3 days after administration compared to the control and base gel group ($P<0.0001$), however, gel 5% treated group increased SOD level significantly 10 days after administration compared to the control and base gel groups.

According to our data (Tables 2 and 3), the activity of GPx and CAT was affected significantly at an earlier time in *P. atlantica* (*subsp. mutica*) treated groups (especially in gel 10%) compared to control and base gel treated groups during wound repair.

In contrast to the antioxidant parameters, the serum levels of MDA (Table 4) in blood samples revealed remarkable increases in the control and base gel treatment animals but decreased significantly in *P. atlantica* (*subsp. mutica*) treated groups, especially in gel 10% treated group during wound repair.

Discussion

The result of the present study showed that topical administration of *P. atlantica (subsp. mutica)* gels causes remarkable changes in antioxidant parameter during wound closure, especially in gel 10% during the first 3 days of the wound healing.

The balance between free radicals and antioxidants may disrupt in many diseases. This disruption may be attributed to a number of factors such as the inability of the cells to produce sufficient amounts of antioxidants or the excess production of ROS.[25] Wounding is another condition that results in a decrease in antioxidants.[19] Guo and DiPietro suggested vitamin E, an anti-oxidant, maintain and stabilize cellular membrane integrity by providing protection against destruction by oxidation.[26] Vitamin E also has anti-inflammatory properties and has been suggested to have a role in decreasing excess scar formation in chronic wounds. Another study suggested vitamin E,

Table 1. Mean ± standard error of changes in superoxide dismutase activities of Bene oil gels compared with control and base gel on different days post injury.

Days post injury	Control	Base gel	5% Bene gel	10% Bene gel
3	347±3.39^{d}	346±3.43^{d}	360±2.74	369±2.92^{ab}
7	346±1.87^{d}	347±2.30^{d}	362±3.39	372±3^{ab}
10	329±1.87^{cd}	328±1.72^{cd}	399±5.57^{ab}	412±4.06^{ab}
14	322±2.55^{cd}	321±2.56^{cd}	409±4.58ab	422±4.36^{ab}
21	321±3.67^{cd}	319±3.44^{cd}	430±6.32^{ab}	445±2.74

abDifferent letters (a=control, b= base gel, c=5% Bene oil gel, d=10% Bene oil gel) represent significant differences for that variable between the associated groups, at different days.

Table 2. Mean ± standard error of changes in plasma glutathione peroxidase activities of Bene oil gels compared with control and base gel on different days post injury.

Days post injury	Control	Base gel	5% Bene gel	10% Bene gel
3	461±1.87^{d}	462±1.50^{d}	474±2.45	482±2^{ab}
7	458±2.55^{d}	459±2.46^{d}	476±2.92	487±2.55^{ab}
10	446±1.87^{dc}	445±3.34^{cd}	507±4.36^{ab}	525±4.74^{ab}
14	436±1.87^{dc}	436±2.08^{cd}	514±6^{abd}	541±3.67^{abc}
21	429±2.92^{dc}	427±2.77^{cd}	528±8^{abd}	552±2.55^{abc}

Different letters (a=control, b= base gel, c=5% Bene oil gel, d=10% Bene oil gel) represent significant differences for that variable between the associated groups, at different days.

Table 3. Mean ± standard error of changes in catalase activities of Bene oil gels compared with control and base gel on different days post injury.

Days post injury	Control	Base gel	5% Bene gel	10% Bene gel
3	1811±5.1^{d}	1813±4.79^{d}	1898±11.58	1922±8.6^{ab}
7	1810±4.47	1811±3.47	1898±8.6	1915±7.07
10	1758±17.72^{cd}	1754±16^{cd}	2008±23.54^{ab}	2063±21.42^{ab}
14	1650±22.36^{cd}	1666±20.15^{cd}	2054±38.03^{ab}	2154±37.5^{ab}
21	1580±25.5^{cd}	1575±27.39^{cd}	2101±21.3^{abd}	2212±33.82^{abc}

Different letters (a=control, b= base gel, c=5% Bene oil gel, d=10% Bene oil gel) represent significant differences for that variable between the associated groups, at different days.

Table 4. Mean ± standard error of changes in malondialdehyde of Bene oil gels compared with control and base gel on different days post injury.

Days post injury	Control	Base gel	5% Bene gel	10% Bene gel
3	14.7±0.25	14.72±0.29	13.70±0.25	13.94±0.26
7	14.8±0.25^{d}	14.94±0.26^{d}	14.20±0.12	13.30±0.07^{ab}
10	15.8±0.25^{cd}	15.84±0.2^{cd}	12.60±0.19^{ab}	12.14±0.15^{ab}
14	16.1±0.24^{cd}	16.12±0.25^{cd}	11.40±0.19^{ab}	11.28±0.16^{ab}
21	17.1±0.19^{cd}	17.30±0.18^{cd}	10.60±0.19^{ab}	10.14±0.36^{ab}

Different letters (a=control, b= base gel, c=5% Bene oil gel, d=10% Bene oil gel) represent significant differences for that variable between the associated groups, at different days.

the term for a group of tocopherols and tocotrienols, is the major lipid soluble antioxidant in skin; it protects cell membranes from peroxidative damage and the deleterious effects of free radicals.[27,28]

Also in agreement with our finding, Schäfer and Sabine Werner suggested compounds present in food, such as vitamins E and C (ascorbic acid), carotinoids, and phenolic compounds have antioxidant properties.[29] Another study by Shukla *et al*.[19] showed in acute rodent wounds, the levels of vitamin E, ascorbate and glutathione decreased by 60-70% as compared to normal skin and only the levels of glutathione recovered completely within 14 days after wounding.[19] It has also been shown that strongly reduced levels of glutathione, Vitamin E and ascorbate were observed in normal and, in particular, in wounded skin of immunosuppressed rats as compared to immunocompetent animals.[30]

Consistent with other findings, palm vitamin E extract, which contains a mixture of 60% tocotrienol and 40% tocopherol, enhances wound healing in diabetic rats and increases the activity of the GPx enzyme.[31] Musalmah *et al*.[32] showed that basal GPx levels were lower in diabetic rats, confirming the earlier reports.[31,33,34] Musalmah *et al.*[32] showed that daily oral supplementation with α-tocopherol increased the level of SOD and GPx.[32] These enzymes scavenge free radicals and prevent oxidative damage.[32]

Schäfer and Werner suggested SOD activity was reduced upon skin injury in rats, and the activities did not fully recover within 14 days.[29] Panchatcharam *et al.*[35] showed that antioxidant treatment, using curcumin, resulted in increased levels of SODs and also of catalase and glutathione peroxidase activities at the wound site. To determine the function of SODs in wound repair, ischemic rat wounds were treated with recombinant SOD. Interestingly, wound breaking strength was increased and wound edema was reduced.[36] In another study, hydrogels of carboxymethyl cellulose were prepared and loaded with bovine SOD1. When applied to rat wounds, the SOD-hydrogel was able to enhance the wound-healing rate.[37]

Similar to our study, Shukla *et al.*[19] showed that activities of catalase and glutathione peroxidase decreased upon skin injury in rats, possibly due to ROS-mediated inactivation. This was also observed in immunocompromised rats, where catalase and glutathione peroxidase activities declined within 2 days after skin injury.[30]

Consistent with our findings, Musalmah *et al*.[32] showed that Plasma MDA levels were increased in untreated diabetic rats. This probably reflects the increase in lipid oxidation due to either increased production of free oxidative radicals,[38] or decreased antioxidant defense mechanisms or both.[31,34,39] They showed supplementation with α-tocopherol decreased the level of MDA in both the normal and diabetic groups, confirming the role of α-tocopherol as a powerful antioxidant.[32] Another finding showed Vitamin E or α-tocopherol supplementation reduced the levels of lipid peroxides in the wound tissue of diabetic rats, and the wound healing process was enhanced, especially in the Vitamin E-treated animals.[40] Several findings suggest that phytosterols, such as b-sitosterol are responsible, at least in part, for preventive effects on the development of diseases due to ROS.[41] Moreover, Yoshida and Niki reported the antioxidant effects of the phytosterols b-sitosterol, stigmasterol, and campesterol, against lipid peroxidation.[42]

Conclusions

In conclusion, the results of this study suggest that excision of the wound leads to oxidative stress and topical administration of *P. atlantica* gels causes remarkable changes in antioxidant parameter during wound closure (especially gel 10%) via pro-oxidative and antioxidant activity which can improve oxidative stress.

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