

Antioxidant, Antimicrobial And Wound Healing Activity Of *Salvadora Persica* Twig Extracts



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Abstract

Wound healing is a complex multifactorial process that results in the contraction and closure of the wound and restoration of a functional barrier. *Salvadora persica*, commonly known as Miswak was found to contain constituents such as tannins, saponins, flavonoids and sterols. Hence it is thought to evaluate wound healing activity of *Salvadora persica* since phytoconstituents like tannins, saponins and flavonoids are known to promote the wound healing process due to their antioxidant and antimicrobial activities. Antimicrobial and antioxidant activities of *Salvadora persica* extracts were studied to understand mechanism of wound healing process. Total phenolic content (TPC) was estimated to screen the prepared extracts by using Folin-Ciocalteu phenol reagent method. Methanol extract showing highest TPC was undertaken for detailed antioxidant, antimicrobial and wound healing activities. Methanol extract showed moderate antioxidant activity on scavenging DPPH, ABTS radicals and by pyrogallol red bleaching method. Methanol extract also showed antimicrobial activity against wound pathogens by agar diffusion method. Methanol extract was formulated into gel and wound healing activity of gel was evaluated using incision and excision wound models in rats. Topical application of prepared gel on the excision wound in rats caused higher rate of contraction and reduced the period of epithelialization when compared to control group animals. In incision wound model, breaking strength of animals treated with the gel containing methanol extract of Miswak twig was found to be significantly ($p < 0.001$) higher as compared to the control group animals. Hence, the present study revealed that gel containing methanol extract of Miswak twig possess wound healing activity.

Keywords: Incision wound; Excision wound; Antioxidant; Antimicrobial activity; Phytomedicines; Cholesterol plasma levels; DPPH; Ascorbic acid

Abbreviations: TPC: Total Phenolic Content; DPPH: 1,1-Diphenyl-2-picryl-hydrazyl; AA: Ascorbic Acid; IAEC: Institutional Animal Ethic Committee

Introduction

Wound healing is an interaction of complex cascade of cellular and biochemical actions healing to the restoration of structural and functional integrity with regain of strength of injured tissues [1]. It involves continuous cell - cell interaction and cell matrix interactions that allow the process to proceed in different overlapping phases including inflammation, wound contraction, re epithelialization, tissue remodeling and formation of granulation tissue with angiogenesis [2]. These events are regulated by several mediators including platelets, inflammatory cells, cytokines and growth factors, and matrix metalloproteinases and their inhibitors [3].

Several factors such as bacterial infection, oxidative stress, necrotic tissue and interference with blood supply, lymphatic blockage and disease condition such as diabetes mellitus delay or reduce the wound healing process. Generally, if the above factors could be altered by any agent, an increased healing rate could be achieved [4].

Nature has gifted us with many herbs having mystical healing properties that are used widely in number of ailments.

The use of herbs and medicinal plants as the first medicine is a universal phenomenon. Today, as much as 80% of the world's population depends on traditional medicine as primary health care needs [5]. Many Plants and their extracts being antioxidant and/or antimicrobial actions have immense potential for the management and treatment of wounds. The phytomedicines for wound healing are not only cheap, well tolerated and affordable but are also purportedly effective and safe as hyper sensitive reactions are rarely encountered with the use of these agents [6]. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. Herbal medicines in wound management also involve disinfections, debridement and provide a moist environment to encourage the establishment of the suitable environment for natural healing processes [7].

Salvadora persica (family *Salvadoraceae*) is an upright evergreen small tree or shrub. It is commonly known as Miswak or Tooth brush tree and is widely distributed in India, Africa, Saudi Arabia, Iran, Israel and Pakistan. It has been claimed in traditional literature to be valuable against a wide variety of diseases [8].

The traditional medicinal use of *Salvadora persica* as antimicrobial toothbrush stick for oral hygiene, and to treat gum inflammation, is a centuries old practice and a part of Greeco-Arab system of medicine [9]. Pharmacological studies indicated that *Salvadora persica* L. plant possess anti-microbial, anti-plaque, aphrodisiac, alexiteric, analgesic, anti-inflammatory, anti-pyretic, astringent, diuretic and bitter stomachic activities. It has great medicinal use in the treatment of nose troubles, piles, scabies, leucoderma, scurvy, gonorrhoea, boils and toothache, to treat hook worm infections, venereal diseases, for teeth cleaning, in rheumatism, cough and asthma, to lower cholesterol plasma levels, reestablishment of the components of gastric mucosa, and as a laxative [10]. It contains important phytoconstituents such as vitamin C, salvadorine, salvadouria, alkaloids, trimethylamine, cyanogenic glycosides, tannins, saponins and salts mostly as chlorides [11].

However, there is no previous report on wound-healing activity of *Salvadora persica* twig in literature. The purpose of the present study was to investigate *in vivo* wound healing activity of *Salvadora persica* twig. Since antioxidant and antimicrobial agents play an important role in wound healing process, antioxidant and antimicrobial activities of *Salvadora persica* twig were carried out to find the mechanism behind wound healing process.

Materials and Method

Materials

Gallic acid, 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid (AA), Folin-Ciocalteu phenol reagent, pyrogallol red, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate and all other substances used were obtained from Sigma- Aldrich Co. Ltd. Nutrient agar was obtained from Himedia (Mumbai, India). All chemicals used, including the solvents were of analytical grade.

Plant materials

The twig of *Salvadora persica* were collected from Malvani area in Malad, Mumbai, India and authenticated by Agharkar Institute, Pune, India.

Methods

Extraction of plant material

Authenticated plant material was further dried in shade, powdered and used for extraction. Extraction was carried out using various solvents such as Petroleum ether (60-80°), chloroform, methanol, 50% aqueous alcohol and water. The extracts were concentrated in a rotary evaporator under pressure, were kept in desiccators and used for further studies.

Determination of extractive value

10 gm of powdered material were extracted with 100ml solvent using Soxhlet extraction apparatus. The % yield of each extract was determined.

In vitro antioxidant assay methods

Phenolic compounds could be a major determinant of

antioxidant potentials of foods and could therefore be a natural source of antioxidants [12]. Hence total phenolic content of the prepared extracts was determined to screen the bioactive extract.

Determination of total phenolic content (TPC)

The total phenolic content was measured using Folin-Ciocalteu reagent as per procedure described by Singleton et al., with some modifications [13]. Test mixture consists of 1ml of extract solution (0.1 or 1mg/ml), 0.5ml of Folin Ciocalteu reagent and 5ml of distilled water. The mixture was incubated at room temperature for 10min. Then 1.5ml of anhydrous sodium carbonate solution (10% w/v) was added and the final volume was made upto 10ml. The final mixture was allowed to stand at room temperature for 2 hr. The absorbance was measured at 725nm using UV-Vis spectrophotometer. The experiment was carried out in triplicate. Gallic acid was used for preparing the standard curve (10µg/ml to 100µg/ml). The total phenolic content in the plant extract was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of extract.

The extract showing maximum TPC was further used for various *in vitro* antioxidant assays, antimicrobial activity and *in vivo* pharmacological activities.

DPPH radical scavenging activity

The free radical scavenging activity of active extract was measured by DPPH using the method of Blios [14]. An aliquot of 1ml of the extract solution in various concentration range was added to 3 ml of 0.1 mM DPPH solution. The decrease in the absorbance was determined at 517 nm after 30 min. The percentage scavenging activity was calculated from $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/ standard. A blank is the absorbance of the control reaction (containing all reagents except the test compound). The % scavenging activity and IC₅₀ value of each extract was calculated for the various concentrations. Ascorbic acid was used as standard antioxidant for comparison.

Peroxyntirite pyrogallol red bleach method

Pyrogallol Red solution (100µM) was prepared in 100mM phosphate buffer, pH 7.4. 1ml of extract solution was added to 2ml of 100µM Pyrogallol Red solution. 0.5ml of 200µM/liter peroxyntirite solution was added to the mixture and vortexed immediately. After 15 minutes the absorbance was measured using UV-Vis spectrophotometer at 540nm. The % inhibition of pyrogallol red bleaching was determined using the formula $[(A_1-A_2)/A_1] \times 100$, where A_1 is the absorbance in presence of antioxidants and A_2 is the absorbance in absence of antioxidants. The IC₅₀ values yielding 50% inhibition of Pyrogallol Red bleaching were estimated. Ascorbic acid was used as standard antioxidant for comparison [15].

ABTS assay

ABTS was dissolved in water to a 7mM concentration. ABTS radical cation (ABTS⁺) was produced by reacting ABTS stock solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 hr.

The ABTS⁺ solution was diluted with a phosphate buffer (2mM, PH 7.4) to achieve an absorbance of 0.8 ± 0.014 at 734nm. Extract solutions were mixed with ABTS⁺ solution, and after 1 min the absorbance was read using UV-vis spectrophotometer at 734 nm. Phosphate buffer solution was used as a blank. The % radical-scavenging activity of the samples was determined using the formula $[(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$, where A_{control} is the absorbance of the control (ABTS⁺ solution without test sample) and A_{test} is the absorbance of the test sample (ABTS⁺ solution with extract). The IC₅₀ values scavenging 50% of ABTS⁺ were estimated. Ascorbic acid and Trolox were used as standard antioxidants for comparison [16].

Antimicrobial activity

In vitro antibacterial and antifungal activities of methanol extract of bark of Miswak twig were determined by the agar diffusion method against wound pathogens [17]. Bacteria such as *Pseudomonas aeruginosa* (NCIM 2200), *Staphylococcus aureus* (NCIM 5022), *Streptococcus pyogenes* (NCIM 2608), *Clostridium perfringens* (NCIM 2677), *Escherichia coli* (NCIM 2065), *Klebsiella pneumonia* (NCIM 5082), *Klebsiella aerogens* (NCIM 2239) and fungal such as *Candida albicans* (NCIM 3471), *Aspergillus niger* (NCIM 1196) were used as test organisms. The cultures of organisms were procured from NCL (National Chemical laboratory) Pune, India and tested. The petri plates were prepared by pouring melted nutrient agar inoculated with 16 to 18 hr old culture test organisms in a sterile petri dish. Cups were bored in agar by means of sterile cork borer and were filled with either extract to be tested or standard or control and incubated at 37 °C for 18-20 hours. Mixture of dimethyl sulfoxide and water were used as control. Chloramphenicol was served as standard when efficacy was tested against bacteria while fluconazole was served as standard for fungi. Diameter of each zone of inhibition was measured and compared with standard.

In-vivo pharmacological activities

Methanol extract of *Salvadora persica* twig was formulated into 1.5 % Carbopol 971 P NF gel by using extract (1%), ethanol, propylene glycol, triethanolamine and distilled water. Prepared gel was evaluated for skin irritation and wound healing activities.

Animals

Albino Wistar rats of either sex weighing 180-200g were used for the study. The animals were procured from Haffkine Biopharmaceuticals, Mumbai, India. All animals were housed in polypropylene cages under standard experimental conditions with 26 ± 2 °C ambient temperature and 12 h light-dark cycle. The animals were fed standard pellet diet and were provided water *ad libitum*. All experimental protocols were approved by the Institutional Animal Ethic Committee (CUSCP/IAEC/10 /2013) of C. U. Shah College of Pharmacy, Santacruz (w), India.

Skin irritation studies

Skin irritation study was conducted on albino rats as per OECD guide lines No. 404 (OECD, 2004) in order to evaluate safety of prepared topical gel [18]. The back of the albino rats was

shaved to remove the fur carefully, 24 hours before application of the sample. Prepared topical Carbopol gel containing methanol extract of *Salvadora persica* twig was applied on the skin patches of albino rats and the site of application in terms of erythema and edema was examined at 24, 48 and 72 hours for changes in any dermal reactions. The irritation index was calculated to assess the irritation potential of the prepared Carbopol gel according to Draize Test [19].

In vivo evaluation of wound healing

Incision and excision wound models were used to evaluate the wound-healing activity of prepared topical Carbopol gel containing methanol extract of *Salvadora persica* twig.

Grouping of animals

For excision and incision wound study, male Wistar rats (160-180g) were selected and were divided into four groups of six animals each. Rats were anesthetized with sodium pentobarbitone injection (45mg/kg, i.p.) and depilated at the predetermined site before wounding. Animals were divided into four groups of six animals each.

Treatment (Group I): Received with topical application of Carbopol gel containing methanol extract of *Salvadora persica* twig

Positive control (Group II): Received topical application of standard drug ointment i.e. Betadine

Negative (vehicle) control (Group III): Received with topical application of plain Carbopol gel

Negative control (group IV): Animals were left without any treatment

For both excision and incision wound models, the treatment groups were classified and treated in the same manner.

Excision wound model

An excision wound was inflicted by cutting away approximately 500mm² full thickness of the predetermined area on the anterior-dorsal side of each rat. Each rat was kept in a separate polypropylene cage and was provided with food and water *ad libitum*. All the test formulations were applied starting from day 0 till complete epithelialization. Wound-healing property was evaluated by % wound contraction percentage and time of wound closure. The wound area was measured immediately by placing a transparent paper over the wound and tracing it out, area of this impression was calculated using the graph sheet. The same procedure is employed every fourth day and wound contraction was expressed as percentage of contraction. The period of epithelialization was calculated as the number of days required for falling off of the dead tissue remnants without any residual raw wound [20].

Incision wound model

A paravertebral long incision of 6 cm length were made through the skin and cutaneous muscle.

After the incision was made, the two ends of the wound were closed with interrupted sutures with stitches 1cm apart using sterile surgical thread and a curved needle. Carbopol gel containing methanol extract of *Salvadora persica twig*, plain Carbopol gel and Betadine were applied for 9 days. The sutures were then removed on the 8th post - wounding day and the breaking strength of 10-day old wound was measured by tensiometer [21].

Statistical analysis

Results were expressed as means ± SEM (Standard Error of The Mean). Comparisons between groups were performed using one way ANOVA followed by Turkey’s pair-wise comparison test on Graph Pad Instat 3 statistical software.

Results and Discussions

Extraction

The extraction process yielded 1.062 % w/w of petroleum ether extract, 2.24 % w/w of chloroform extract, 17.07 % w/w of the methanol extract, 16.09 w/w % of the of water extract and 5.18 % w/w of 50% aqueous alcoholic extract (Table 1 & 2).

Table 1: Determination of % yield of extracts of *Salvadora persica twig*.

Sr. No.	Solvents	Yield (% w/w)
1	Petroleum ether (60-80 °C)	1.062
2	Chloroform	2.24
3	Methanol	17.07
4	Water	16.09
5	50% aqueous alcohol	5.18

Table 2: Determination of Total Phenolic Content (TPC) of extracts of *Salvadora persica twig*.

Sr. No.	Solvents	mg GAE/gm of extract ± SD
1	Petroleum ether (60-80°C)	13.1 ± 2.504
2	Chloroform	16.35 ± 1.132
3	Methanol	125.6 ± 0.7
4	Water	36.33±1.053
5	50% aqueous alcohol	55.3 ± 0.8752

In vitro antioxidant assay methods

Determination of Total Phenolic Content (TPC): The estimation of total phenolic content of the different extracts revealed a high phenol content in the methanol extract i.e. 125.6±0.7mg/g gallic acid equivalent (GAE) followed by 50% aqueous alcohol extract (55.3 ± 0.8752mg/g GAE), water extract (36.33±1.053mg/g GAE), chloroform extract (16.35 ± 1.132mg/g GAE) and petroleum ether extract (13.1 ± 2.504mg/g GAE) by reference to standard curve (y=0.011x+0.011and r2=0.998) (Table 1).

DPPH radical Scavenging activity: Anti-oxidant activity of Miswak twig was studied by inhibition the stable free radical DPPH. The amount of extract/standard needed for 50% inhibition (IC50). Methanol extract of *Salvadora persica twig* showed DPPH

scavenging activity at higher IC₅₀ value of 63.88 µg/ml as compared to standard ascorbic acid (10.99 µg/ml).

Assessment of pyrogallol red bleaching by peroxy nitrite:

The plant extract and standard exhibited inhibition of bleaching by Pyrogallol Red method indicating peroxy nitrite scavenging activity. Standard ascorbic acid was able to inhibit bleaching of Pyrogallol Red at IC₅₀ value of 38.08µg/ml. However methanol extract of *Salvadora persica twig* showed less inhibitory activity with IC50 value of 783.48µg/ml as compared to standard ascorbic acid.

ABTS scavenging assay: Standard ascorbic acid was able to scavenge ABTS radical at IC₅₀ values of 7.23µg/ml. Methanol extract of *Salvadora persica twig* exhibited moderate free radical scavenging activity by ABTS method with IC₅₀ values of 108.24µg/ml.

In the present study antioxidant activity levels were found to be relatively high in the methanolic extract of Miswak twig by DPPH, ABTS and Pyrogallol bleaching method; hence oxidative stress may be reduced which is associated with impaired or delayed wound healing process (Table 3).

Table 3: Free radical scavenging activity of methanolic extract of *Salvadora persica twig* by DPPH, ABTS scavenging and pyrogallol red bleaching methods.

Sr. No.	Samples	IC ₅₀ Values of Samples (µg/ml)		
		DPPH Scavenging Assay	ABTS Scavenging Assay	Pyrogallol Red Bleaching Assay
1	Methanolic extract of <i>Salvadora persica twig</i>	85.67 ± 1.85	49.10 ± 2.07	336.50 ± 5.74
2	Ascorbic acid	10.99 ± 0.22	7.23 ± 0.61	38.08 ± 0.44

Antimicrobial activity

The methanol extract from *Salvadora persica twig* has shown inhibition effects on the growth of all the organisms tested. Amongst the test organisms used, *Clostridium perfringens*, *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* were found to be most sensitive to methanol extract of a *Salvadora persica twig* followed by *Klebsiella pneumoniae*, *Aspergillus niger*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella aerogens*. Microbial infection of wounds delays healing and causes a more pronounced acute inflammatory reaction which can lead to further tissue injury and damage. The antimicrobial activity of the extract on wound pathogens partly contribute to the wound healing effect by eliminating infection thus allowing the natural tissue repair processes to start. Hence the results of this study confirm that the herbs possess anti-bacterial activity and this will help keep the wound area sterile, thus promoting wound healing. This fact supports a faster wound healing in the treated groups compared with the control group (Table 4).

Table 4: Antimicrobial activity of methanol extract of *Salvadora persica* twig against wound pathogens.

Sr. No.	Microorganisms	Zone of inhibition (mm)	
		Methanol Extract of <i>Salvadora Persica</i> Twig (50mg/well)	Standard
1	<i>Pseudomonas aeruginosa</i>	5.67 ± 0.58	10 ± 0.00
2	<i>Staphylococcus aureus</i>	5.00 ± 0.00	14.33 ± 0.58
3	<i>Streptococcus pyogenes</i>	2.33 ± 0.58	12.67 ± 0.58
4	<i>Escherichia coli</i>	3.33 ± 0.58	17 ± 0.00
5	<i>Klebsiella pneumonia</i>	2.00 ± 0.00	11 ± 1.00
6	<i>Klebsiella aerogens</i>	1.33 ± 0.58	12 ± 1.73
9	<i>Clostridium perfringens</i>	17 ± 0.00	18 ± 0.00
7	<i>Candida albicans</i>	6.00 ± 0.00	13 ± 0.00
8	<i>Aspergillus niger</i>	Nil	Nil

In vivo pharmacological activities

Skin irritation study: Carbopol gels containing methanol extract of *Salvadora persica* twig showed no erythema or oedema

on intact rat skin. The primary skin irritation index of the gels was calculated as 0.00. The results indicated that all Carbopol gels did not cause any skin reaction after examining at 24, 48 and 72 hrs. Since the primary skin irritation index of the creams was calculated as 0.00, it can be classified as non-irritant and were found to be safe for topical application.

Excision Wound Study: Table 5 records the reduction of wound area of the different groups over the period of 24 days. It was seen that the faster healing of wound took place in case of animals, which received Carbopol gel containing Miswak extract of *Salavadora persica* twig and standard. The least rate of wound healing was seen in control group (no treatment) and vehicle control group which received plain Carbopol gel (without extract). A very rapid closure of the wound in the both Carbopol gel containing Miswak extract of *Salavadora persica* twig and standard treated groups observed between 4 and 8 days of post surgery. After day 8 of post surgery, wound closure was gradual till the total closure of the wound. Total wound closure was observed by the 22 day of post surgery in Carbopol gel containing methanol extract of *Salavadora persica* twig and by 25 day in control group. On 16th day, wound contraction of standard group was found to be significant (p < 0.001) in comparison to control group. The period of epithelization of standard group (16 days) was also found to be significantly (p<0.001) low as compared to control group (25 days).

Table 5: Effect of topical application of Carbopol gel containing methanol extract of *Salvadora persica* twig on wound contraction of excision wound.

Sr. No.	Post Wounding Days	% Wound Contraction			
		Group 1	Group 2	Group 3	Group 4
		<i>Salvadora persica</i> extract	Betadine	Plain gel	control
1	4	54.77 ± 12.24	56.04 ± 20.10	28.59 ± 7.29	30.47 ± 16.65
2	8	81.55 ± 8.40	83.47 ± 17.162	62.36 ± 8.36	59.52 ± 9.13
3	12	88.33 ± 5.293	95.92 ± 3.80	78.98 ± 6.01	76.21 ± 4.805
4	16	91.85 ± 6.63	98.7 ± 2.06***	88.52 ± 3.00	84.05 ± 3.40
5	20	95.46 ± 5.45	100	93.06 ± 1.38	89.61 ± 2.66
6	24	100		100	96.61 ± 2.85

Incision wound model: Table 6 compares the tensile strength of the healing skin treated with different gels measured on 10th days. The results of the incision wound healing studies are presented as mean weight in gram ± SD required to break open the resutured wound. The animals treated with methanolic extract and standard showed significant (p<0.001) increase in breaking strength (607.33±8.07gm and 614.33±8.80 respectively) as compared to the control group animals (406.50±9.16gm). This observation confirms that the methanol extract of *Salavadora persica* twig possesses excellent wound healing property so far as tensile strength of wound healing tissue is concerned.

Table 6: Effect of topical application of Carbopol gel containing methanol extract of *Salvadora persica* twig on breaking strength of incision wound.

Sr. No.	Samples	Breaking strength (gm)
1	Methanolic extract of <i>Salvadora persica</i> twig	607.33 ± 8.07***
2	Standard (Betadine)	614.33 ± 8.80***
3	Vehicle control (Plain carbopol gel)	409.17 ± 9.56
4	Control	406.50 ± 9.16

Conclusion

Results obtained in the present study have shown the antioxidant and antimicrobial activity of methanol extract of Miswak twig. Thus, the external application of methanol extract of *Salvadora persica* twig on the wound prevented the microbes to invade through the wound, resulting protection of wound against the infections of the various microorganisms. At the same time, external application of the extract entrapped the free radicals liberated from the wound surrounding cells, which are having inherent machinery to protect the cells from the microbes. The faster rate of wound closer in excision wound model indicates the better efficacy of medication the increase in tensile strength of wounded skin indicates the promotion of collagen fibers. The increased tensile strength reveals that the disrupted surfaces are firmly knit by collagen. The synergistic effect of both antimicrobial and antioxidant activity, increased wound contraction and breaking strength accelerated the wound-healing process. Hence, present study confirms the promising wound healing activity of *Salvadora persica* twig.

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