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Wound Healing Activity of Alcoholic Extract of *Tamarixaphylla L.* on Animal Models

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To evaluate the wound healing activity of ethanolic extract of TamarixaphyllaL. on animal model.Wound creation like circular excision and linear incision method were considered for this study. The various parameters were studied like DNA estimation, total protein estimation, estimation of Hexosamine and Uronic acid, estimation of lipid peroxides and antioxidant activity, Tensile Strength of tissues from incision wounds, Antioxidant activity, Antimicrobial activity, Period of epithelialization and finally TNF-a concentration in the wound tissue homogenate were estimated. The treatment groups with the extract showed significant antimicrobial activity with compare to the standard drug. Significantly, 93. 86% increase in the collagen content and significant 52% up regulationin tensile strength was observed in the treated group. 40% reduction was observed in epithelialization period of the treated wounds. The results of the current study confirm that the ethanolic extract of T. aphyllahaspotent wound healing capacity.

Keywords: Tamarixaphylla, tensile strength, epithelialization period, TNF-α, wound healing.

Wound healing is a series of conjugated process in which the damaged tissue at the site of injury is replaced by newly formed tissue. Healing is a five step process that includes haemostasis and blood clotting, fibroplasia and neovascularization, granulation tissue formation, re-epithelialization and finally the formation of new extracellular matrix and tissue remodeling[1,2]. Several naturally occurring plant products are reported to up drive the process of healing[3,4], which are rich in active constituents such as triterpenes, tannins, flavonoids and alkaloids[5] as well as other biomolecules [6]. All these active phyto-principles are found to upregulate one or more steps of wound healing process.

In this study, we aimed to screen the alcoholic extract of *Tamarixaphylla L.* for possible wound healing activity on animal models. *Tamarixaphylla*,iscommonly known as Athel or Tamarisk in English and Abal, Tarfaa, Ghaz or Athel in Arabic. It is mainly found as trees or tall shrubs that grow up to 12 m in height in alluvial or

saline soil and has reddish brown or grey colored bark. Mostly, plants from this family are found to grow well in sub-tropical and temperate regions. These are the halophytes which can tolerate a variety of abiotic stresses such as draught, temperature, and saline impacts easily[7]. *Tamarix* is a genus with over 55 species that are native to Middle East, Mongolia, North Africa, India, China and Europe with saline soils[8,9,10]. In Saudi Arabia, the plant is reported to have natural vegetation properties in colder regions of Asir province such as Abha and Al-Baha[11]. Although, the distribution is not restricted to the selective regions but the salty soils with low temperature range climate is suitable for its growth. This plant is widely available in Jazan province and commonly used for wound healing as a folklore medicine. The genera *Tamarix* is believed to possess a significant role in the phyto-remediation process and contributes to the reduction of pollutants from environment[12]. Wound healing and anti-inflammatory action of *Tamarixaphylla*is also mentioned in Islamic literature as well as other sources from remote areas in Saudi Arabia. In Al-Qassim region of Saudi Arabia, dried powder of all parts of the plant was used to treat skin diseases of camel (allergic or mycotic dermatitis) by applying it on the affected skin for at least one week[13]. Burnt smoke of the dried powdered leaves of the plant has the history to be used as wound healing agent as well as dental analgesic[14]. Plant leaves when boiled in water and tied on the affected skin, works miraculously for wound healing, abscesses and rheumatism[15].

Our current approach was to explore the wound healing capacity of this plant in a scientific manner by using modern techniques against the standard animal models. Many researchers previously reported various other activities of this plant but none of them explored the wound healing potential at molecular level and measure the role of TNF-á in wound healing. In view of these facts regarding medicinal values of the plant, we chose to examine the wound healing potential of an alcoholic extract of the leaves of *Tamarixaphylla*on rats.

MATERIALS AND METHODS

Animals

Male albino rats weighing 140–210 g body weight were selected for study. Animals were kept in clean, sterile, polyvinyl cages and fed with commercial rats feed. Food and water were provided frequently to the animals *ad libitum*. All experimental procedures were carried as per institutional guidelines on ethics for animal handling.

Chemicals

L-hydroxyproline, pepsin, glucuronic acid, calf thymus DNA, chloramine-T and bovine serum albumin were purchased from Sigma Aldrich, Saudi Arabia. *p*-dimethylaminobenzaldehyde and Folin's Phenol reagent were purchased from LobaChemie, Mumbai, India. Methyl cellulose was also obtained from Sigma Aldrich, Saudi Arabia. Anti-TNF-á mAb was purified using a protein G column kit (Kirkegaard& Perry Lab., Gaithersburg, MD) from the culture supernatants of hybridoma cells (clone MP6-XT2.2-11).

Preparation of alcoholic extract of *Tamarixaphylla*

The leaves of *T. aphylla* were collected from Jazan Region, Saudi Arabia. Leaves were cut into very small pieces, weighed, dried, powdered and homogeneously mixed in 10–20 volumes (by weight) of 70% ethanol and filtered to yield a viscous supernatant. This viscous supernatant liquid used as the crude alcoholic extract. Lyophilization of an aliquot of the extract was carried out and weighed.

Animal grouping, drug administration and Wound creation

Hairs present at the back side of the rats were removed and a 4 cm^2 full thickness open excision wound was created by removing a patch of skin under anesthesia. Animals were segregated in two groups of six rats each. Control group (group 1) received 200 µL of unbuffered physiological saline, once a day, for a period of 15 days. The experimental group (group 2) received 200 µL of the alcoholic extract of *T. aphylla*, applied topically once a day for a period of 15 days. The granulation tissues formed were removed on 3, 6, 9 and 15 days post-wounding and used for analyses.

Incision wounds:Rats were distributed in two groups of six animals each. Rat skin was shaved and a six cm linear incision was made with a sterile sharp blade. Surgical sutures were used to close the incision maintaining a distance of approximately 1 cm between two. Extract was

applied topically for the excision group animals. Sutures were removed on day 7 post-wounding. Dumbbell shaped tissue pieces were removed from the wound site on day 10 post wounding and used for tensile strength measurements.

Biochemical parameters

Nasir *et al.*(2017)[16] method was utilized to extract Nucleic acids. Homogenization of 100 mg of granulation tissue was carried out in 5 mL of ice cold distilled water. To this, was added 5 mL of 10% trichloroacetic acid (TCA), and kept in an ice bath for 0.5 h in order to precipitate proteins and nucleic acids. Contents were then centrifuged and the pellets were first washed with 1 mL of 10% TCA followed by 3 mL of absolute alcohol to remove lipid content. To separate nucleic acids, the lipid free sediment was resuspended in 5 mL of 5% TCA and heated at 90° C for 15 min. The contents were centrifuged and the supernatant was used for the estimation of DNA by the method reported previously [17]. Estimation of total protein was performed according to Lowry *et al.*, (1951) method []. Pellets were resuspended in 0.1M Tris-HCl (pH 7.4) and the total collagen content in granulation tissues was estimated based on the hydroxyproline index by the method of Wold*et al.*, (1999)[18]. Levels of Hexosamine and Uronic acid were estimated by following the methods of Saeed *et al.*, 2016 and Mojica *et al.*, 2010 respectively[19,20].

Estimation of lipid peroxides and antioxidant activity

The thiobarbituric acid reaction method was used to determine Lipid peroxide levels (as nanomoles of malondialdehyde) in granulation tissues as described by Santos *et al.*, (1980)[21]. To test the antioxidant potential of the plant, radical analysis was carried out by ESR measurements at room temperature in X-band using a Varian type ESR spectrophotometer Model E112 with 100 KHz field modulation. DPPH free radicals were measured according to Santiago *et al.*, (1992) method[22]. To 10 µL of the *T. aphylla* extract, was added 500 µL of 50 µM DPPH in methanol, mixed well and immediately transferred to the ESR spectrophotometer cell and analyzed exactly after 60 s. Blank was performed using 10 µL of methanol.

Biophysical parameters

To determine the rate of contraction of wounds, wound surface was traced on a transparent graph paper and the surface area was measured by planimetry. The period of epithelialization was considered as the total number of days needed for eschar shedding without any remains of raw wound. Muehlberger (2005)[23] method was utilized for the measurement of tensile strength of wound tissues.

Measurement of cytokine concentrations

To neutralize TNF-á biological activity, mice were injected intraperitoneally with mAb against cytokine at 400 ìg one day prior to and three days after the wound creation. Rat IgG was used as the control Ab. An amount of 25 ìg/kg TNF-á, purified from a human B-cell lymphoblastoid cell line (BALL-1) was injected into the peritoneal cavity once daily beginning on the day of wound

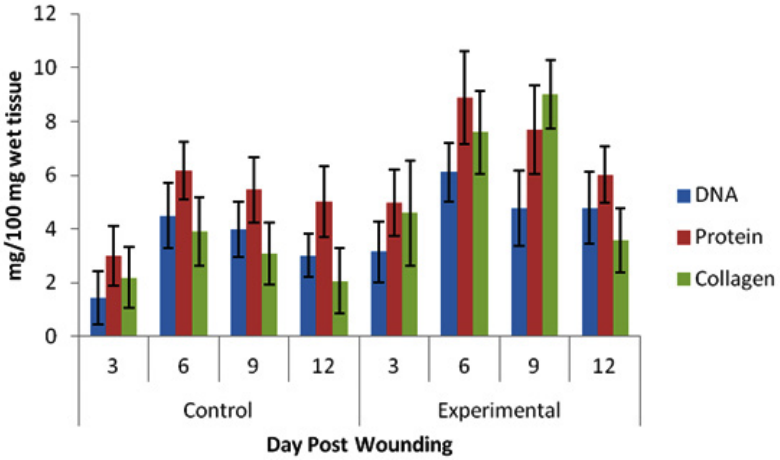

Fig. 1. DNA, protein and collagen contents in the granulation from control and experimental rats (all values are mg/ 100mg wet tissue) mean ± SD, n = 6

creation[24]. PBS was used as the control vehicle. Wound tissues were homogenized in PBS, and the TNF-á concentration in the supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Cambridge, MA). Results were expressed as the values per one wound with a detection limit 5.1 pg/mL.

Antibacterial activity

The antibacterial activity of the alcoholic extract was measured by reported method[25,26]. 1 mm^2 wells were made on nutrient agar plates, using a cork borer, and 20 µL of the extract was applied to the wells. Microorganisms to be tested were streaked on the agar plates and grown

Table 1. Tensile Strength of tissues from incision wounds of the control and treated rats (measured as kg/cm^2), Mean ± SD, n=6

	Control	Treated
Tensile strength (Kg/cm^2)	8.35 ± 1.66	12.71 ± 1.35

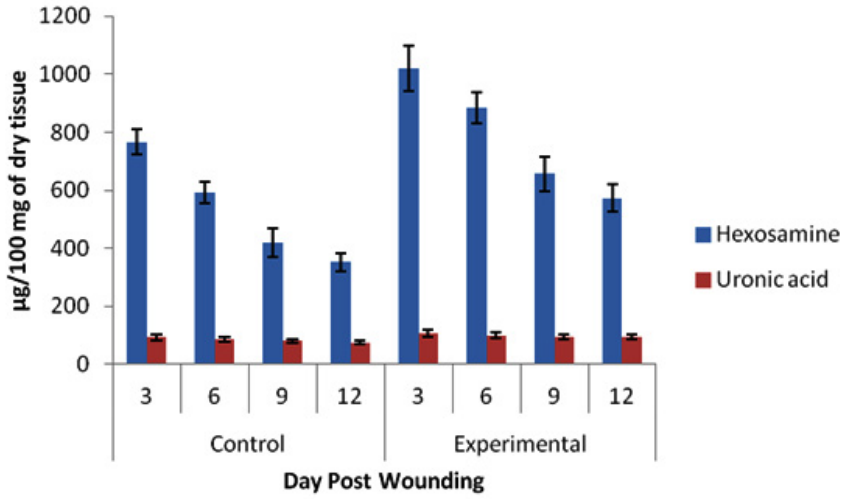

Fig. 2. Hexosamine and uronic acid content in dry granulation tissue (µg/100mg of dry tissue) mean ± SD, n = 6

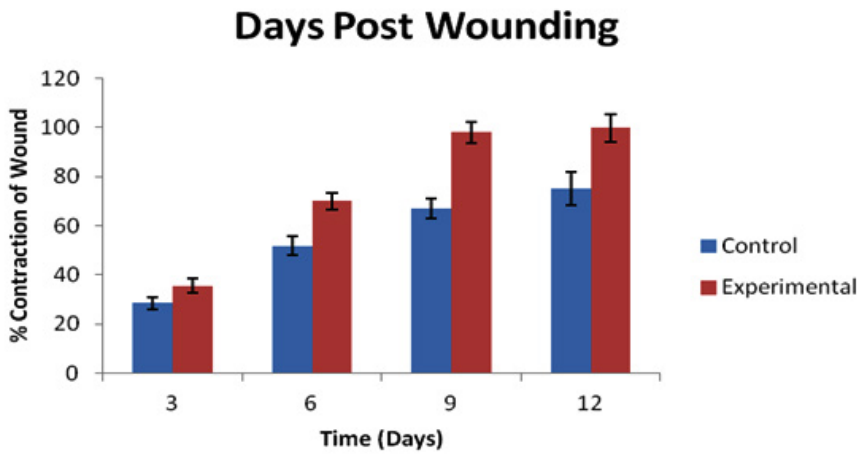

Fig. 3. Progression of wound percentage contraction Vs time

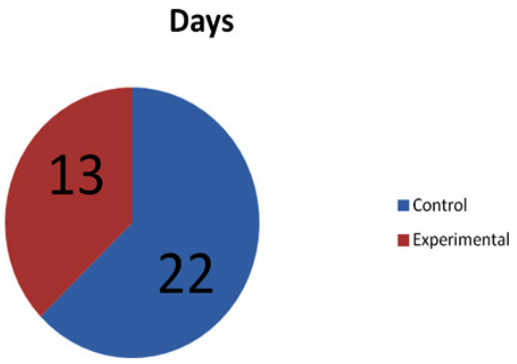

Fig. 4. Period of epithelialization in days for control and experimental wound

overnight at 37 °C. The presence of clear zones of inhibition was taken to represent antibacterial activity. Equal amount of alcohol (vehicle) were inoculated into another set of similar plates, to serve as controls.

RESULTS

Estimated results of the total protein, DNA and collagen content in the granulation tissues of control and treated wounds are summarized in Figure 1.

Significantly, 93. 86% increase in the collagen content was observed in the treated group at the day 6 of wound creation. Similar pattern was noticed in case of DNA content, with a parallel increase in total protein content. The findings of Hexamine and uronic acid levels in the granulation tissues of control and experimental wounds are shown in Figure 2.

The ground substance level was up regulated upto day 12 post-wounding in the treated group, and fell back to normal afterwards. Figure

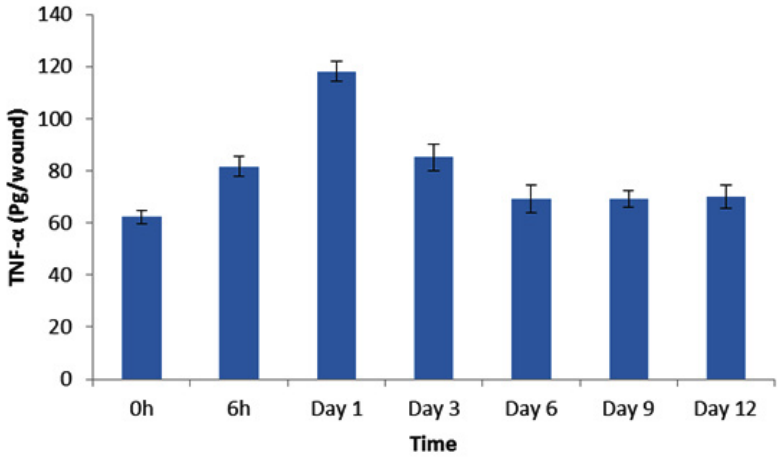

Fig. 5. TNF-a concentration in the wound tissue homogenate was measured at 0 and 6 h and on days 1, 3, 6, 9 and 12 after wound creation. Each column represents the mean ± SD of 3 homogenates, each of which was prepared from 6 wounds

Table 2. Antioxidant activity of *T. phyllus* methanolic extract quantified through ESR measurements of DPPH radical spin reduction

	Control	Treated
DPPH radicals ($\times 10^{17}$ spins/mL)	0.301 ± 0.0193	0.163 ± 0.0121

Table 3. Antimicrobial activity of control and treated groups against *S. aureus* and *P. aeruginosa*

Organism	Zone of inhibition (mm)	
	Control	Treated
S. aureus	5.36 ± 0.0136	22.29 ± 0.0438
P. aeruginosa		10.03 ± 0.0235

3depicts results of the rates of contraction of control and experimental wounds.

The contraction of wound for treated group was found to be much faster. Interestingly, a sharp (40%) reduction was observed in epithelialization period of the treated wounds Figure4. The tensile strengths of tissues for the control and experimental incision wounds are compared in

A significant 52% up regulationin tensile strength was observed in the treated group. Table II reveals the antioxidant activity of *Tamarixaphylla*plant extract, estimated by ESR measurement technique.

Upto 41% reduction of DPPH spins from the control value was observed in the treated group. The TNF-a concentration in the homogenate supernatants of the collected wounds at various time intervals estimated as shown in Figure5.

TNF-a was detected just after wound creation, and rapidly increased in concentration during the first several hours. The peak concentration level was reached on day 1, and levels declined thereafter to the basal level. TNF-a production was detected in the homogenate of wound tissues with a peak level on day 3 after wound creation; wound healing was significantly delayed on day 3, but not on day 6, in mice treated with anti-TNF-a mAb compared with mice that received control IgG; and TNF-a accelerated wound repair on day 3, but not on day 6.Table III summarizes the extent of antibacterial activity of *Tamarixaphylla*. The drug was found to be potentially active against *S. aureus* and *P. aeruginosa*.

DISCUSSION

This work is aimed to assess the beneficial effects of plant, *Tamarixaphylla*, on rat dermal wound healing process using standard animal models. Plant metabolites are reported to be promising agents for wound healing and are preferable because of their availability, non-toxic nature, easy administration, lesser side effects and effectiveness as crude preparations. Previously, many plants and their products are reported to have significant wound healing activities such as *C. asiatica*, a sweet, acrid, tropical weed/green, was found to be effective in wound healing[6]. Likewise, the positive influence of atropical cactus *Aloe vera* on wound healing was also documented[4]. These findings encouraged us to further examine other plants which had other reported medicinal values, for wound healing. Therefore, systematic *in vivo* studies on *Tamarixaphylla*for wound healing were conducted. Cellular hyperplasia indicated that there was an increase in DNA content in the treated wounds. Simultaneously, there was also an increase in the total protein content representing active synthesis and deposition of matrix proteins in the granulation tissues. Collagen constitutes an essential component of the extracellular matrix and the healing process depends, to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation. The type III collagen levels are particularly increased during early stages of wound healing[6]. Assessment of collagen content in granulation tissues indicated that there is an enhanced production of new collagens in treated wounds post treatment with *Tamarixaphylla*. Hexosamine and uronic acid, commonly known as matrix molecules, are involved in the synthesis of new extracellular matrices. During early stages of wound healing, an increase in the levels of these components was reported, following which normal levels were restored (Dunphy and Udupa, 1955). Similar results were obtained in case of*Tamarixaphylla*treated wounds, where the levels of hexosamine and uronic acid increased significantly. Tissue damage can be aggravated by free radicals and oxidative reaction products which arise through lipid peroxidation. These play a major role in various tissue disorders[27] and are predominantly noticed during fibrosis as well as during wound healing[28]. In view to these contexts, several plant products having antioxidant action such as curcumin, vitamin E, etc. have been testified to reduce oxidative damage to tissues [29]. In fact, the use of antioxidants became very popular in recent years for effective strategy for therapeutic approaches to such type of disorders. Numerous medicinal plants have been reported to possess antioxidant properties [30]. Our studies on the lipid peroxide status and DPPH radical reducing properties were carried out through ESR technique. It was observed that *Tamarixaphylla*extract has significant antioxidant activity, which would help inhibit oxidative damage and stimulate the healing process. Wound contraction rate appeared to be faster in treated groups due to the larger availability at the wound site when applied

topically. The period of epithelialization was also found to be lesser for the treated wounds. All these results additionally supported the effectiveness of *Tamarixaphylla*in wound healing process. Newly synthesized collagens deposited at the wound site increases the collagen amount per unit area as well as the tissue tensile strength[31]. *Tamarixaphylla* treated incision wound tissues exhibited higher tensile strength, indicating larger collagen content at the wound site. Post-operative wound infection is frequently encountered in several surgical procedures owing to the exposure of wound to microorganisms. Several plant products are known to possess intrinsic antibacterial activity, improving their medicinal values. The results indicate that TNF-a is closely involved in the very early process of wound healing in skin. TNF-a was produced in the wounded tissues and reached a peak level on day 1, after which it returned to the basal level. However, this increase in TNF-a level was not statistically significant even at the time point of maximal production. This may be a result of the high TNF-a level detected just after wounding (0 h), whichthis constitutive expression of TNF-a is thought to be involved in the regulation of homeostasis in skin tissue[32]. Thus, TNF-a levels measured in the wound tissues were likely higher than the normal basal levels, which suggests that this cytokine may be involved in the process of wound healing.

Our studies indicated that *Tamarixaphylla*is a potent inhibitor of growth of microorganisms such as *S.aureus*and *P. aeruginosa*. These data are in accordance with previously reported study on the antifungal[33] and antibacterial[34] activities of other medicinal plants, without cellular toxicity. The outcome of the present study strongly indicated that *Tamarixaphylla*is a potential candidate for wound healing owing to its positive influences on various phases of the process. Antioxidant and antimicrobial properties additionally played an important role in their wound healing capacity.

CONCLUSION

It was concluded that *Tamarixaphylla* may be the potential wound healing agent and it may be free from other problem which was associated with synthetic drug.

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