The effect of *Cotinus coggygria* L. ethanol extract in the treatment of burn wounds

Buşra ERTAŞ 1, Betül OKUYAN 2, Ali ŞEN 3, Feriha ERCAN 4, Hüseyin ÖNEL 1, Fatih GÖGER 5, Göksel ŞENER 6*

1 Department of Pharmacology, School of Pharmacy, Marmara University, İstanbul, Turkey.
2 Department of Clinical Pharmacy, School of Pharmacy, Marmara University, İstanbul, Turkey.
3 Department of Pharmacognosy, School of Pharmacy, Marmara University, İstanbul, Turkey.
4 Department of Histology and Embryology, School of Medicine, Marmara University, İstanbul, Turkey.
5 Department of Pharmacognosy, School of Pharmacy, Anadolu University, Eskişehir, Turkey.
6 Department of Pharmacology, School of Pharmacy, Fenerbahçe University, İstanbul, Turkey.

* Corresponding Author. E-mail: goksel.sener@fbu.edu.tr (G.S.); Tel. +90-(0216) 910 19 07.

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**ABSTRACT:** The overall aim of the present research is to evaluate for the first time the curative effect of *Cotinus coggygria* leaves on burn injury in an experimental burn model along with its anti-inflammatory and antioxidant activity potential. Also, phenolic compounds of *C. coggygria* were characterised by LC-MS/MS. Wistar albino rats weighing 200-250 g were exposed to 90°C bath for 10 s to induce burn injury, involving 30% of the total body surface area. In the treatment groups, 5% *C. coggygria* ethanol extract was applied topically as a cream immediately after the burn. Blood and skin tissue samples were taken after decapitation at the 4th and 48th hours following the burn procedure. Interleukin 1-β (IL-1β) and tumour necrosis factor (TNF-α) were determined in serum samples, and hydroxyproline, prostaglandin E2 (PGE2), and myeloperoxidase (MPO) activity and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels were determined in skin tissue samples. Increased levels of serum cytokines were decreased with *C. coggygria* treatment in both periods. MPO activity, prostaglandine (PGE2), and 8-OHdG levels increased, while hydroxyproline levels decreased due to burn damage. On the other hand, these parameters were returned to its normal levels with *C. coggygria* treatment. In addition, the tissue histology of animals treated with *C. coggygria* showed a complete epithelialization with increased collagenation. As a result, *C. coggygria* may be an alternative treatment approach for burns-induced skin damage and wounds.

**KEYWORDS:** Antioxidant activity; anti-inflammatory activity; burn wound healing activity; *Cotinus coggygria*; phenolic compounds

1. **INTRODUCTION**

Burn injury is among skin injuries in which the clinical situation progress to include systemic complications such as systemic inflammatory response syndrome, multi-organ failure and sepsis due to injury of cells and blood vessels and impaired blood supply to the wound [1]. The healing process begins with inflammation and continues with proliferation and migration of adjacent epithelial cells, production of extracellular matrix, and finally ends with wound contraction [2]. The presence of various cytokines such as IL-1β and TNF-α is the major factor for the initiation of the healing process [3]. The irregularity that can be experienced in this complex biological process triggers the production of reactive oxygen species (ROS) and reactive nitrogen species and delays the wound healing process [4].

Nowadays, the delay in burn wound healing is a serious problem for patients and healthcare providers worldwide. Since the delay in healing processes of severe burn wounds may result in serious infection, treatment should be carried out as soon as possible [5]. Inadequate efficacy and side effects of current topical preparations lead to the search for new drugs in the treatment of burns [6]. There have been ongoing efforts over the years to develop new agents from an ethnomedical source for effective of treating burns [7]. The use of traditional medicinal drugs and herbs in the treatment of burns and wounds has advantages in terms of easy accessibility, low financial burden and biocompatibility [8]. However, the use of herbal products in treatment requires attention and should be applied in special pharmaceutical presentation forms [9]. In other words, the active ingredients in the product used must be determined. Accordingly, the extracts obtained from
plants are first evaluated in terms of their predicted effects in the in vitro environment, and then studied in animal models [10]. After these studies, it is important to identify the active ingredients of the extracts of the promising plants.

*Cotinus coggygria* (smoke tree), a member of the Anacardiaceae family, is a small tree or shrub common mainly in southern Europe and western China [11]. *C. coggygria* exhibits astringent, anti-inflammatory, and antiseptic properties through its high content of tannins [12]. The leaves of the *C. coggygria* plant are used as a folk medicine through its antiseptic, anticoagulant, antipyretic, antidiarrheal and anti-inflammatory effects [13]. *C. coggygria* has traditional uses for the treatment of skin and mucosal lesions [14]. In addition, a decoction obtained from different parts of *C. coggygria* has uses in pharyngitis and stomatitis [15]. *C. coggygria* leaves aqueous extract had hepatoprotective properties. Moreover, an extract from the *C. coggygria* leaves and flowers had antigenotoxic and anticancer activities [16]. Various solvent extracts of this species showed antibacterial properties [17].

To the best of our knowledge, there is no previous study on the burn wound healing effect of *C. coggygria*. Thus, we investigated the effect of wound healing activity of *C. coggygria* in rats. After analyzing the phenolic compounds of *C. coggygria* extract, we evaluated the in vitro anti-inflammatory and antioxidant activity of the extracts by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and lipoxygenase inhibition tests, respectively. The burn wound healing efficiency of *Cotinus coggygria* (Cot. Cog.) extract was evaluated with an experimental burn injury rat model.

2. RESULTS


Cot. Cog. extract with an IC₅₀ value of 11.60 µg/mL showed a remarkable antioxidant activity when compared with the standard agent, ascorbic acid (IC₅₀: 2.50 µg/mL) against the DPPH radical. Cot. Cog. Extract exhibited a significant anti-inflammatory activity with an IC₅₀ value of 72.04 µg/mL against a 5-lipoxygenase enzyme (IC₅₀ for standard indomethacin: 18.05 µg/mL) (Table 1).

Table 1. Anti-inflammatory and antioxidant activity of CCE

<table>
<thead>
<tr>
<th>Assays</th>
<th>Cot. Cog. Extract*</th>
<th>Ascorbic acid</th>
<th>Indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH radical scavenging activity (IC₅₀, µg/mL)</td>
<td>11.60±0.34b</td>
<td>2.50±0.24a</td>
<td>18.05±0.95a</td>
</tr>
<tr>
<td>Anti-lipoxygenase activity (IC₅₀, µg/mL)</td>
<td>72.04±2.12b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cot. Cog. Extract: Ethanol extract of *Cotinus coggygria* leaves
** Each value in the table is represented as mean ± standard deviation (SD) (n=3). Statistical analyses of data were carried out by Student’s t-test. Different letter superscripts in the same line indicate significant differences (p<0.05).

2.2. Metabolite profiling of Cot. Cog. Extract by LC-MS/MS

The LC-MS spectral data demonstrated the presence of quinic acid, gallic acid, protocatechuic acid, methylgallate, myricetin glucoside, pentagalloylglucose, myricetin rhamnoside, quercetin rhamnoside, ethylgallate, ethyl ester of digallic acid and myricetin in the Cot. Cog. Extract. Also, pentagalloylglucose was found as major compound in extract (Figure 1, Table 2).
Table 2. Characterization of phenolic compounds in the Cot. Cog. Extract

<table>
<thead>
<tr>
<th>Rt</th>
<th>[M-H]</th>
<th>Fragments</th>
<th>Identified as</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3</td>
<td>191</td>
<td>173,127</td>
<td>Quinic acid</td>
<td>[18]</td>
</tr>
<tr>
<td>4.7</td>
<td>169</td>
<td>125</td>
<td>Gallic acid</td>
<td>[18]</td>
</tr>
<tr>
<td>6.3</td>
<td>153</td>
<td>109</td>
<td>Protocatechuic acid</td>
<td>[18]</td>
</tr>
<tr>
<td>8.0</td>
<td>183</td>
<td>168, 124</td>
<td>Methylgallate</td>
<td>[18]</td>
</tr>
<tr>
<td>8.8</td>
<td>479</td>
<td>316, 297, 271, 179, 11</td>
<td>Myricetin glucoside</td>
<td>[18]</td>
</tr>
<tr>
<td>9.9</td>
<td>939</td>
<td>479, 469, 383, 317</td>
<td>Pentagalloylglucose</td>
<td>[18]</td>
</tr>
<tr>
<td>10.2</td>
<td>463</td>
<td>315, 287, 271</td>
<td>Myricetin rhamnoside</td>
<td>[18]</td>
</tr>
<tr>
<td>12.2</td>
<td>447</td>
<td>300, 271, 255, 245</td>
<td>Quercetin rhamnoside</td>
<td>[18]</td>
</tr>
<tr>
<td>13.8</td>
<td>197</td>
<td>168, 140, 125</td>
<td>Ethylgallate</td>
<td>[19]</td>
</tr>
<tr>
<td>14.8</td>
<td>349</td>
<td>197, 169, 125</td>
<td>Ethyl ester of digallic acid</td>
<td>[20]</td>
</tr>
<tr>
<td>14.0</td>
<td>317</td>
<td>289, 179, 151, 137</td>
<td>Myricetin</td>
<td>[21]</td>
</tr>
</tbody>
</table>

2.3. Biochemical parameters

As shown in Figure 2, serum TNF-α and IL-1β levels were significantly increased in the burn-induced group compared with healthy controls at 4th and 48th hours following thermal burn. 4 h after burn injury, TNF-α and IL-1β levels were significantly lower in the Cot. Cog.-treated group than in the based-cream group (p<0.01-p<0.001), (Fig. 2a-2b). And similarly, 48 h after burn injury, increased TNF-α level in the based cream-treated group was reduced by Cot. Cog. treatment (p<0.05-p<0.001).

![Figure 2](http://dx.doi.org/10.29228/jrp.153)

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8-hydroxy-2-deoxyguanosine (8-OHdG) levels, which were measured as an indicator of oxidative DNA damage, increased significantly at the 4th and 48th hours after burn (p<0.05-0.001). On the other hand, Cot. Cog. treatment decreased the DNA oxidation significantly at 48th hour (p <0.01, Fig. 3a). Similarly burn injury significantly increased MPO activity (p <0.05-0.01) in skin tissue at 4th and 48th hours, while Cot.Cog. treatment decreased the enzyme activity significantly at the 48th hour after burn (p <0.01, Fig. 3b).

Increased hydroxyproline indicates the formation of collagen that promotes burn healing. Therefore, the hydroxyproline levels was measured in both formulation treated and untreated burn tissues. Hydroxyproline levels of burn tissues are found to be decreased. While the treatment with Cot. Cog extract slightly increased hydroxyproline levels at the 4th hour, it prevented the decrease significantly in hydroxyproline levels by protecting the tissue at the 48th hour (p<0.01). The values of hydroxyproline levels in the treatment at 48th hour was found to be significantly higher compared to the levels at the 4th hour (p<0.05, Fig. 3c).

Inflammation induced by burn trauma occurred in the skin caused a significant increase in the levels of PGE2 at the 48th hour. When comparing burn and Cot cog-treated groups 4 hours after burning, close levels of PGE2 were observed between groups. Animals treated with the ointment formulation of Cot. Cog. extract showed a significantly (p<0.05, Fig. 3d) decreased levels of PGE2.

![Figure 3](image_url)

**Figure 3.** Effect of ethanolic Cot. Cog. extract on the 8-hydroxy-2-deoxyguanosine (8-OHdG) (a), MPO (b), hydroxyproline (c) and PGE2 (d) levels on skin at 4th and 48th after burn induced skin injury * p <0.05, **p<0.01, ***p<0.001 compared to control group; + p <0.05, ++p <0.01 compared to based cream-treated group; & p <0.05 compared to Cot. Cog.-treated group at 4th

### 2.4. Histopathological observations

Regular epidermis and dermis were observed in control group (Fig. 4A). Desquamation of epidermis, cellular damage, degeneration in hair follicles and irregularity in distribution of collagen fibers and bleeding were seen in burn induced skin injury group after 4 hours (Fig. 4B). Local thickening of the epidermis,
moderate recovering of hair follicles and quite regular distribution of collagen fibers were observed in burn induced skin injury group after 48 hours (Fig. 4C). Local thickening of the epidermis, moderate bleeding, recovering of hair follicles and quite regular distribution of collagen fibers were observed in burn induced skin injury group after Cot. Cog. treatment with 4 hours (Fig. 4D). Quite regular epidermis, hair follicles and distribution of collagen fibers in dermis were seen in burn induced skin injury group after Cot. Cog. treatment with 48 hours (Fig. 4E).

3. DISCUSSION

Burn injury healing is a very complex process that includes renovating of close to normal skin structure depending on time and burn severity [22, 23]. Using of medicinal plants based ointments in the treatment of burn injury healing among the public dates back to centuries and continued till today [24]. Especially, preparations made from natural products containing antioxidants are frequently preferred for this purpose [25]. Cotinus coggygria (smoketree) is widely used in traditional public health due to its wound healing effect [26, 27]. Aksoy et al. [26] confirmed the wound healing efficacy of Cot. Cog. in the excision wound model in diabetic rats. Although Cot. Cog. has been studied in wound model, it has not been studied in the thermal trauma before. The present study was done to investigate the wound healing potential of topically administered Cot. Cog. extract in the treatment of 4 and 48 hours thermally-induced burn injury.

In our study, we determined the in vitro anti-inflammatory and antioxidant activity of Cot Cog. ethanol extract. According to our results, Cot. Cog. ethanol extract showed significant antioxidant and anti-inflammatory activity compared to control groups.

Figure 4. Representative light micrographs of skin in the experimental groups. Normal epidermis, dermis with hair follicles and distribution of collagen fibers are seen in the control group (A). Desquamation of epidermis and cellular damage (arrow), disorganisation of collagen distribution (*) and bleeding (arrowhead) are seen in burn induced skin injury group after 4 hours (B). Local thickening of the epidermis (arrow), moderate recovering of hair follicles (f) and quite regular distribution of collagen fibers (*) are seen in burn induced skin injury group after 48 hours (C). Local thickening of the epidermis (arrow), moderate bleeding (arrowhead), recovering of hair follicles (f) and quite regular distribution of collagen fibers (*) are seen in burn induced skin injury group after 4 hours (D). Quite regular epidermis (arrow), hair follicles (f) and distribution of collagen fibers (*) in dermis are seen in burn induced skin injury group after 48 hours (E). Masson’s trichrome staining. Original magnification: x100.
Based on the aforementioned evidence, in this study, the ability of Cot. Cog. extract to heal tissue damage was evaluated for the first time in an experimental burn model. Increased serum levels of TNF-α and IL-1β, which are markers of inflammation after thermal burn, were decreased with the application of simple cream containing 5% Cotinus coggygria ethanol extract. The study also showed that application of 5% Cotinus coggygria ethanol extract contained simple cream reduced both increased 8-OHdG level, which indicates oxidative DNA damage, and increased MPO level, that indicates neutrophil infiltration in tissue. Similarly, increased PGE2 levels due to burn trauma and especially burns induced after 48th hour, were reduced with treatment. Hydroxyproline, an important component of the skin, decreased with damage but recovered with treatment. The curative effects of simple cream application containing 5% Cot. Cog. ethanol extract on the burn tissue were also demonstrated by the results of histological examinations.

Thermal injury is a process that starts with symptoms of local inflammation in the tissue and creates significant systemic damage involving different organs [28]. Proinflammatory cytokines are released rapidly and last for a few days after the burn, and acute phase proteins increase [29]. In our study, increased serum TNF-α and IL-1β levels after 4 and 48 hours of thermal burn injury decreased with treatment Cot. Cog. extract. These results suggest that, Cot. Cog. has a preservative function against thermal injury associated inflammation. In a recent study, the anti-inflammatory activity of the ethyl-acetate fraction obtained from the dried bayonets of Cot. Cog. was investigated in a carrageenan-induced rat model [16]. The results of the study proved the significant anti-inflammatory activity of the Cot. Cog. ethyl-acetate fraction depending on the dose [16]. It was concluded that 100 mg/kg dose fraction caused a reduction in edema by 76.7% and showed a relatively more significant efficacy than indomethacin (53.8%). In another study, Matić et al. [30] evaluated the protective effect of a single dose of intraperitoneal injected Cot. Cog. methanol extract on the induction of acute phase response in terms of the levels of acute phase proteins in the liver. In this study, extract administration promoted the highest increase in acute phase reactants Hp and α2M 24 h after Cot. Cog. extract [30]. Based on our results supporting the literature, it can be said that burn healing effect of Cot. Cog. extract is achieved by inhibiting the release of proinflammatory cytokines.

MPO is a type of enzyme that is stored in neutrophils and macrophages [31]. As a biomarker, it is followed to determine the degree of inflammatory infiltration in the burn area [32]. The elevation of 8-OHdG in skin tissue is indicative of oxidative DNA damage [33]. Recent study has reported that antioxidants have a fundamental role in removing the products of inflammation [34]. They show these effects by preventing the destruction of fibroblasts and other cells caused by ROS and can heal burn lesions [35]. In the present study, MPO activity and 8-OHdG level was significantly increased in tissue samples following thermal burn. On the basis of results of our study we demonstrated that Cot cog extract when applied topically improves burn healing by reducing 8-OHdG level and burn-induced increased level of MPO in vivo burn injury model. These results suggest a potential application of Cot. Cog. extract in ointment form for the treatment of clinical burn wound healing.

Collagen, the main component of the extracellular matrix, liberates its constituents hydroxyproline and other peptides [36], and also plays an important role in the successful completion of wound healing [37]. The hydroxylation of proline in collagen determines the stability of the helical structure of collagen [38]. Hydroxyproline is a complementary part of collagen fiber, a predominant extracellular protein in granulation tissue [39]. Therefore, the measurement of hydroxyproline content is considered an index for collagen turnover [40]. An increase in hydroxyproline level points out increased collagen synthesis, indicating wound healing [41]. According to our results supporting the literature, thermal injury caused a decrease in hydroxyproline levels. It has been observed that the level of hydroxyproline is increased in burnt tissues treated with Cot. Cog. These results may indicate that the improvement in the skin with Cot. Cog. treatment may be due to the increase in hydroxyproline.

During the wound healing process, M2 macrophages suppress inflammation and provide angiogenesis [42]. PGE2, one of the eicosanoids, is released from M2 macrophages in burn injuries and increases inflammation in the tissue [43]. Although PGE2 has a role in the initial phases of wound healing, it is released at opposite stages of the healing process and has a role in the initiation of inflammation [44]. The findings observed in the current study show that it induces PGE2 production in skin tissue in a thermal burn model Cot. Cog. extract treatment decreased this increased PGE2 level, indicating its role in wound healing by inhibiting some mediators and cytokines as we noticed earlier.

As a result of the phytochemical analysis of the extract, it was observed that it contained phenolic compounds such as quinic acid, gallic acid, protocatechuic acid, methylgallate, myricetin glucoside, pentagalloyl glucose, myricetin rhamnoside, quercetin rhamnoside, ethylgallate, ethyl ester of digallic acid and myricetin. Gallic acid and methylgallate were also isolated from this species in a previous study by our team [45]. Phytochemicals, especially phenolics in medicinal plants, are the major bioactive compounds known for burn healing [46]. Also, pentagalloyl glucose [47], myricetin rhamnoside [48], quercetin rhamnoside...
analyses. Collected groups, respectively. Twice daily. Th shaved dorsums were dipped in a 25 ºC water bath for 10 s. With physiological saline (10 m

Cover burn groups (Cot. Cog.) Twenty

Animals Application and Research Center. All rats were fed with standard rat chow and tap water ad libitum, observed light/dark cycle and at room temperature at 25 ± 2ºC and humidity at 50%. The study was approved by Marmara University Animal Experiments Local Ethics Committee (Ethics committee no: 31.2017.mar).

Material (100 g) were placed in a Soxhlet apparatus and extracted with ethanol (96%) up to 24 h. The ethanol extract was obtained as powder residue after evaporation process in vacuo. This powder extract (Cot. Cog. Extract, yield: 38.94%, w/w) was stored at 4 °C and in the dark.

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5 MATERIALS AND METHODS

5.1 Plant material

The leaves of Cotinus coggygria were collected in the flowering periods from the wild from Kirklareli province, located in north-eastern Turkey and identified by Dr. Sukran Kultur (Department of Pharmaceutical Botany, Faculty of Pharmacy, University of Istanbul). Voucher specimens were kept at the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE No: 22260).

5.2 Extraction

Cotinus coggygria leaves were air-dried and grounded in an electric grinder into a fine powder. Ground material (100 g) were placed in a Soxhlet apparatus and extracted with ethanol (96%) up to 24 h. The ethanol extract was obtained as powder residue after evaporation process in vacuo. This powder extract (Cot. Cog. Extract, yield: 38.94%, w/w) was stored at 4 °C and in the dark.

5.3 Phytochemical analysis of Cot. Cog. Extract

Phytochemical analysis of Cot. Cog. Extract was carried out according to our previous study [56].

5.4 In vitro antioxidant activity of Cot. Cog. Extract

To determine the DPPH inhibition, the method described by Zou et al. [57] was used.

5.5 In vitro anti-inflammatory activity of Cot. Cog. Extract

The anti-inflammatory activity was evaluated by 5-lipoxygenase inhibition assay [58, 59].

5.6 Animals

Female/male Wistar Albino rats (300-350g) were obtained from Marmara University Experimental Animals Application and Research Center. All rats were fed with standard rat chow and tap water ad libitum, observed light/dark cycle and at room temperature at 25 ± 2°C and humidity at 50%. The study was approved by Marmara University Animal Experiments Local Ethics Committee (Ethics committee no: 31.2017.mar).

6.1 Thermo injury and experimental design

In the current study, the thermal injury model was constructed following a previous method [60]. Twenty-four rats were divided into three group (n=8): Control (C), vehicle-treated burn and Cotinus coggygria (Cot. Cog.)-treated burn groups. Under light ether anesthesia, after shaving of the dorsal region, rats in the burn groups were exposed to a 90 ºC water bath for 10 seconds. As a result, the exposure to heat damage covered 30% of the body and created a second-degree burn [61]. After this procedure, all rats were resuscitated with physiological saline (10 mL/kg; s.c.) due to their fluid loss. Control rats were also anesthetized and the shaved dorsums were dipped in a 25 °C water bath for 10 s.

After the thermal injury took place, a 5% of Cot. Cog. extract (w/w) ointment was applied topically twice daily. This ointment formulation was made by mixing 5 g of Cot. Cog. extract and 95 g simple ointment base. After that, the ointment was administered in aliquots of 0.5 g to the vehicle and the Cot. Cog-treated groups, respectively. Ointment treatment was applied both 4 and 48 hours after induced burn injury. Samples collected from dissection of the wound skin before decapitation took place, were divided to be used at analyses.

[49], etilgallate [50], gallic acid [50, 51], myricetin [52], quinic acid [53], protocatechuic acid [54, 55] have been previously reported to have wound healing activity. Therefore, these compounds, along with other compounds found in this plant, may be responsible for the burn wound healing activity of the extract.

4. CONCLUSION

In this study, ethanolic extract of Cotinus coggygria L. showed anti-inflammatory and antioxidant effects in both in vitro and in vivo, where these effects are attributed to the presence of phenolic compounds in the extract. To our knowledge, this is the first study to demonstrate that the topical application of extract of Cot. Cog. may provide burn-induced wound healing activity in rats, probably by inhibiting inflammatory cytokines and oxidative damage. Since inflammation, oxidative damage and collagenation are all interrelated and cascading processes, Cot. Cog. extract is believed to have the potential to heal burn-induced wounds by affecting one or more of these processes.

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Dorsal skin area (1 cm²) of rats with full-thickness was carefully excised, and then, excised samples washed with saline were weighed (g/cm²). Skin tissues were homogenized to obtain a 10% (w/v) homogenate solution with IKA brand Ultra-Turrax T25 (USA) homogenizer in cold PBS solution. After the homogenate samples were centrifuged at 3000 g for 10 minutes, their supernatants were carefully removed and stored at -80°C for biochemical analysis. Blood samples were collected via cardiac puncture while rats were under mild ether anesthesia after the treatment of 4 h and 48 h induced burns. Blood samples were centrifuged at 1800 g for 15 minutes at room temperature, and immediately after, serum samples were separated and stored at -20°C before analysis.

6.8. Biochemical analyses

Serum inflammatory biomarker levels tumour necrosis factor (TNF-α) and interleukin 1β (IL-1β) were evaluated using enzyme-linked immunosorbent assay (ELISA) kits (LifeSpan Biosciences). The resulting color conversion was measured at 450 nm (BIOTEK, Epoch Elisa Reader). Results are given in ng/mL. Myeloperoxidase (MPO) activity, 8-hydroxy-2′-deoxyguanosine (8-OHdG), prostaglandine (PGE2) and hydroxyprolin levels were evaluated in skin tissues homogenates using commercially available kits (cat no; E0031Ra, E0511Ra, E0504Ra, Bioassay Technology Laboratory, China). All procedures were performed according to the manufacturer’s instructions.

6.9. Histological Preparation of Skin Samples

Skin tissue samples for light microscopic evaluation were fixed in 10% formaldehyde solution and processed routinely for embedding in paraffin. Paraffin sections (5 µm thick) were stained with Masson’s trichrome to indicate histological evaluation. Stained sections were examined with light microscope (Olympus BX51, Tokyo, Japan) and photographed with a digital camera (Olympus DP72, Tokyo, Japan).

6.10. Statistical analysis

The data were given as means ± standard error of mean (SEM) and analyzed by one-way analysis of variance (ANOVA) and the Tukey’s multiple comparison tests using GraphPad Prism 8.0. Values were considered significantly different at \( p < 0.05 \).

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