



Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: <http://www.elsevier.com/locate/jtcme>Efficacy of topical application of standardized extract of *Tragopogon graminifolius* in the healing process of experimental burn wounds

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ARTICLE INFO

Article history:

Received 13 February 2017

Received in revised form

26 January 2018

Accepted 18 February 2018

Available online 27 April 2018

Keywords:

Tragopogon graminifolius

Second-degree burn

Oxidative stress

Medicinal plant

ABSTRACT

Tragopogon graminifolius DC. is a perennial plant from the family Asteraceae which grows in West parts of Iran. Several biological activities like antimicrobial, antioxidant and anti-inflammatory effects are reported for the plant. The aim of this study was to assess the wound healing activity of standardized extract from *T. graminifolius* (TG) aerial parts. Topical standardized TG extract with 5% and 10% concentrations in eucerine base was assessed for its healing properties on second degree burn in rats during a 14-day period. Biomarkers of oxidative damage including total antioxidant power, lipid peroxidation and total thiol molecules of the skin tissue samples were also evaluated. Results showed that 10%TG had the best efficacy with $80 \pm 3\%$ wound closure and tissue repair in comparison to negative control ($p < 0.05$). Significant reduction of tissue oxidative stress biomarkers was also observed. Histological analyses confirmed wound healing activity of TG extract, as well. Considering the antioxidative stress and anti-inflammatory activities of TG, explained by the high content of phenolic compounds of the plant, standardized TG extract could be considered as a natural remedy for the treatment of burn wounds. Further clinical studies are suggested to confirm the effectiveness of TG as a wound healing agent.

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1. Introduction

Skin is a vital organ which performs several functions such as thermoregulation, homeostasis, metabolic, neurosensory and immunologic functions. It is also a physical barrier against infections; thus, when it is injured, pathogens can have a direct access to the deep tissues.¹

Burns, defined as tissue damage by heat or corrosive chemicals,

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

are one of the most common causes of skin injuries. Extensive tissue damaged during burns impairs angiogenesis, collagen reorganization, granulation tissue formation and induces free radical-mediated damage which results in delayed tissue repair. Recovery from burn wound needs long hospital stay, expensive medications and long rehabilitation period.² As one of the first lines of treatment, topical antibacterial agents are used to prevent infection; though, they can cause allergic reactions which postpone the healing process.^{3,4} Another treatment approach is to use recombinant growth factors and tissue engineered wound dressings which are too expensive for a relatively high number of the patients; thus, investigations for the discovery of new wound healing agents are still required.

One of the available options in burn wounds management are

medicinal plants which provide a wide area of research for scientists due to the vast diversity of phytochemicals with antioxidant, anti-inflammatory and immunomodulatory effects.^{5–7}

Tragopogon graminifolius DC. is a perennial plant from the family Asteraceae which grows in the West parts of Iran and is locally known as “Sheng”. In Persian medicine, *T. graminifolius* (TG) has been known as a hemostatic, wound healing, detoxifying and hepatoprotective agent, and is used for several gastrointestinal complications. Studies showed that different species of the genus *Tragopogon* contain phytochemicals such as flavonoids including luteolin, vitexin, isovitexin, vicenin –1 and 2, and orientin.^{8–10} Farzaei et al. reported the presence of five phenolic compounds including *p*-coumaric acid, ferulic acid, catechin, gallic acid and caffeic acid in the hydroalcoholic extract of *T. graminifolius* aerial parts.¹¹ TG essential oil contains *n*-hexadecanoic acid, β -carophyllene, heneicosane and nonanal as major components. The plant also showed significant antioxidant properties.¹¹ As oxidative stress has a crucial role in the pathogenesis of burns and wounds, potent antioxidant activity is of a great interest for the treatment of skin tissue injuries. TG have demonstrated different pharmacological activities in recent studies including amelioration of TNBS-induced inflammatory bowel disease as well as ethanol-induced peptic ulcer disease in rats.^{12,13}

Due to previously reported anti-inflammatory and antioxidant properties of TG, we hypothesized that the plant can be used as a wound healing agent in skin damage. The goal of the present study is to investigate the wound healing properties of standardized extract from TG aerial parts in animal model of second-degree burn wound along with the assessment of oxidative damage biomarkers in skin tissue.

2. Method and materials

2.1. Chemicals

Ethanol, distilled water, formalin 10%, ketamine, xylazine, normal saline (0.9%), eucerine (400%), hematoxylin, eosin, silver sulfadiazine 1% cream USP, and sodium bicarbonate were supplied from Merck company (Germany).

2.2. Plant material and preparation of standardized extract

TG was collected in July 2012 from Kermanshah province, West of Iran and was authenticated by Dr. F. Attar (Department of Biology, Faculty of Sciences, University of Tehran). A voucher specimen (No.43603) was deposited in the Central Herbarium of University of Tehran.

Aerial parts of TG were air-dried in shadow and powdered by mechanical grinder. Powdered aerial parts (100 g) were extracted 3 times with 80% ethanol. The obtained extracts were mixed and the solvent was evaporated under reduced pressure using rotary evaporator.¹⁴ As described in our previous report,¹¹ the extract was standardized based on five phenolic compounds (gallic acid, *p*-coumaric acid, Ferulic acid, catechin and caffeic acid) using high performance liquid chromatography (HPLC) method and the standardized extract was used for further experiments.

2.3. Ointment preparation

Eucerine is a well-known emulsion base which can absorb a high portion of aqueous or oily solutions and thus, can be used for the preparation of several types of creams and ointments.¹⁵ Ointments were prepared with concentration of 5% and 10% of the extract in eucerine. Silver sulfadiazine 1% and eucerine were used as positive and negative controls, respectively.

2.4. Burn wound induction

Twenty four male Wistar rats, weighing 200–250 g, were divided into 4 groups of 6 animals in each group. The rats were individually housed under standard animal lab condition ($25 \pm 1^\circ\text{C}$, humidity 60%, 12 h light/12 h dark cycles) and fed with standard laboratory food and water *ad libitum*. Treatments include TG 10% extract (TG10), TG 5% extract (TG5), silver sulfadiazine (C+) and eucerine (C-). All animal experiments were according to laboratory animal ethics guidelines.

Anesthesia was induced by intraperitoneal injection of pentobarbital (50 mg/kg). Dorsal parts of animals were shaved and burn wounds were created on the shaved area of rats using a burn set with an aluminum rod (1.5 cm) with 110°C heat and exposed for 10 s under 1 atm pressure. Animals were kept separately (one in a cage). Treatment started 24 h after burn wound induction. Ointments were daily applied to cover all over the wound and the wound dressed by a regular dressing. Dressing was changed every 24 h for 14 days.

Using a digital camera, wound areas were photographed every 24 h and a ruler was used as scale. At the end of the study, pictures of each day were analyzed by Adobe Photoshop CS3 software to measure wound contraction. Wound contraction rate was calculated by the following formula:

$$\text{Wound contraction (\%)} = \left[\frac{(\text{wound size of the induction day} - \text{wound size of the specific day})}{\text{wound size of the induction day}} \right] \times 100$$

At the day of 14, all rats were sacrificed and skin tissues of the burnt area were collected. Each sample were cut into pieces; one part was kept in formalin 10% for evaluation of histological changes. The other half was preserved in -80°C for assessing total thiol molecules (TTM), lipid peroxidation (LPO) and total antioxidant power (TAP).

2.5. Histological analysis

Hematoxylin and eosin were used to stain tissue sections and microscopic images of tissue were taken under $\times 400$ magnification.¹⁴

2.6. Tissue oxidative stress biomarkers assessment

Samples were floated in phosphate-buffered saline and then homogenized with Heidolph Silent Crusher M (Germany). Then, the samples were centrifuged at 3000 g at 4°C for 15 min and supernatant fluid was kept for further analyses.¹⁶

2.7. TTM assessment

For TTM assessment, the collected supernatant fluid was mixed with Tris-EDTA buffer (EDTA 20 mM, Tris base 0.25 M pH: 8.2) and

Table 1
Wound contraction rate (%) in the 14th day of animal experiment.

14th day	Treatment
57.80 ± 5.71	C-
$78.80 \pm 3.96^{**}$	C+
$73 \pm 0.1^*$	5% TG
$80 \pm 0.3^{**}$	10% TG

Values are expressed as mean $\pm$ standard deviation for each group. Significant differences in comparison to C-: *: $P < 0.05$, **: $P < 0.01$, TG5: *Tragopogon graminifolius* extract (5%), TG10: *T. graminifolius* extract (10%).

the DTNB reaction with total sulfhydryl groups to achieve a chromogen material and the absorbance was measured at 412 nm.^{16,17}

2.8. LPO assessment

For the determination of LPO, reaction of 2-thiobarbituric acid reducing substance (TBARS) method with lipid peroxides was used. Tissue extract was mixed with trichloroacetic acid (20%) and the precipitant was dissolved with H₂SO₄ (0.05 M). After that, 2-Thiobarbituric acid (TBA, 0.2% in 2 M sodium sulfate) was added and heated for 30 min in boiling water bath. The final mixture was reacted with *n*-butanol and the absorbance was measured at 532 nm (ELISA reader, Biotek, Germany).¹⁶

2.9. TAP assessment

Total antioxidant power was measured by assessing the ability of tissue extract to reduce Fe³⁺ to Fe²⁺. FRAP reagent (25 ml of sodium acetate (300 mM), 2.5 ml 2,4,6-tripyridyl-*s*-triazine (TPTZ) (10 mM) and 2.5 ml FeCl₃·6H₂O (20 mM) (pH 3.6)) was mixed with super natal fluid and the absorbance was determined at 593 nm.^{16,18}

2.10. Statistical analysis

Results were expressed as the mean ± standard deviation. All collected data was analyzed with one-way ANOVA test (SPSS 15 software) and *P* < 0.05 showed statistical significance.

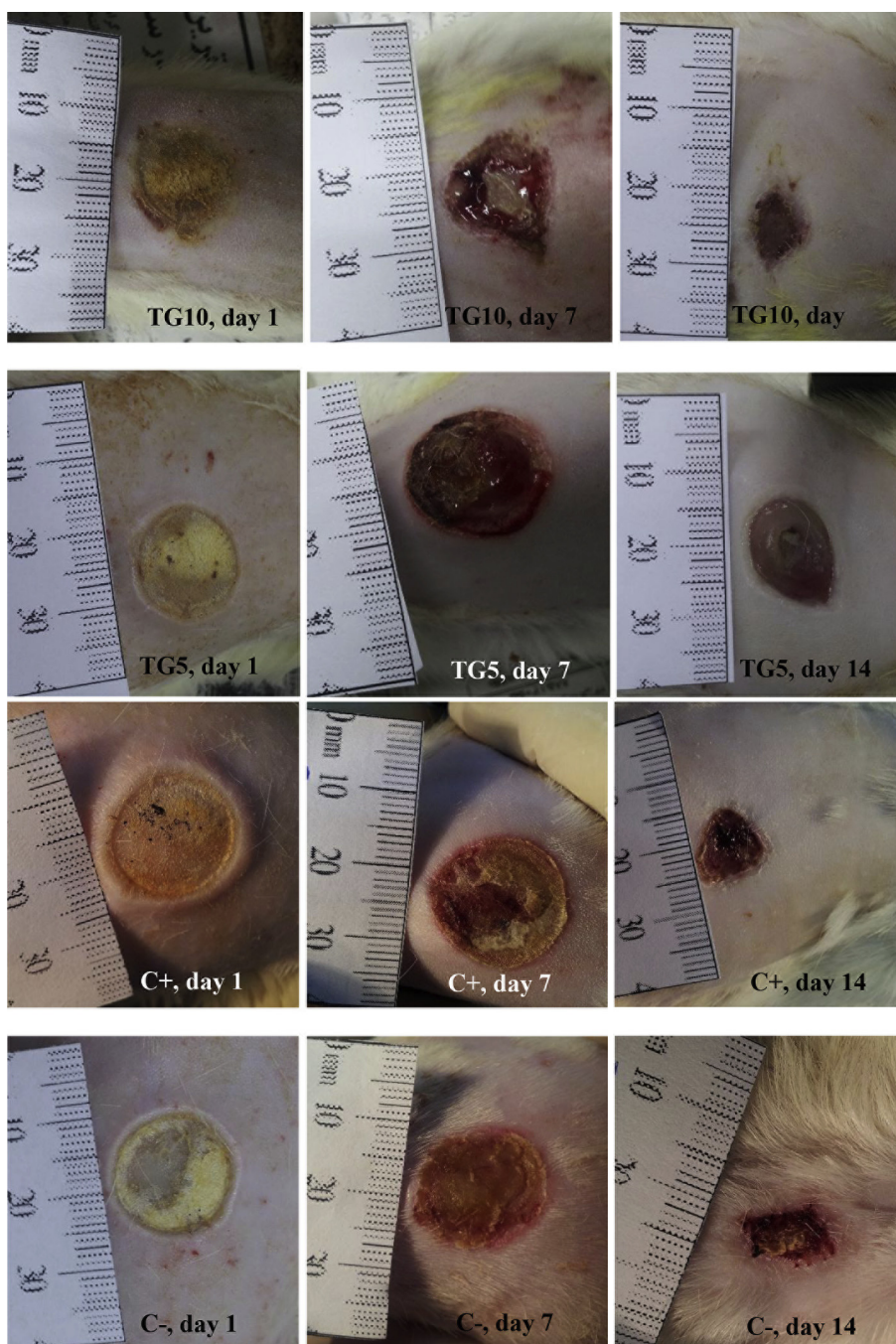


Fig. 1. Macro-area of wounds in 1st, 7th, and 14th day of experiment. TG: *Tragopogon graminifolius*.

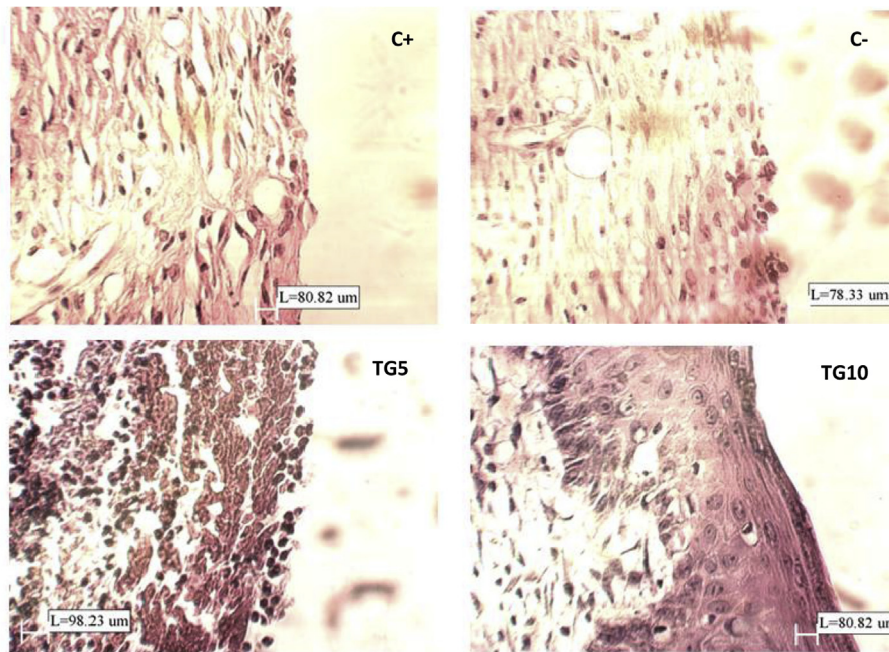


Fig. 2. Microscopic images of skin tissue samples of 14th day with magnification of $\times 400$.

C+: PMNs population decrease and epithelium regeneration is observable in wound edge but not in wound center (neovascularization: +).

C-: high density of blood vessels and no re-epithelialization, only reduction in PMNs and macrophages (neovascularization: +).

TG5: high density of blood vessels and connective cells, no healing and extreme bleeding in dermis layer (neovascularization: ++++).

TG10: re-generation is almost complete, only stratum keratinosum need to be complete.

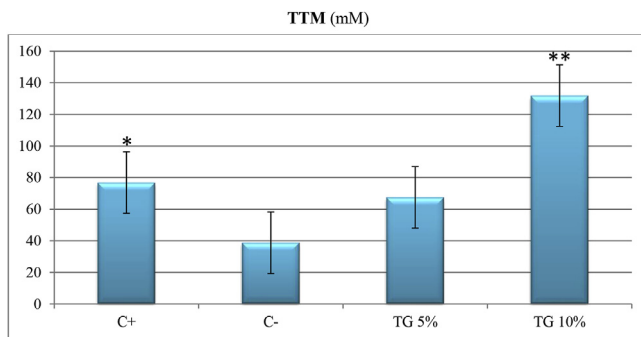


Fig. 3. Comparing effect of different treatments on total thiol content of skin tissue on day 14.

TTM: total thiol molecules. C-: negative control, C+: positive control, TG5: 5% extract ointment, TG10: 10% extract ointment. Significant difference in comparison to negative control: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

3. Results

3.1. Wound closure and healing process

Table 1 shows the wound closure percentage in the last day of experiment. In negative control group, the wound area reached to nearly half of the zero day wound size. Ointment of 5% TG showed a significantly better wound healing effect in comparison with negative control ($p < 0.05$). TG 10% ointment showed the best wound healing effect with 80 ± 0.3 reduction in wound size compared with the day of wound induction which was significantly higher than negative control ($p < 0.01$). This effect was numerically (but not significantly) better than silver sulfadiazine (Table 1). Fig. 1 shows the macro-area of the wounds in different groups.

3.2. Histological analysis

The collected tissue samples of each group on 14th day were assessed under $\times 400$ magnification. One photograph of each group is shown in Fig. 2.

In C+ group, the number of polymorphonuclears (PMNs) was decreased in the wound edge. Basal epithelium regeneration was observed in wound margins, but not in wound center. Fibroblasts density was relatively high and collagen bundles had an irregular appearance.

In C-, re-epithelialization was not occurred, but the number of macrophages and PMNs were started to decrease and blood vessels had a high density.

In TG5 group, despite the 14 days of treatment, there was an extreme bleeding and no healing in dermis layer was occurred. Blood vessels and connective cells density was high.

In TG10 group, epidermis was regenerated and different layers of dermis was repaired, but stratum keratinosum was not yet completely formed.

3.3. Tissue oxidative stress biomarkers

Silver sulfadiazine 1% and TG extract with concentration of 10% increased TTM compared with negative control with $P < 0.05$; whereas TG5 could not significantly increase TTM in comparison with C- ($P > 0.05$). As shown in Fig. 3, TG10 increased TTM even more than positive control ($P < 0.05$) (Fig. 3).

In LPO test, TG5, TG10 and C+ significantly decreased lipid peroxidation in skin tissue compared with negative control ($P < 0.01$) (Fig. 4).

In TAP test, neither TG5 nor TG10 were able to increase total antioxidant capacity of skin tissue ($P > 0.05$). Positive control significantly increased total antioxidant capacity of skin tissue in comparison to all other groups ($P < 0.001$) (Fig. 5).

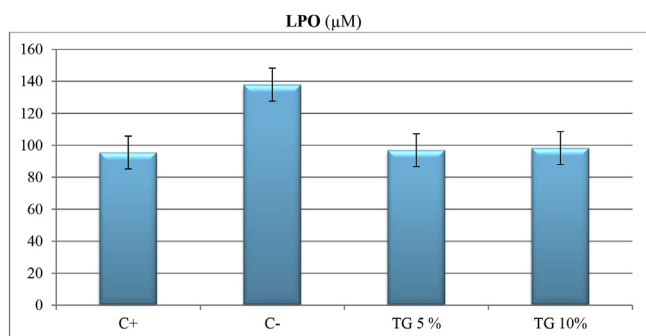


Fig. 4. Comparing effect of different treatment on lipid peroxidation in skin tissue on day 14.

LPO: lipid peroxidation, C-: negative control, C+: positive control, TG5: 5% extract ointment, TG10: 10% extract ointment. Significant difference in comparison to negative control: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

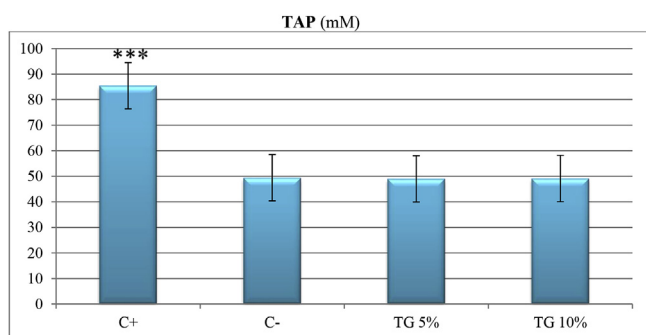


Fig. 5. Comparison of different treatments on total antioxidant power of skin tissue samples on day 14.

TAP: total antioxidant power, C-: negative control, C+: positive control, TG5: 5% extract ointment, TG10: 10% extract ointment. Significant difference in comparison to negative control: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

4. Discussion

Burn wound healing is a complicated process with several stages which finally results in dermis and epidermis tissue repair. Inflammatory response, occurring in a short time after injury, mainly starts with infiltration of granulocytes or PMN leukocytes and creates free radicals in inflammation site which could damage cells and postpone the healing process.^{19,20}

Topical antibiotics such as silver sulfadiazine are among the drugs used for the management of burn wound; however, finding new drugs with higher efficacy and lower adverse effects is still considered as a priority.²¹

About 1–3% of modern medicines are specified to wound repair; whereas nearly 30% of preparations in traditional medicine are proposed for skin disorders which indicates high potential of traditional medicine to suggest new drugs for wound care.^{22,23}

Different species of *Tragopogon* have been used for their astringent, antitussive and antiulcer properties, and demonstrated beneficial effects in wound healing.^{24,25} *T. graminifolius* aerial parts and roots have been used in Persian medicine for their anti-inflammatory, antiseptic, anti-hemorrhagic and for skin damages, especially wounds.^{13,26,27}

To the best of our knowledge, there is no previous study on the wound healing properties of *T. graminifolius* or other species of the genus *Tragopogon*. In the present study, the wound healing activity of TG extract was assessed on second degree burn for the first time. Considering wound closure percentage, both concentrations of TG

topical ointment showed significant healing effect compared with negative control group. In addition, TG 10% showed the best wound closure effect among all assessed treatments. Histological analysis also confirmed wound healing activity of the extract as TG 10% represented the best repair and regeneration in different layers of skin in comparison to the other treatments.

Oxidative stress has an important role in the pathogenesis of several types of tissue damage, including burn wounds and ideal wound healing agents are supposed to show potent antioxidant activity in order to overcome the burst of free radicals produced as the result of tissue damage.²⁸ Despite of no significant change in TAP test, marked alteration in other biomarkers of oxidative stress measured in the skin tissue of wound area, including LPO and TTM, represented *in vivo* antioxidant activity of TG extract. It is demonstrated that a normal wound healing process depends on a balance between free radicals and antioxidants. Overproduction of free radicals cause delayed and impaired wound healing; thus, antioxidants play a crucial role in healing process of skin injuries.²⁹ Sakarcan et al. (2005) also investigated the role of oxidative stress in animal model of burn wound using systemic *Ginkgo biloba* L. and demonstrated lower level of lipid peroxidation and improved endogenous antioxidant defense mechanisms; thus, our study is in line with previous reports describing the importance of antioxidant activity for wound healing agents.³⁰

As previously mentioned, TG extract is rich in polyphenolic compounds like gallic acid, *p*-coumaric acid, ferulic acid, caffeic acid and catechin which have demonstrated antioxidant and anti-inflammatory effects. There are several reports on wound healing effects of polyphenol-rich herbal extracts or their isolated polyphenolic compounds.³¹ Vafi et al. reported wound healing activity of *Hypericum scabrum* L. and *Lythrum salicaria* L. which contained a high amount of flavonoids.³² Another study on *Malva sylvestris* L. also reported wound healing properties of the plant to be partially attributed to its polyphenolic compounds.³³ A polyherbal preparation containing *M. sylvestris*, *Rosa damascena* Mill., and *Solanum nigrum* L. which is rich in polyphenolic compounds such as tannins were also effective for the treatment of burn wounds.³⁴ Therefore, the healing activity of TG extracts, at least in part, can be attributed to its high polyphenolic content. Future studies on the polyphenol-rich fractions or isolated compounds are needed to confirm this hypothesis.

5. Conclusion

TG aerial parts extract ointment with concentration of 10% had significant beneficial effects on animal second-degree burn wound model in regard to wound closure, histological evaluations and biomarkers of oxidative stress. Thus, the extract can be considered as a future treatment for healing of burn wounds. Future animal studies, as well as clinical trials are essential to demonstrate the safety and efficacy of TG extract as a topical wound healing agent in human.

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