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Incision wound healing activity of pine bark extract containing topical formulations: A study with histopathological and biochemical analyses in albino rats

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The present study was designed to identify and compare the *in vivo* wound healing capacity of a bark extract from *Pinus brutia* and Pycnogenol® in an incision wound model in rats. O/W cream formulations were prepared incorporating 2% Pycnogenol® and *P. brutia* bark extract. The rats were divided into three groups (n = 8). Subsequently placebo and test formulations were applied to animals once a day from day "0" until the 9th day. Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) were studied in addition to histopathological examinations. Treatment with *P. brutia* extract containing cream inhibited lipid peroxidation by a 35 % decrease in MDA and 46.8 % increase in SOD activity, whereas 19.3 % decrease in MDA and 34.7 % increase in SOD activity were attained with Pynogenol® compared to control. The histological data revealed a better performance of *P. brutia* extract enriched formulation in terms of degeneration of hair roots, increased vascularization and a decrease in necrotic area. Consequently, a high wound healing activity was observed in animals treated with *P. brutia* extract significantly accelerating the wound healing process.

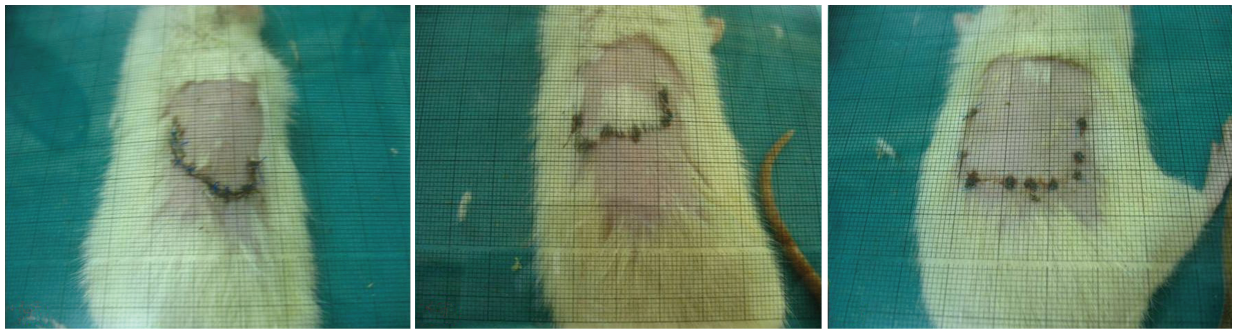
1. Introduction

Wounds are physical injuries that result in an opening or break of the skin. The healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin and it is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis (the deepest skin layer) begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin) is regenerated. There are three stages in the process of wound healing: inflammation, proliferation, and remodeling (Yoshinori et al. 2004; Nayak et al. 2007a; Diegelmann and Evans 2004; Kumar et al. 2007; Karodi et al. 2009; Guo and DiPietro 2010).

Although the use of pine bark dates back to ancient times, it has recently found wide application in the fields of nutrition, health and medicine. Pine bark extracts contain numerous phenolic compounds such as catechin, epicatechin, taxifolin and phenolic acids. The most common commercially available pine bark extract is Pycnogenol®, a standardised extract of French maritime pine bark (*Pinus maritima*), which is probably the most studied phenolic tree extract containing proanthocyanidins. Pycnogenol® has been reported to have cardiovascular benefits, the ability to enhance microcirculation by increasing capillary permeability, strong free radical scavenging activity against reactive oxygen and nitrogen species, the potential to regenerate the ascorbyl radical and to protect endogenous vitamin E and glutathione from oxidative stress. Pycnogenol® also accelerates wound healing processes and is a potent active ingredient for the treatment of minor injuries (Packer et al. 1999; Blazsó et al. 2004; Gulati 2005).

P. brutia extracts were analyzed and were reported to contain procyanidins and (+)-catechin. Furthermore, the bark of *P. brutia* was regarded to be an effective source of taxifolin as it contained considerably high amounts of this secondary metabolite. Extracts of *P. pinea*, *P. brutia* and *P. nigra* exhibited strong radical scavenging activities and possessed high amounts of total phenols (Yesil-Celiktas et al. 2009a).

The aim of this study was to identify and compare the *in vivo* wound healing capacity of a bark extract from *Pinus brutia* and Pycnogenol® in an incision wound model in rats. Levels of oxidative status markers such as malondialdehyde (MDA) which is an end product of lipid peroxidation, activities of antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) were studied in addition to histopathological examinations (hematoxylin and eosin and Mallory Azan staining) order to investigate the possible protective effect of *Pinus brutia* and Pycnogenol® against oxidative stress during wound healing. To the best of our knowledge, as confirmed through a review of the literature, wound-healing effect of *Pinus brutia* bark extract has not yet been studied.



(A) (B) (C)

Fig. 1: Macroscopic appearance of necrotic area in the dorsal flap. (A) Placebo group (B) Pycnogenol® group (C) *P. brutia* group

2. Investigations and results

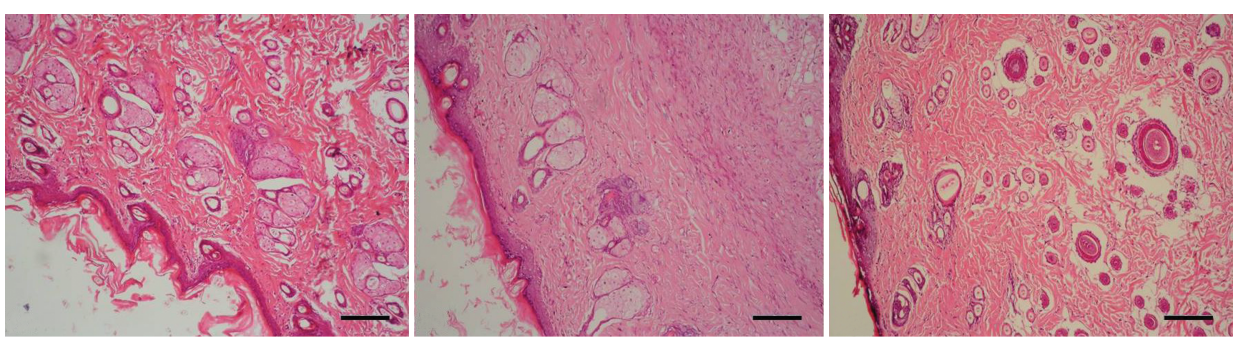
2.1. Wound healing and histological analysis results

Cream formulations were applied over the flaps on a daily basis and repeated for 9 days. The rats were anesthetized on the 10th postoperative day and the dorsal skin flaps were photographed (Fig. 1). The wound site was less compact, immature (pale pink color) and disorientated (Fig. 2A) in the placebo group, whereas more compact and more mature wound sites were observed in the formulation groups (Fig. 2B, 2C).

The comparison of histological data obtained from all groups revealed that degeneration of hair roots was the highest under *P. brutia*, whereas Pycnogenol® and placebo groups were similar. There were signs of degeneration in sebaceous glands which were decreased in the *P. brutia* group compared to control and Pycnogenol® group. There was no significant difference between the groups for the connective tissue. More collagenous fibers and connective tissue cells were found at the placebo group than in the Pycnogenol® and *P. brutia* groups. Hypodermis showed normal thickness and the number of vessels was increased compared to placebo group (Fig. 3). Epithelial thickness provides scar tissue formation at the incision area. If the epithelial thickness is low after wound healing, the chance of a remaining scar at the epidermis is high. The epidermal thickness was found similar for placebo and *P. brutia* groups but was found significantly higher in the Pycnogenol® group than in the other groups (Fig. 4). However, vascularization was increased under *P. brutia* compared to Pycnogenol® and placebo. Thus, treatment with *P. brutia* enriched cream resulted in an acceleration of new blood vessel formation. Adipocytes exist at hypodermis layer in the normal histological structure. A decrease in adipocytes indicates that wound healing is delayed or wound healing is not regular. It was noticed that the thickness of the adipose tissue of *P. brutia* and Pycnogenol® preserved its approximately natural histological integrity without any degenerative changes in the adipocytes (Table 1). The percentage of necrotic area was found highest for the placebo group and lowest for the *P. brutia* group indicating better recovery of the wound (Fig. 5).

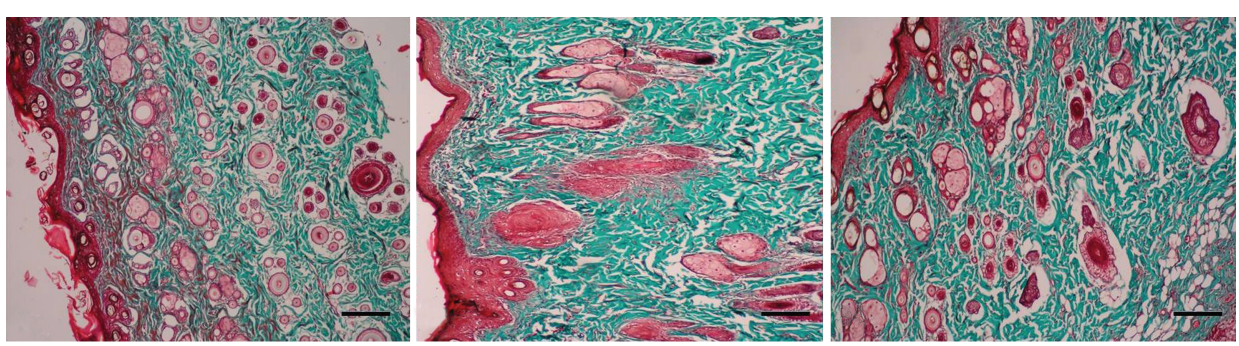
2.2. Biochemical analysis results

Tissue MDA, SOD and catalase levels are presented in Table 2. Application of placebo the wound borders significantly increased MDA level in the skin, while it resulted in a marked decrease in the SOD activity without a significant change in the catalase activity. Application of Pycnogenol® also significantly



(A) (B) (C)

Fig. 2: Light microscopic images of dorsal skin flap (A) Placebo group (B) Pycnogenol® group (C) *P. brutia* group. H&E staining, Original magnification X20, Scale bar = 250 µm



(A) (B) (C)

Fig. 3: Masson Trichrome staining of the sections of dorsal skin flap. (A) Placebo group (B) Pycnogenol® group (C) *P. brutia* group. Original magnification X20, Scale bar = 250 µm

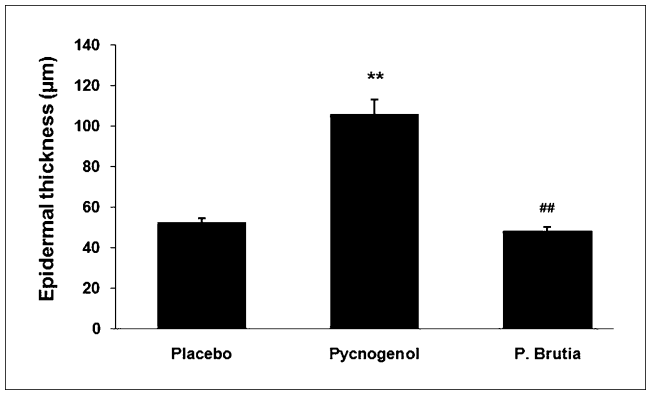


Fig. 4: Comparison between groups of epidermis thickness. Data are presented as mean ± SEM of 8 individual experiments. ** $p < 0.001$ Pycnogenol® vs placebo; ##$p < 0.001$ *P. brutia* vs Pycnogenol®

Table 1: Histopathological findings in the skin incision of Placebo, Pycnogenol® and *P. brutia* groups

	Placebo	Pycnogenol®	*P. brutia*
Epithelial thickness	++	++++	++
Hair roots	++++	+	+++
Sebaceous gland	+++	++	+
Connective tissue	+++++	++++	++++
Collagenous fibers	+++++	++++	+++
Subcutaneous Adipocytes	++	+++	+++
Vascularization	++	++	+++

+, minimal; ++, mild; +++, moderate, ++++, less severe; +++++, severe

increased MDA level, however this was accompanied by a significant increase in the SOD activity as well; catalase activities remained unchanged in the Pycnogenol® group. This variation can be explained based on the chemical composition of the samples; *P. brutia* soxhlet extract contained 10.8 mg catechin, 1.98 mg epicatechin, 0.30 mg catechin gallate and 162.7 mg taxifolin per g extract (Fig. 6), whereas Pycnogenol® contained 7.9 mg, 4.8 mg, 0.85 mg and 17.0 mg of respective compounds per g extract (Ince et al. 2009).

On the other hand, application of *P. brutia* decreased MDA levels in the skin around the wound area of *P. brutia* group and SOD activity was significantly higher than in the placebo group. Application of *P. brutia* did not affect catalase activity in the wounded skin. MDA and SOD levels of *P. brutia* group were better than that of the placebo group.

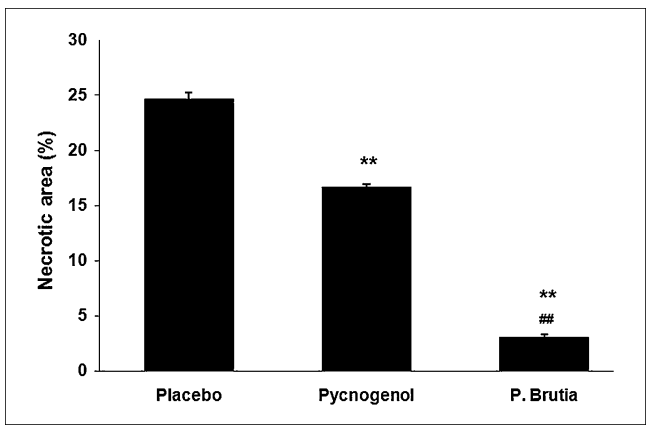


Fig. 5: Comparison of the percentage of necrotic area. Data are presented as mean ± SEM of 8 individual experiments.** $p < 0.001$ Pycnogenol® vs placebo; ##$p < 0.001$ *P. brutia* vs Pycnogenol®

Table 2: Malonyldialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) activities in the wound area of the experimental groups

	MDA (nmol/g protein)	SOD (U/g protein)	CAT (U/g protein)
Placebo	2.74 ± 0.22	2.18 ± 0.12	0.93 ± 0.07
Pycnogenol®	2.21 ± 0.097	2.94 ± 0.13 ##	1.07 ± 0.10
P. brutia	1.78 ± 0.21 ##	3.20 ± 0.19 ##	1.14 ± 0.15

Data are presented as mean ± SEM, n = 5–6
$p < 0.01$, when compared to placebo

3. Discussion

The repair of wounds involves different phases including contraction, the formation of epithelialisation and fibrosis (Mukherjee et al. 2000) and these are three stages in the process of wound healing: inflammation, proliferation, and remodeling (Nayak et al. 2007).

During the anti-inflammatory phase, neutrophils and monocytes at the wound site carry out various tasks, such as the removal of bacteria to prevent the wound from becoming infected. *P. brutia* enhanced the inflammatory stage of the wound-healing process based on its excellent radical scavenging properties with an EC_{50} value of 8.1 µg/ml thereby advancing the production of antioxidative enzymes contributing to the anti-inflammatory effect (Yesil-Celiktas et al. 2009b).

Considering proliferation of the wound healing process, Grimm et al. (2004) showed that Pycnogenol® binds preferentially to matrix proteins and inhibits their enzymatic hydrolysis by matrix metalloproteinases. The binding toward elastin was more pronounced than the binding toward collagen. With an attempt to make an analogy, notably high concentrations of taxifolin combined with catechin in *P. brutia* bark extract might have displayed a high affinity to collagen and elastin as well. The ability of Pycnogenol® to reduce oxidized ascorbate will probably extend the activity of the vitamin in the wound vicinity, supporting collagen formation. The pronounced binding of Pycnogenol® constituents to collagen (and elastin) and their significant inhibitory effect on matrix metalloproteinases (MMPs) are likely to represent a key function in wound healing.

Collagen matrix remodelling occurs throughout the healing process and requires abundant amounts of ascorbic acid for the post-translational hydroxylation of proline. The constituents of Pycnogenol® were stated to recycle oxidized ascorbate which might enhance remodelling. A similar mechanism might also well apply in the case of *P. brutia* constituents (Grimm et al. 2004).

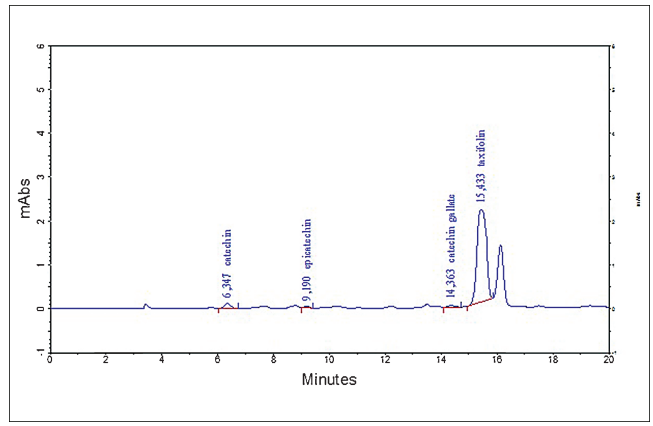


Fig. 6: HPLC chromatogram of *P. brutia* extract

When a skin flap is raised the sensitive equilibrium that regulates the blood flow to the flap is destroyed completely and important changes occur. Flap is in a vascular disequilibrium situation. The most important condition in the beginning phase is the lost of functionality of sympathetic innervations and ischemia. For the living of a skin flap, there should be an adequate feeding circulation and reduction in the damage of ischemia (Kerrigan and Daniel 1982).

Traditionally, medicinal plants have been used for many years as topical and internal preparations to promote wound repair. Medicinal plants have a great potential and have been shown to be very beneficial in wound care, promoting the rate of wound healing with minimal pain, discomfort, and scarring to the patient (MacKay and Miller 2003). Some of these plants owe their activities to a direct effect on the wound healing processes and some to their anti-inflammatory and anti-microbial properties. A combination of these properties is also possible in some of the medicinal plants used in wound care (Odimegwu et al. 2008).

Kupeli-Akkol et al. (2011) used *Achillea biebersteinii Afan.* (Asteraceae) extract for wound healing. According to their results, a *n*-hexane extract of *A. biebersteinii* was found to have good activity in wound healing experimental models. In another study, Baie and Sheikh (2000) concluded that *Channa striatus* (haruan) incorporated in a cream enhanced wound healing, which was argued to be due to an increase in the tensile strength. Odimegwu et al. (2008) reported that *Dissotis theifolia* has antibacterial and wound healing effects when formulated as ointment and could therefore explain the successes claimed in the folk use of the plant in the treatment of sores, boils and wounds. It has been demonstrated that an ethanol extract of *Hibiscus rosa sinensis* has accelerated wound healing activity by enhancing wound contraction, shortening epithelialization period, increasing tensile strength and hydroxyproline content (Nayak et al. 2007b).

Furthermore, the results of a preclinical study indicated that aqueous leaf extract of seabuckthorn possesses a significant wound-healing activity in the rat model by an increase in SOD, catalase, and glutathione peroxidase enzyme activities (Gupta et al. 2005). Another study focused on the curcumin treatment of wounds, which were found to heal much faster as indicated by improved rates of epithelialisation, wound contraction and increased tensile strength confirmed by histopathological examinations. Curcumin treatment was shown to decrease the levels of lipid peroxides, while the levels of SOD, catalase and glutathione peroxidase activities were significantly increased exhibiting the antioxidant properties of curcumin in accelerating wound healing (Panchatcharam et al. 2006). Another study dealt with the wound healing capacity of an aqueous extract of *Myrica esculenta*. The results indicated that *M. esculenta* extract possessed potent antioxidant activity by inhibiting lipid peroxidation and increase in SOD and catalase activities, besides antioxidant activity to understand the mechanism of wound healing capacity (Nainwal and Kalra 2009). In our study, treatment with a *P. brutia* extract containing cream also inhibited lipid peroxidation by a 35 % decrease in MDA and 46.8 % increase in SOD activity, whereas 19.3 % decrease in MDA and 34.7 % increase in SOD activity were attained with Pynogenol® compared to control. More profound activity of *P. brutia* extract compared to Pycnogenol® can be due to a 9.6-fold higher concentration of taxifolin present in the extract. Blazsó et al. (2004) applied Pycnogenol® to rats in an experimental wound model and found that this was a potent treatment of minor injuries because of proanthocyanidins.

However, there was no study with *P. brutia* bark extract related to wound healing in the literature. Therefore, the activities of compounds such as catechin, epicatechin and catechin gallate present in *P. brutia* bark extract were of interest. Kapoor et al. (2004) investigated the effects of epicatechin gallate (ECG), on scar formation in a full thickness incisional model of wound healing in rats. ECG showed a significant improvement in the quality of scar formation both in terms of maturity and orientation of the collagen fibers. In addition, an increase in the number of new blood vessels was observed in the ECG-treated group. Likewise in our study, better vascularization was attained with *P. brutia* group, whereas control and Pycnogenol® groups were at the same levels. Another study with epigallocatechin gallate (EGCG), the major polyphenolic compound present in green tea, has demonstrated that the residual wound size of the mice treated with 10 ppm EGCG-incorporated collagen sponge decreased significantly faster than that of the other mice after 14 days. The results suggested that a collagen sponge (CS) incorporated with EGCG at low concentrations could enhance wound healing in diabetic mice by accelerating reepithelialization and angiogenesis as well as improving the cellular reorganization of granulation tissue (Kim et al. 2008).

In the present study, the epidermal thickness was found similar for placebo and *P. brutia* groups. But in the Pycnogenol® group it was significantly higher than in the other groups. However, vascularization was increased under *P. brutia* compared to Pycnogenol® and placebo groups. Both Pycnogenol® and *P. brutia* applications caused a significant increase in antioxidant SOD enzyme activity; only *P. brutia* application resulted in a significant decrease in MDA levels in comparison to placebo group, reflecting a suppressed lipid peroxidation in the *P. brutia* group. These findings support the suggested antioxidant effect of *P. brutia* during wound healing.

Consequently, a profound wound healing activity was observed in animals treated with *P. brutia* extract compared with those treated with Pycnogenol® and placebo, significantly accelerating the wound healing process. Hence, the extract may be suggested for treating wounds in human beings.

4. Experimental

4.1. Materials

Ethanol and acetonitrile were supplied by Merck (Darmstadt, Germany) and stearic acid was supplied by Sigma-Aldrich (St. Lois, USA). Pycnogenol® was kindly provided by Horphag Research Ltd. (UK). All other chemicals and reagents were of analytical grade.

4.2. Plant material and Pycnogenol®

Pinus brutia Ten. has spread out in eastern Mediterranean countries and is distributed widely in several regions in Turkey (Davis PH 1978–1988). A pine bark specimen (*P. brutia*) was collected from Izmir- Deliomer (N: 38 ° 10' 17.0", E: 27 ° 03' 46.7", altitude: 120 m) in August 2006. The specimen was dried at room temperature, ground by using a conventional grinder and stored at + 4 °C. A voucher specimen is kept at IZEF Herbarium (IZEF 5762) (Ince I et al 2009). Pycnogenol® was gently donated by Horphag Research Ltd., UK.

4.3. Preparation of solvent extracts

Pycnogenol® was gently donated by Horphag Research Ltd., UK. *P. brutia* barks were collected from nature and were extracted in the Supercritical Fluid Technologies lab. of Bioengineering Department at Ege University. About 100 g of ground pine bark were extracted with 1000 ml of ethanol (Merck; ≥ 99 purity) for three cycles where the whole extraction duration was 6 h using a soxhlet (500 ml) apparatus. The extracts were concentrated to dryness at 60 °C *in vacuo* by a Laborato 4001, Heidolph rotary evaporator and subsequently lyophilized.

4.4. HPLC analysis

Sample solutions were prepared by dissolving the extracts in methanol at a concentration of 5.00 mg/ml in Ultrasonic LC30 (Germany). The flavonoids were analyzed using a HPLC method (Yesil-Celiktas 2009; Ince et al. 2009). A Varian Chrom Sep column (SS 250 × 4.6 mm) was used. The mobile phase

comprised 2% acetic acid (Merck) in water (A) and acetonitrile (Merck) (B). Gradient elution was performed starting with 92A/8B, changing the composition to 72A/28B in 20 min, followed by 65A/35B in 10 min, and held for 2 min. Each run was finished to permit an equilibration with 92A/8B for 5 min. The detection wavelength, flow rate and column temperature were set to 280 nm, 1 ml/min, and 30 °C, respectively.

4.5. O/W cream formulation

O/W creams were prepared by dissolving oil phase (stearic acid, 18%) at 70–80 °C. Oil and water phases were mixed together at the same temperature without vortexing to avoid the entrapment of air. After cooling to 30–40 °C, 2% Pycnogenol® and *P. brutia* bark extract were added to the cream.

4.6. Animals

About 24 male Wistar-Albino rats weighing 100 to 150 g were used in this study. The experiments were performed in accordance with the regulations specified by Ege University, Animal Ethical Committee and conformed to national guidelines on the care and use of laboratory animals. The animals were maintained under standard conditions (25 ± 1 °C, 12-hour light/dark cycles) and fed with pellet diet and water *ad libitum*. The rats were divided randomly into three groups, consisting of eight rats per group. The rats were treated immediately with control or test formulations. Group 1 (placebo group), application of placebo cream; group 2, application of Pycnogenol® cream; group 3, application of *P. brutia* cream. Formulations were applied to animals once a day from day "0" until the 9th day.

4.7. Incision wound model

The rats were anaesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine hydrochloride (75 mg/kg) and xylazine (8 mg/kg) anesthesia intraperitoneally. A single dose of ceftriaxon disodium (60 mg/kg) was administered intramuscularly as a prophylaxis against infection. The dorsal fur of the animals was shaved with a razor blade. On the dorsal side of rats, a caudally based 2 × 2 cm flap was drawn and was pulled up by sharp dissection as described by Khouri et al (1986). Incisions were made and the entire flap was undermined below the level of the *panniculus adiposus*. After the incision, surgical sutures were applied to the parted skin at intervals of 1 cm with 4/0 silk suture. The wounds were left undressed. Total operation time was 15 min for every rat (Ehrlich and Hunt 1968).

4.8. Wound healing

Cream formulations were applied over the flaps on a daily basis and repeated for 9 days. All cream formulations were applied evenly in sufficient amounts to cover all wound areas once a day.
Flap viability was evaluated 9 days after the initial operation, at which time a certain amount of necrosis in the distal part of all dorsal flaps was noted. On the 10th postoperative day the rats were reanesthetized for evaluation of flap viability. The dorsal skin flaps were photographed with a digital camera (Canon A 610, Japan). A mechanism was set up by the aid of tripod to standardize all images and images were taken from equal distances. The necrotic skin (defined by the necrotic skin borders) and total flap (defined by the surgical borders) areas were delineated, and surface areas were calculated.
The animals were sacrificed by high dose ketamine hydrochloride administered intramuscularly. When the animals were sacrificed skin biopsy was taken from the beginning of the 3 cm distal of the flap base and 1 cm width. These skin samples were washed with physiological saline and wrapped with aluminum foil to be kept at −80 °C for biochemical evaluation of the wound healing parameters (malondialdeyhde, catalase, superoxide dismutase). For the histopathological analysis, the investigations of tissue degeneration of skin biopsy samples by light microscope were fixed in 10% buffered formalin.

4.9. Histopathological analysis

Skin biopsy samples were fixed in 10% buffered formalin for 24 h and routine paraffin wax embedding procedures were used and blocked. Standard 5 μ sections (Leica RM 2145 model microtome, Germany) were cut, then stained with both hematoxylin and eosin (H&E) and Mallory Azan staining. Sections were investigated at a magnification of 20X. After taking their digital photos (Olympus BX51 Light Microscope, Olympus C5050 Digital Camera, Japan) at a magnification of 20X, images were transferred to computer and the thickness of epithelium, necrotic skin and total flap area's border were determined with the aid of a software programme (Image Pro Express Ver.4.5.1.3., Media Cybernetics Inc., 2002, USA). Surface areas (cm^2) were calculated and epithelium thickness was measured. Necrotic surface area was divided to total flap surface area and results were given as percent of skin necrosis.

4.10. Biochemical analysis

The biopsy materials allocated for biochemical analysis were washed with saline and kept at −80 °C. Frozen flap tissue biopsy samples (100 mg) were fractionated into pieces and homogenized for 5 min in phosphate buffer (1:10, w/v) with homogenizator (B. Braun Biotech International; Potter S, USA). The homogenates were centrifuged for approximately 5 min (Hettich; Universal 16R, Germany). After this procedure; malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) analysis were carried out in upper phase homogenates.
SOD activities were measured spectrophotometrically as described by Sozmen et al (2001) based on the inhibition of epinephrine auto oxidation by SOD at 480 nm with a UV plate reader (Thermo Lab systems; Multiskan EX, USA). The assay was calibrated using purified SOD, and 1 unit of enzyme was defined as the amount that inhibits 50% of auto oxidation of epinephrine. CAT activities were determined as described by Aebi (1984). The degradation of hydrogen peroxide (H_2O_2) by catalase enzyme was recorded spectrophotometrically at 240 nm (Shimadzu UV-1208, Japan). One unit of CAT was defined as the amount of enzyme that decomposes 1 μmol H_2O_2/ min under specific conditions. Protein levels were measured by the Lowry method (Lowry et al. 1951) and activities of both enzymes were given per g protein.
The MDA levels were measured by using the commercial kit (LPO-586) from OXIS Research. The assay is based on achromogenic reaction, *N*-methyl-2-phenylindole with MDA at 45 °C. The results were calculated as nmol/g protein with respect to the calibration curve prepared with 1,1,3,3-tetraethoxypropane.

4.11. Statistical analysis

Data were presented as mean ± SEM. Six slices were randomly selected from each individual animal in a group and five separate data were collected from each slice for the determination of epidermal thickness and necrotic area. Statistical comparison of the groups was performed using SPSS Ver. 12.0 (SAS Institute, Cary, NC, USA). Non-parametric Kruskall Wallis and Mann-Whitney U tests were used to analyze the significant difference between groups in necrotic area around the flap, biochemical analysis of SOD, CAT and MDA levels in the flap biopsy and the histological examination of the tissue biopsy material. When significancy was determined, *post hoc* analysis with the Bonferroni test was carried out for the between group comparisons. A *P* value below 0.05 was considered statistically significant.

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