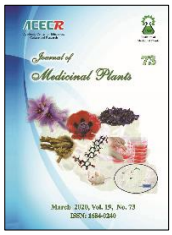
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Research Article

Wound healing activity of the flowers of *Lilium candidum* L. in burn wound model in rats

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ABSTRACT

Background: Ethnomedicinal plants are of interest for treatment of various wound types. **Objective:** This study aimed to evaluate burn wound healing potential of the hydro-alcoholic extract of the flowers of *Lilium candidum* L. in rats. **Methods:** DPPH free radical scavenging power, total flavonoids, and the phenolic content of the extract were evaluated. Following burn wound induction, 30 Wistar rats were divided into 5 groups; silver sulfadiazine cream 1% (reference standard), eucerin (control), and 5%, 10% and 20% ointments of *L. candidum* flowers extract (treatment groups). Wound contraction and the percentage of wound healing were measured every day until 14th day and the histological changes were evaluated. **Results:** DPPH assay of *L. candidum* extract indicated high antioxidant activity. Total phenolic content of *L. candidum* extract was equal to 157 mg gallic acid equivalent/g; while total flavonoid was measured as 32.4 mg quercetin equivalent/g dried extract. The mean of wound area was significantly different in three 5%, 10% and 20% extract-treated rats in comparison with the control. Although, 20% ointment of *L. candidum* flowers extract demonstrated the best wound healing improvement, even better than silver sulfadiazine. Histopathological analysis confirmed the wound healing activity of *L. candidum* and promoting tissue repair, especially for the ointment containing 20% extract. **Conclusion:** Regarding the high content of phenolic compounds and flavonoids, besides potent antioxidant properties of the flowers of *L. candidum*, the use of its ointments led to significant decrease of burn wound area, suggesting that these extract ointments can treat burn wounds and improve the healing processes.

Abbreviations: HSV, Herpes Simplex Virus-1; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; BHA, Butylated hydroxylanisole; $AlCl_3$, Aluminium chloride; NaOH, Sodium hydroxide; SSD, Silver Sulphadiazine; IP, intra peritoneal; VEGF, vascular endothelial growth factor; TGF-β, Ang-1/Tie-2, transforming growth factor beta

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1. Introduction

Burn is a kind of skin or tissue injury caused by heat, electricity, chemicals, or radiation [1]. The severity of burns can be specified by the depth and the degree of skin damage [2]. Burns that only involve the outer layer of the skin (epidermis) is known as the first-degree burn. In this type of burn, skin usually become red and very painful with an obvious dry appearance [3]. In second-degree burns, both the epidermis and the second layer of the skin (dermis) are involved, inducing white or red color, pain, swelling, and dryness. In third-degree burns, there is a destruction in all layers of the skin with dry appearance and no pain [4]. Burn injuries may cause physical disabilities as well as mental and emotional illness, even sometimes there is a possibility of mortality [5, 6]. The healing process includes inflammatory, proliferative and remodeling phases [7], nevertheless, burn healing is an ongoing challenge in modern medicine [1]. Despite the existence of various chemical products for burn treatment, there is a growing trend for natural products and traditional medicine worldwide [8]. In a comprehensive review, Bahramsoltani et al. reported that single herbal preparations including *Allium sativum, Aloe vera, Centella asiatica,* and *Hippophae rhamnoides* were the most effective species for burn wound healing [9]. Herbal-based remedies/medications with potent antioxidant and anti-inflammatory activities are frequently used to treat burn injuries, and can accelerate the healing process [10].

Lilium candidum L. (popularly known as the Madonna lily) from Liliaceae, is confirmed to improve the symptoms of a verity of ulcers, inflammations, wounds, and burns. Yet, there are limited data about the phytochemical composition of the plant [11, 12]. *L. candidum* contains important phytochemicals; mainly flavonoids (quercetin, isorhamnetin, kaempferol) and other phytochemicals such as organic acids, glycosides, nitrogenous, steroidal compounds, saponins, and jatropham [11, 13, 14]. Moreover, white lily flowers are rich in carotenoids, anthocyanins, phenyl propanoids, and essential oil components [15, 16]. The extracts of *L. candidum* were found to possess antibacterial, antifungal, antioxidant, anti-tumor, and anti-yeast activities [17-20]. In traditional medicine, the flowers of *L. candidum* were known to have anti-inflammatory properties [21]. It is believed that the flavonoid constituents of lily flowers are responsible for its anti-inflammatory activities, which was attributed to the cyclooxygenase inhibition [22].

It was evidenced that the leaf extract of *L. candidum* strongly inhibited the Herpes Simplex Virus-1 (HSV) - 1, while slightly affected HSV-2 [23]. In folk medicine, the whole parts of the plant were used to lighten the skin, especially for periocular hyperpigmentation [11, 24]. The steroidal glycosides isolated from Easter lily (*L. longiflorum*) bulbs enhanced the dermal fibroblast migration activity, a model for the early events in wound healing [7]. In this paper, the wound healing property of an ointment containing the hydro-alcoholic extract of *L. candidum* in rats, besides, its antioxidant effects and phenolic content are reported.

2. Materials and Methods

2.1. Chemicals

2,2- diphenyl-1- picryl- hydrazyl- hydrate (DPPH), Folin ciocalteu, Butylated hydroxylanisole (BHA) were purchased from Sigma Chemical Company (USA). Aluminium chloride ($AlCl_3$), Sodium hydroxide (NaOH), Sodium nitrite ($NaNO_2$), Sodium bicarbonate, Gallic acid, Ethanol, Formalin, Ketamine, Xylazine were provided by Merck company

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(Germany). Eucerin (Farabi, Iran) and Silver Sulphadiazine (SSD) 1% cream (Najo, Iran) were purchased from Iranian companies.

2.2. Plant materials

Flowers of *L. candidum* were collected during February 2015 from a local flower market in south of Tehran province, Iran, and were identified by Professor Gh. Amin. Voucher specimen was deposited at the herbarium of the Faculty of Pharmacy, Tehran University of Medical Science, Tehran, Iran (No: 7072-TEH).

2.3. Extraction

Flowers were shadow-air-dried at room temperature and 120 g of dried flowers were pulverized, and extracted using the percolation method. Every 24 hours, three sequential extractions were performed, using a mixture of ethanol/water (70:30 v/v). The extract was concentrated by a rotary vacuum evaporator (50 °C, 100 rpm) [20].

2.4. DPPH free radical scavenging activity

The DPPH scavenging activity was measured following the method published by Fahimi et al., 2015 [5, 25]. The experiment was carried out in triplicate and methanol was used as blank. The radical scavenging activity was calculated as the percentage of DPPH discoloration using following formula:

Inhibition (%) = 100 - [(sample absorbance - control absorbance)/ (blank absorbance)]

Where the sample absorbance is the absorbance of different extract concentrations mixed with DPPH, the control absorbance is the absorbance of the mixture of the extract and methanol, and the blank absorbance considered as the absorbance of DPPH with ethanol without any extract.

2.5. Total phenol assay

The phenolic content of the plant extract was determined using Folin-ciocalteu reagent, and the total phenolic content was expressed as gallic acid equivalent/g extract [26].

2.6. Total flavonoids assay

The reaction mixture contained the extract, 70% ethanol, and $NaNO_2$. After a while, $AlCl_3$ and NaOH were added, and later, the absorbance was measured at 510 nm against the blank [27]. Total flavonoid content was expressed as microgram (µg) quercetin equivalent in mg extract (µg quercetin/mg extract).

2.7. Preparation of the ointment

The ointment was prepared using 5%, 10%, and 20% of *L. candidum* extract in an eucerin base (25%), while SSD cream 1% was used as the reference standard. The concentrations of the extract were selected based on data obtained by our pilot study.

2.8. Animal study

Male Wistar rats (N= 30, 6 in each group, 200 ± 30 g) were kept under standard conditions; temperature (25 ± 1 °C), humidity (55% ± 10%), and 12/12 hours (h) light/dark cycle with full access to the standard pellet diet and tap water. Animals were kept in accordance with the instructions provided by the Tehran University of Medical Sciences Review Board and the experiments were performed following the approval of ethical committee of animal experimentation, Tehran University of Medical Sciences (TUMS), Tehran, Iran. Animals were anaesthetized by intra peritoneal (IP) injection of ketamine/xylazine. The dorsal part of the skin was shaved and a full-thickness circular 1cm diameter second degree burn wound was created using an electrical heater (110 °C heat for 10 sec) [5].

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Rats were divided into 5 groups including; SSD cream 1% as the reference standard, eucerin as the control, and 5 %, 10 % and 20 % ointments of *L. candidum* flowers extract as the treatment groups, starting right after burn wound induction. Ointments were used topically over the wounds every day for 14 days [5]. The wounds areas were cleaned, photographed with a digital camera and calculated using Adobe Photoshop CS5. The wound contraction rate was measured according to this formula:

Wound contraction % = 100 × [(first day wound area - specific wound area)/ first day wound area]

On the day 14 (the end of experiment), animals were sacrificed, the granulated tissues were collected, and preserved in buffered formalin 10% to evaluate the histological changes. Series of 3-4 µm thickness sections were prepared for each sample, stained with hematoxylin/eosin, and microscopic photographs were captured under × 400 magnification.

2.9. Statistical analysis

Data were analyzed using the SPSS 20 software (one-way ANOVA and Tukey`s *Post hoc* test). $P \leq 0.05$ were statistically considered significance.

3. Results

3.1. Plant material yield

The extraction procedure yielded to 51.1% dry extract.

3.2. DPPH free radical scavenging activity

The 50% inhibitory concentration of DPPH radical scavenging activity was equal to 248 mg/ml (Table 1).

Table 1. The antioxidant content of *L. candidum* extract

DPPH IC_{50} (mg/ml)	Total phenol (mg of gallic acid equivalent/g extract)	Total flavonoids (mg of quercetin equivalent/g extract)
248	157	32.4

DPPH: 1, 1-diphenyl-2-picrylhidrazyl, IC_{50}: inhibitory concentration 50%

3.3. Total phenols

Total phenolic content of *L. candidum* extract was calculated using standard curve of gallic acid ($y=0.454x+0.139$, $R^2=0.997$), which was equal to 157 mg gallic acid /g dried extract (Table 1).

3.4. Total flavonoids

Total flavonoid content was measured as 32.4 mg quercetin equivalent/g dried extract using this formula:

$$y = 0.002x + 0.0227$$

Y is the absorbance of solution at 510 nm, X is total flavonoid content of the extract as µg quercetin equivalent in mg extract (Table 1).

3.5. Rate of wound healing

The percentage of wound healing enhanced in all animal treated groups during 14 days of the study. The wound area was significantly reduced in rats treated with *L. candidum* ointment compared with both positive and negative controls ($P < 0.001$). On the 14th day, 20% extract group exhibited the best wound healing effect in comparison with other groups ($P < 0.001$). Table 1 shows the rate of wound healing in *L. candidum* ointment treated groups (Table 2).

3.6. Histopathological analysis

In SSD treated group, the wound has almost been repaired and the epidermal layer is completely formed. In some areas, layers were

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thin. Epidermal and dermal junction folder (that support the skin), and the auxiliary parts such as hair follicles were not formed. Collagen bundles were formed in most parts of the dermal layers, although it was less than that of the 20% group. Tissue inflammation was significantly diminished; but the inflammatory cells are still observed in some areas. In non-treated group, the wound area has not been repaired with significant depth. Extensive hyperemia and inflammation and copious exudate are seen. Granulation tissue at rim of the wound is under development (Fig. 1).

Wound was covered with granulation tissue in rats treated with ointment containing 5% extract. Wound depth was less than the control group; however, the inflammation and hyperemia are still significant. Epidermal repairing was started at rim of the wound and collagen bundles are not observed in dermal layers. The application of 10% extract caused a complete wound healing in this group. The epidermis was completely formed, but in some areas was thin. Epidermal and dermal junction folder and the auxiliary parts were not formed. Collagen bundles have been disappeared in dermal layers. Tissue inflammation and hyperemia are still observed in most areas. In group of ointment with 20% extract, wound was repaired, and the epidermal layer was completely formed. Epidermal and dermal junctions were formed in most areas, and a few hair follicles grown. Collagen bundles were formed in most parts of the dermal layers, and tissue inflammation was significantly diminished (Fig. 1).

Table 2. Wound healing percentage in experimental groups

Groups	14th day
Control	56 ± 0.035
SSD	73.5 ± 0.018[a]
5% Extract	80.6 ± 0.027[a]
10% Extract	89 ± 0.066[a,b,c]
20% Extract	94 ± 0.048[a,b,d]

Values are presented as Mean ± SEM
Abbreviations: SSD, silver sulfadiazine
[a] significantly different from control, $P < 0.001$
[b] significantly different from SS, $P < 0.001$
[c] significantly different from 5%, $P < 0.05$
[d] significantly different from 5%, $P < 0.001$

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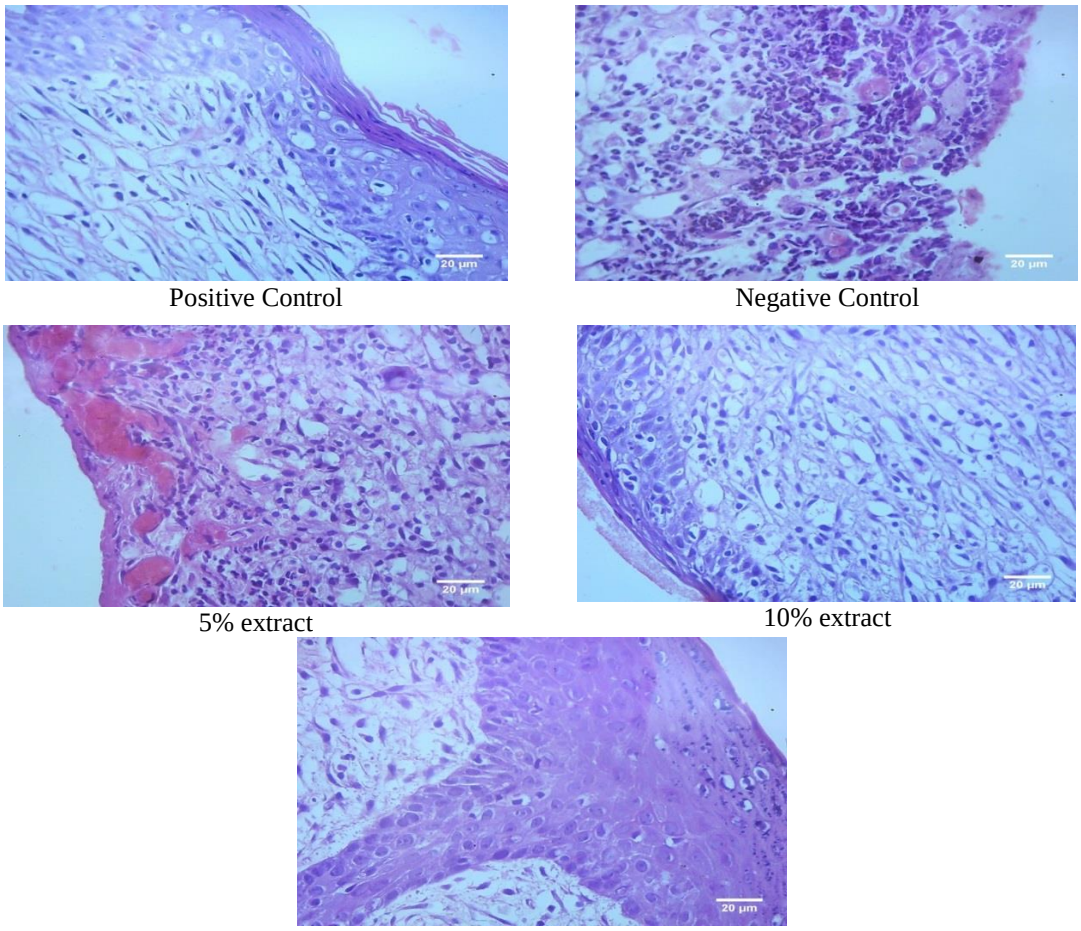


Fig. 1. Microscopic examination of skin tissue samples with magnification of ×400 (Day 14th).

4. Discussion

Wound healing refers to a sequence of complicated events including inflammation, re-epithelialization, granulation, neovascularization, and wound contraction. Upon a thermal injury, neutrophils accumulate at the lesion site, which stimulate the reactive oxygen species (ROS), and the proteases overproduction. This can trigger the release of the interstitial fluid, leading to the endothelial cells, and skin damage. Excessive production of free radicals and proteases impairs the production and release of growth and angiogenic growth factors from macrophages and keratinocytes, delaying skin repair.

In this manner, herbal-based formulations containing a variety of phytochemicals with remarkable efficacy and safety can interfere the wound healing process by means of antimicrobial, anti-inflammatory, antioxidant, collagen synthesis stimulation, cell proliferative, and angiogenic effects [9]. Well-orchestrated alliance between various cells (i.e. immunocytes, macrophages, neutrophils, lymphoid cells), local release of various growth factors, and cytokines can affect the rate of wound repair; while disruption in this process causes delayed wound healing [28]. Due to their broad vulnerability for infections, burn wounds require fast treatment. Up to date, many topical preparations have been formulated; however, instead of wound healing properties, most of them possess antimicrobial activities and may have insufficient efficacy and

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can induce toxicity (i.e. SSD) [29]. Both plant extracts and a series of phytochemicals (i.e. alkaloids, flavonoids, terpenes, glycosides) showed pro-wound healing effects *in vivo* and *in vitro* models, and are of great interest, mainly due to their non-toxic properties. In addition, herbal preparations are able to interfere in each and every steps of wound healing phases [30]. Therefore, there is constant request for therapeutic strategies to treat general and chronic ulcers, particularly burn wounds, diabetic foot ulcers, venous and arterial leg ulcers, and pressure ulcers.

Topical application of the ointment of 20% extract showed the highest wound healing properties *in vivo* in comparison with all other test materials. In addition to the better result compared with the control, the ointment of 20% *L. candidum* extract demonstrated a significant enhancement of wound closure percentage, even higher than that of the positive control group. In the context of wound areas, there were considerable developments of re-epithelialization, cell proliferation, and migration in 20% *L. candidum* extract treated group.

As mentioned, flavonoids are the main constitutes of the genus *Lilium*. In addition, flavonoid compounds are famous for their significant antioxidant potentials. There are various evidence confirming the effectiveness of flavonoids in managing the chronic foot ulcers [31]. Treatment with hesperidin, a flavonoid compound abundantly present in citrus species, enhanced the angiogenesis, and vasculogenesis via up-regulation of relevant genes (i.e. vascular endothelial growth factor (VEGF), Ang-1/Tie-2, transforming growth factor beta (TGF-β), and Smad-2/3) mRNA expressions, representative of wound healing in chronic diabetic foot ulcers in rats [32]. Lidhi et al. suggested that an ointment containing flavonoid-rich fractions of *Martynia annua* and *Tephrosia purpurea* were more effective than isolated flavonoids in wound healing, which was attributed to the synergistic interactions between the flavonoids and other constituents [33]. The main mechanism that flavonoids coordinate in wound healing is proposed to be collagen synthesis and/or degradation including the acceleration of collagen synthesis, decrease of soluble collagen degradation, and inhibition of catabolism of soluble collagen. It is worth mentioning that clinically, collagen deposition in wound sites is the most important phase of healing [34], whereby the high flavonoid content of *L. candidum* might be a possible explanation for its wound healing property. On the other hand, DPPH radical scavenging activity and the high phenolic content of the extract, make it an eligible candidate for burn wound healing through an antioxidant mechanism. In accordance, previous studies have also confirmed the antioxidant properties of many species of genus *Lilium* [35, 36]. It has been well established that oxidative stress impairs the wound healing process, in turn, the antioxidant agents accelerate the repair stages and increase the healing potential [37, 38]. Overall, special chemical composition of the flowers of *L. candidum* in concomitant with its antioxidant, antibacterial, and anti-inflammatory assets leads to wound healing improvement in rats.

5. Conclusion

In terms of histopathological evaluations, the ointment of 20% *L. candidum*-treated wounds showed better granulation tissue, empowered by thick regenerated epithelial layer. These findings reveal that the high phenolic and flavonoid contents of *L. candidum*, besides its known antioxidant potential caused significant burn wound healing in rats. However, future studies

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should focus on the antibacterial and anti-inflammatory properties of this species. Further, the mechanistic examination of the molecular and cellular pathways behind the wound healing effect of *L. candidum* is emerging.

Author contributions

Mahshid Dibaj, Roodabeh Bahramsoltani, and Parvin Mahdaviani: Conduction of practical parts, collection and/or assembly of data and interpretation, manuscript writing; Alireza Abdollahi: Pathophysiological assessment; Gholamreza Amin: plant material collection and identification; Saeideh Momtaz, Mohammad Abdollahi and Amir Hossein Abdolghaffari: provision of study material, conception and design, and final approval of manuscript. All the authors have read and approved the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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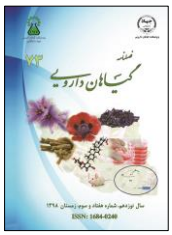
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جهاد دانشگاهی

مقاله تحقیقاتی

اثر عصاره گل‌های گیاه *Lilium candidum* L. بر بهبود زخم در مدل زخم سوختگی در موش صحرایی

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اطلاعات مقاله

گل واژگان:
Lilium candidum L.
سوختگی
فلاونوئید
فنل‌ها
ترمیم زخم

چکیده

مقدمه: گیاهان دارویی برای درمان انواع مختلف زخم مفید هستند. **هدف:** این مطالعه با هدف ارزیابی پتانسیل بهبود زخم سوختگی توسط عصاره هیدروالکلی گل‌های *Lilium candidum* L. در موش صحرایی انجام شد. **روش بررسی:** ظرفیت آنتی‌اکسیدانی عصاره به روش DPPH، توتال فلاونوئید و محتوای فنلی عصاره مورد بررسی قرار گرفتند. پس از القای زخم سوختگی، موشها به ۵ گروه تقسیم شدند: کرم سولفادیازین نقره ۱ درصد (استاندارد مرجع)، اوسرین (کنترل)، و عصاره گیاه با دوزهای ۵، ۱۰ و ۲۰ درصد که پماد آنها بر پایه اوسرین تهیه شد. میزان انقباض و درصد ترمیم زخم تا روز چهاردهم اندازه گیری شد و تغییرات بافت‌شناسی مورد بررسی قرار گرفتند. **نتایج:** فعالیت آنتی‌اکسیدانی بالای عصاره توسط تست DPPH نشان داده شد. توتال فنل عصاره ۱۵۷ میلی‌گرم معادل اسید گالیک/گرم بود. در حالی که توتال فلاونوئید عصاره خشک معادل ۳۲/۴ میلی‌گرم کورستین/ گرم اندازه‌گیری شد. میانگین سطح زخم در سه گروه موش تحت تیمار با عصاره نسبت به شاهد تفاوت معنی‌داری داشت. اگرچه، پماد حاوی ۲۰ درصد عصاره بالاترین میزان بهبود زخم در مقایسه با دیگر گروه‌های درمانی، حتی بهتر از سولفادیازین نقره نشان داد. نتایج بافت‌شناسی، فعالیت ترمیم زخم و بافت توسط عصاره را تأیید نمود، به ویژه برای پماد حاوی ۲۰ درصد عصاره. **نتیجه‌گیری:** با توجه به محتوای بالای ترکیبات فنولیک و فلاونوئیدها در عصاره، به علاوه خاصیت آنتی‌اکسیدانی بالای گل‌های لیلیوم که موجب کاهش چشمگیر سطح زخم سوختگی شده است، پیشنهاد می‌شود که پماد حاوی این عصاره می‌تواند زخم‌های سوختگی را درمان کرده و روند بهبودی را تسریع نماید.

مخففها: VHS، ویروس هرپس؛ DPPH، ۲،۲- دی فنیل-۱- پیکریل هیدرازیل؛ BHA، هیدروکسیل آنیزول بوتیله؛ $AlCl_3$، کلرید آلومینیوم؛ NaOH، هیدروکسید سدیم؛ SSD، سولفادیازین نقره؛ IP، تزریق داخل صفاقی؛ VEGF، فاکتور رشد اندوتلیال عروقی؛ TGF-β، فاکتور تبدیل رشد بتا

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