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Research Article

In-vivo and *In-vitro* Diabetic Wound Healing Effects of *Salvadora persica* Twig Extract and its Mechanisms of Action

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ABSTRACT

Due to unclear etiology, the healing impairment of diabetic patients is still a serious clinical problem for physicians worldwide. The impaired wound healing in diabetic wound has brought attention to promoting healing process and prevent complications arising from diabetic wounds. *Salvadora persica* (*S. persica*) is reported to possess wound-healing activity in fresh rats. However, there is no scientific report on the wound-healing activity of *S. persica* twigs extract on diabetic wounds. Hence, the aim of the present study is to evaluate *in-vivo* wound healing activity of methanol extract of *S. persica* twigs in alloxan-induced diabetic rats. Antioxidant, antimicrobial, angiogenesis and fibroblast proliferation activities of *S. persica* twigs extract were investigated to understand the mechanism of action behind the wound healing process. Methanol extract of *S. persica* twigs extract was formulated into topical gel using carbopol 971 P NF polymer as gelling agent and wound healing activity of the developed gel was evaluated using incision, excision and dead space wound models in diabetic rats. The developed topical gel accelerated the wound healing process as assessed by increased wound contraction, breaking strength, hydroxyproline and hexosamine content. Such investigation was encouraged by the efficiency of the methanol extract of *S. persica* twigs as antimicrobial, antioxidant, fibroblast proliferating and angiogenic properties. Hence, it can be concluded that topical gel containing methanol extract of *S. persica* twigs may be helpful in the enhanced healing of diabetic wounds.

Introduction

Wound healing is a very orderly and highly controlled process needs the coordination of various cells, growth factors and cytokines. Cutaneous wound healing consists of three overlapping phases: coagulation and inflammation, proliferation and remodeling.[1] However, this normal wound healing process is impaired in diabetes due to metabolic disorders.[2] Diabetes mellitus is hyperglycemic multistep disorder that interferes with the process of wound healing.[3] High glucose concentration in diabetic patient inhibits wound healing process. In diabetic patient, healing impairment is caused by a number of physiological factors, including infection, oxidative stress, diminished fibroblast proliferation and persistent inflammatory response, increased advanced glycation end-products.[4] The increased inflammation in the wound also hinders angiogenesis, preventing the wound from progressing into the proliferation phase. Other diabetic complications such as neuropathy, nephropathy, atherosclerosis and foot deformities count to the seriousness of the disease and in the evolution of chronic wounds in diabetic patients that might be problematic leading to ulceration, necrosis and amputation.[5] Involvement of all these factors makes the diabetic wound difficult to heal. Besides, this being a challenging task for physicians, it causes massive socio-economic problems. Improper wound healing causes substantial global morbidity and mortality.[6] Hence, it is essential to manage diabetic wounds by controlling all these factors responsible for delayed wound healing.

The current wound care and management utilizes multi-modal approach based on the strategy TIME (T-nonviable

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tissues, I-infection and inflammation, M-moisture balance, E-epithelialization and granulation tissue formation) defined by Wound Healing Society which indicates use of antimicrobials, including topical antiseptics, topical antibacterials and systemic antibiotics, wound debriding agent, anti-inflammatory agents, growth factors, etc. Antimicrobials used in wound debridment are synthetic agents that shows toxicities to the host cells such as human dermal fibroblasts and epidermal keratinocytes.[7,8] Hence there is a dire need to search for efficacious, cost-effective and safe alternative agents for wound healing. Plant extracts with wound healing activity can substitute synthetic agents as they are safe, reliable, clinically effective, low cost, globally competitive and better tolerated by patients.

S. persica (family *Salvadoraceae*) *is* an upright evergreen small tree or shrub. It is commonly known as Miswak or tooth brush tree with and is widely distributed in India, Africa, Saudi Arabia, Iran, Israel and Pakistan. Traditionally, it has been used as an antimicrobial toothbrush stick for oral hygiene and to treat gum inflammation.[9] It is known to possess anti-inflammatory, antimicrobial, analgesic, antipyretic, astringent, diuretic and bitter stomachic activities. It is used in the treatment of scabies, boils and toothache, nose troubles, piles, leucoderma, to treat hook worm infections, for teeth cleaning, re-establishment of the components of gastric mucosa, and as a laxative, in rheumatism, cough and asthma, to lower cholesterol plasma levels.[10] It contains salvadorine, salvadourea, vitamin C, trimethylamine, cyanogenic glycosides, tannins and saponins.[11]

In-vivo wound healing, *in-vitro* antioxidant and antimicrobial activity of methanol extract of *S. persica* twigs has been reported earlier, which revealed that the methanol extract of *S. persica* twigs showed *in-vivo* wound healing activity on fresh wounds in rats.[12] However, according to the literature survey, diabetic wound healing effect of methanol extract of *S. persica* twigs has not been reported. Hence, the present study aimed to investigate wound healing activity of methanol extract of *S. persica* twigs in diabetic rats. The hypothesis of the study is that if extract works on all the phases of wound healing, wound healing will not get impaired and prevents the further wound healing complications. Hence, in addition to wound healing activity, the *in-vitro* antioxidant assays, antimicrobial activity, scratch assay and chick chorioallantoic membrane (CAM) assay were employed to study free radical scavenging, antimicrobial, cell proliferation and angiogenesis effects, respectively, under the treatment of methanol extract of *S. persica* twigs.

MATERIALS AND METHODS

Allopurinol, bovine brain extract (BBE), bovine serum albumin (BSA), L-hydroxyproline were procured from Sigma Chemical Co. (USA). Xanthine oxidase, nitroblue tetrazolium chloride (NBT), xanthine and nutrient agar were procured from Himedia Ltd. Mumbai, India. Folin ciocalteu solution, pyrogallol red, potassium dihydrogen phosphate and thiobarbituric acid, dipotassium hydrogen phosphate, trichloroacetic acid, anhydrous sodium carbonate, ascorbic acid, butylated hydroxytoluene (BHT) potassium persulfate, ferrous ascorbate, ethylene diamine tetra acetic acid (EDTA), alloxan monohydrate were purchased from S.D.Fine Chemicals, Mumbai, India. D (+) glucosamine HCl was purchased from Merck. All other chemicals and solvents used were of analytical grade.

Plant Material and Preparation of Twig Extract

S. persica twigs were collected from Malvani area at Malad, Mumbai, India. The plant material was authenticated at Agharkar Research Institute, Pune, Maharashtra with a voucher specimen no. 3/187/2015/Adm-2799/Auth 15-172. Extraction was carried out using methanol solvent using soxhlet extraction method. A 50 g of the powder was extracted with methanol (250 mL) using a Soxhlet extraction apparatus for 30–32 hours. The obtained extract was concentrated on water bath. This methanol extract was referred to as ME-SPT.

In-vitro Antioxidant Assays

Xanthine Oxidase (XO) Inhibition Assay

The inhibitory effect on XO was measured as per method described by *Azmi.*[13] The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.5), sample solution, freshly prepared enzyme solution (0.2 units/mL of xanthine oxidase in phosphate buffer) and distilled water. The assay mixture was pre-incubated at 37°C for 15 minutes. Then substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37°C for 30 minutes. Next, the reaction was stopped with the addition of 0.5 M HCl. The absorbance was measured at 295 nm using UV-vis spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced with the phosphate buffer. Another reaction mixture was prepared (control) having DMSO instead of test compounds.

Superoxide Anion Scavenging Assay

Superoxide anion scavenging assay was performed according to method described by *Behera.*[14] The reaction mixture consisted of sodium phosphate buffer (pH 7.4), 3 mM Xanthine, 3 mM Ethylene diamine tetraacetic acid (EDTA), 0.15% BSA, 15 mM nitroblue tetrazolium chloride, sample solution. The reaction mixture was incubated at 25°C for 10 minutes. Then reaction was initiated by adding 1.5 U/mL of xanthine oxidase and incubated at 25°C for 20 minutes. After 20 minutes the absorbance was measured at 560 nm using UV-vis spectrophotometer. The inhibtion rate was calculated by measuring the amount of the formazan which was reduced from NBT by superoxide.

Non Enzymatic Protein Glycation Assay

The inhibitory effect of extract on advanced glycation end products (AGEs) formation was evaluated by the Bovine serum albumin (BSA)-glucose assay.[15] BSA (10 mg/mL) was incubated with (200 mM) glucose solution prepared in 200 mM sodium phosphate buffered and extract containing 0.02% sodium azide at 37°C. Catechin was used as a positive control. Reactions without any inhibitor were also setup. Each solution was kept in the dark in a capped tube. After 7 days of incubation, fluorescence intensity (excitation wavelength of 370 nm and emission wavelength of 440 nm) was measured for the test solutions.

Antimicrobial Activity

Antimicrobial activity of *S. persica* twigs (ME-SPT) of methanol extract was determined by the agar diffusion method[16] against wound pathogens. 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 hours old culture test organisms in a sterile petri dish. Cups were bored in agar by sterile cork borer and 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 was 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.

Evaluation of *In-vitro* Wound Healing Activity

In-vitro assays are useful in wound healing research for determining the possible effectiveness of various treatments, particularly antimicrobial and healing enhancing agents. Another advantage of *in-vitro* testing is the ability to screen multiple agents or samples simultaneously.

MTT Viability Aassay:

Viability test was carried out to eliminate cytotoxic concentrations of methanol extract of *S. persica* twigs using MTT [(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazlium bromide)] assay.[17] Cell line used was 3T3 L1 fibroblast. 100 µL of 3T3L1 fibroblast cells ($1x10^5$cells/mL) were seeded in the 96-well plate. A 2 µL of the different concentrations of the plant extract were added to the cell suspension. The volume was made up to 200 µL by adding 98 µL of DMEM (Dulbecco's Modified Eagle's Medium) medium supplemented with 10% FBS (fetal bovine serum). The sterile 96 well plate was then incubated for 24 hours at 37°C in presence of 5% CO_2. Untreated cells (without addition of test drugs) were served as control. After the incubation period, 10 µL of MTT (5 mg/mL) was added and the plate was incubated for 4 hours. After incubation, 150 µL of solution was removed from the well and 150 µL of 1N HCL: Isopropanol (1:24) solution was added to dissolve the formazon crystals formed. Optical density of the dissolved crystals was measured using an ELIZA reader at 570 nm. The absorbance indicated the number of viable cells in each well. The percentage of cell viability was calculated using following formula.

$$\% \text{ Cell viability} = \frac{(\text{Control - Sample})}{(\text{Control})} \times 100$$

Scratch Assay

The extract showing the optimal survival of cells was selected and used for incubation with 3T3L1 fibroblast cells for scratch assay. 100 µL of 3T3L1 fibroblast cells ($1x10^5$cells/mL) were seeded in the 96 well plate. The system was made up to 200 µL by adding 100 µL of DMEM supplemented with 10% FBS. The plate was incubated at 37°C in CO_2 incubator for 24 hours. When 70–80% confluency was reached a straight scratch was made using a 10 µL microtip stimulating a wound. Media was aspirated and the cells were subjected to PBS (phosphate buffer saline) washing to remove detached cells. Images of the each well were captured using TS view image capture software as 0 hour reading. Wells were replenished with DMEM (198 µL) and varying concentration of test drugs (2 µL). Untreated cells (without addition of test sample were served as control. Images of the wound were captured every 24 hours till the gap was filled. Distance between the gaps was measured to evaluate the rate of migration. After 24 hours, images of migrated cells were taken using digital camera connected to inverted microscope to observe the closure of wound area.[18]

Chick *Chorioallantoic Membrane* (CAM) Assay

Fertilized eggs of White Leghorn breed (six d old) were acquired from Goregaon poultry farm. The development of the embryo was ensured using an egg candler. Eggs were carefully surface sterilized with 70% alcohol and incubated at 37°C, with 80% humidity. The eggs were inoculated with 10 µL of standard/test drug/vehicle control and sealed with parafilm. The eggs were kept in vertical position with the air sac upward, for incubation, to prevent inoculated drugs touching the shell membrane. On 12^{th} day the eggs were broken gently from the sides of air sac and inner shell membrane was removed. The CAM was dispensed out on a petri plate containing 2 mL normal saline. Using image analysis software, stereomicroscopic images of four non-overlapping regions of CAM were taken. The images were then analyzed by counting the bifurcation and the average of the count was compared

with that of control and standard drug using '3.1 Analysis Software. The Counting was done as follows: single intact blood vessel was considered as 1 and the bifurcation point of a single blood vessel as 2.[19] The mixture of DMSO and water was used as vehicle control. Adrinomycin was used as a positive control.

Formulation of Gel Containing Methanol Extract of *S. persica* Twigs

1% of methanol extract of *S. persica* twigs was incorporated in preparation of gel formulation using Carbopol 971 P NF (1.5%) polymer as gelling agent. The developed formulation containing methanol extract of *S. persica* twigs was referred to as F2-ME- SPT.

In-vivo Pharmacological Evaluation

Experimental Animals

The animals were procured from Bharat Serum and Vaccines Pvt. Ltd., Thane and housed in the animal house of C. U. Shah College of Pharmacy. Animals were acclimatized to the experimental room for one w and conditioned at room temperature and natural photoperiods. Animals were provided to standard food pellets as basal diet and water *ad libitum*. Study was conducted after obtaining ethical committee clearance from the Institutional Animal Ethical Committee (IAEC) of C. U. Shah College of Pharmacy, S. N. D. T University, Mumbai. (Reg. no. 39/1999/CPCSEA). Male and female albino rats weighing 180–250 g were used.

Grouping of Animals

Four groups of animals containing six each were used for each of the excision, incision and dead space wound models. The animals of group I were treated with topical application of carbopol gel of *S. persica* twigs. The animals of group II served as the reference standard and treated with betadine (Win Medicare Pvt. Ltd.) ointment. The animals of group III were considered as the vehicle control and treated with plain carobopol gel. The animals of group IV were left untreated (Negative control). The experimental protocol for incision and excision wound model was approved by IAEC with Protocol no. CUSCP/IAEC/08/2014 while IAEC approved dead space wound model protocol with protocol number CUSCP/IAEC/11/2016.

Induction of Experimental Diabetes

Diabetes was induced in overnight-fasted rats by a single intraperitoneal (i.p.) injection of alloxan monohydrate 120 mg/kg. The rats were fed with 5% glucose water and basal diet ad libitum during the next 24 hours to avoid sudden hypoglycemia. On day 2, glucose water was replaced with normal distilled water. Blood samples were withdrawn from tail of animals at 72 hours. Fasting blood glucose levels were estimated using glucose strips. Hyperglycemia was confirmed by elevated blood glucose levels determined at 72 hours after injection. Rats with fasting blood glucose levels above 200 mg/dl were considered diabetic and selected for the study.[20,21] These animals were then used for the evaluation of wound healing activity by excision, incision and dead space wound model. The particular skin area of rats was shaved using hair remover cream (Veet). Rats were anaesthetized using sodium pentobarbitone (45 mg/kg, ip) before wound creation of excision, incision and dead space wound models.

Excision Wound Healing Model

A full thickness of the excision wound of circular area (approx. 500 mm^2) and 2 mm depth was made on the shaved back of the rats. F2-ME-SPT, plain Carbopol gel and betadine were topically applied once a day, to different group of rats, starting from day 0 till complete epithelialization. The rate of wound contraction was measured as percentage reduction of wound sizes every 4 days interval. Progressive decrease in the wound size was monitored periodically using transparency paper and a marker, and the wound area was assessed graphically to monitor the percentage of wound closure, which indicates the formation of new epithelial tissue to cover the wound. Number of days required for falling of escher without any residual raw wound was recorded to estimate the period of epithelialization.[22]

Incision Wound Model

Incision of 6 cm was made through the skin and cutaneous muscles on the depilated back of the rat using scalpel blade. The incision was then closed with interrupted sutures with stitches 1-cm apart using black surgical thread and curved needle. F2-ME-SPT, plain carbopol gel and betadine were applied to different groups of rats, once daily from day 0 to day 9 post-wounding. The sutures were then removed on the 8^{th} post wounding day and the breaking strength of 10^{th} day old wound was measured by tensiometer.[23]

Dead Space Wound Model

A longitudinal paraventral incision was made through the skin on the depilated back of the rat using sterile scalpel blade. Dead space wounds were inflicted by implanting two sterilized cotton pellets (10 mg), one on either side of the lumbar region on the ventral surface of each rat. The parted skin was kept together, stitched, and closed with interrupted sutures (black surgical thread and curved needle) 1-cm apart. F2-ME-SPT, plain carbopol gel and betadine were applied to different groups of rats, once daily from day 0 to day 9 post-wounding. On the 10^{th} post-wounding day, granulation tissues formed on cotton pellet were dissected out carefully. The wet weight of the granulation tissue was noted.[24]

The granulation tissues were dried at 60°C for 12 hours, and dry weight was recorded. The dried tissues were then hydrolyzed with 6 N HCl and kept at 110°C for 24 hours in a sealed glass tubes. This acid hydrolysate was used for estimation of hydroxyproline and hexosamine content.

Table1: Inhibition of xanthine oxidase, superoxide anion, protein glycation, lipid peroxidation by S. persica extract

Extracts/ Standards	*Percent inhibition of Xanthine Oxidase (100 µg/mL)*	*Percent scavenging of superoxide anion (1000 µg/mL)*	*Percent inhibition of protein glycation (2000 µg/mL)*	*Inhibition of Lipid peroxidation IC_{50} (µg/mL)*
ME-SPT	3.87 ± 1.72	21.05 ± 4.84	21.81 ± 4.82	1913.70 ± 9.96
Allopurinol (100 µg/mL)	93.25 ± 0.50	-	-	-
Catechin (100 µg/mL)	-	90.00 ± 0.60	98.52 ± 0.53	-
Quercetin (1000 µg/mL)	-	-	98.74 ± 50.21	-
Butylated Hydroxy Toluene	-	-	-	67.64 ± 1.44

Values are represented in Mean ± SD (n=3)

Determination of Hydroxyproline Content

The above acid hydrolysate, neutralized to pH 7 was then added to 1-mL of 0.01 M $CuSO_4$, 1-mL of 2.5 N NaOH and 1-mL of 6% H_2O_2. All the tubes were incubated at 80°C for 5 minutes and tubes are chilled in ice and water bath. Upon cooling, 4 mL of 3 N H_2SO_4 and 2 mL of 5% p-dimethylaminobenzaldehyde were added. The samples were incubated at 70°C for 16 minutes and then cooled. The absorbance was measured at 540 nm using spectrophotometer. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline at the same time.[25]

Determination of Hexosamine content

A 1-mL of acid hydrolyzed fraction was added to 1-mL of acetyl acetone reagent in 0.5 N Na_2CO_3 and heated in boiling water bath for 15 minutes. After cooling, 5 mL of 95% ethanol and 1-mL of ehrlichs reagent were added to it. The reaction was allowed to stand for 30 minutes to complete. Purple red color was developed was measured after 30 minutes at 530 nm against the blank. Hexosamine content of the samples was determined from the standard curve prepared with D (+) glucosamine hydrochloride.[26]

Statistical Analysis

Results were expressed as Mean ± SEM (Standard Error of Mean). Inter comparisions between the groups were made using One way ANOVA followed by Bonferroni post test on GraphPad Instat 3 statistical software and statistical significance was determined.

RESULTS AND DISCUSSION

Wound healing is a very complicated process and requires the collective efforts of many different tissues and cell lineages. Healing process start soon after wounding and it takes place in four phases. The first phase involves coagulation and inflammations which is characterized by platelet aggregation to control excessive blood loss from the damaged vessels. The proliferative phase comprises angiogenesis, granulation tissue formation, collagen deposition and epithelialization.[27] The last phase of the healing process encompasses collagen deposition and remodeling within the dermis.[28]

However, in diabetes, increase in free radicals *in-vivo* is observed and this reduces body's antioxidation capacity. The overproduction of free radicals such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) at wound site during inflammation phase leads to oxidative stress. Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and then uric acid in the presence of O_2 to yield superoxides. These **s**uperoxides further react with nitric oxide to form deleterious reactive peroxynitrite radicals. Superoxide is converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). Release of H_2O_2 may promote formation of other oxidants that are more stable including, hypochlorous acid, chloramines, and aldehydes.[29] The wound site becomes rich in oxidants along with their derivatives such as chloramines. Thus, oxidative stress during prolonged inflammation induces remarkable cytotoxicity within the wound area impairing healing. It was reported that antioxidants improve the healing process of infected and noninfected wounds by lessening the damage caused by oxygen radicals.[30] Hence, antioxidant activity of the plant extract was evaluated by targeting free radicals such as superoxides, advanced glycation end products and xanthine oxidase enzyme.

In results of antioxidant assays, allopurinol showed high inhibition of XO of 93.25% ± 0.50 at 100 µg/mL. Catechin showed maximum scavenging of superoxide anions of 90.00 ± 0.6 % at 100 µg/mL concentration. ME-SPT showed percent inhibition of XO of 3.87 ± 1.72 at 100 µg/mL and percent scavenging of superoxides of 21.05 ± 4.84 at 1000 µg/mL (Table 1) and hence, can prove as effective inhibitor of xanthine oxidase and scavenger of superoxide anions.

Advanced glycation end-products (AGEs) are produced in patients with long-term hyperglycemia and responsible for multiple symptoms including impaired wound healing,[6,31] catechin and quercetin are well-known inhibitor of non-enzymatic protein glycation and hence, showed excellent antiprotein glycation of 98.52 ± 0.53 and 98.74 ± 50.21%, respectively at a concentration of 1000 µg/mL for the BSA/glucose system. The percent inhibition of protein glycation of ME-SPT was found to be 21.81 ± 4.82% at the concentration of 2000 µg/mL (Table 1). Hence, antiglycation activity of ME-SPT might be helpful in treating diabetic wounds.

Table 2: Antimicrobial activity of S. persica extract by cup plate method

Microorganisms	*Zone of inhibition (mm)*		
	Chloramphenicol (50 µg/well)	*ME-SPT (100 mg/well)*	*Fluconazole (50 µg/well)*
Pseudomonas aeruginosa	10.00 ± 0.00	7.00 ± 0.00	-
Staphylococcus aureus	14.33 ± 0.58	8.33 ± 1.53	-
Streptococcus pyogenes	12.67 ± 0.58	6.33 ± 1.53	-
Escherichia coli	17.00 ± 0.00	5.33 ± 1.53	-
Klebsiella pneumonia	11.00 ± 1.00	12.33 ± 0.58	-
Klebsiella aerogens	12.00 ± 1.73	6.66 ± 1.53	-
Clostridium perfringens	18.00 ± 0.00	6.00 ± 1.73	-
Candida albicans	-	3.67 ± 0.58	12.33 ± 0.59
Aspergillus niger	-	1.33 ± 0.58	13.00 ± 0.10

Values are represented in Mean ± SD (n=3)

Table 3: Effect of *S. persica* extract on % cell viability by MTT viability assay

Extracts/sample	*% cell viability at concentration (µg/mL)*				
	100	*500*	*1000*	*1500*	*2000*
Vehicle control	20.93				
ME-SPT	-13.84	-10.4	-6.49	0.82	-1.36

Lipid peroxidation (LPO) is the oxidative degradation of lipids and proceeds by a free radical chain reaction mechanism. This process leads to cell damage during majority of cell injuries like ulcers, burns and infected wounds. The results indicated that ME-SPT showed inhibition of lipid peroxidation with IC_{50} value of 1913.70 ± 9.96 µg/mL. Standard butylated hydroxy toluene (BHT) showed inhibition of lipid peroxidation at IC_{50} value of 67.64 ± 1.44 µg/mL (Table 1). Thus, ME-SPT can inhibit lipid peroxidation process and hence, reduce cell destruction and promote wound healing process. From the results of *in-vitro* antioxidant assays, it can be concluded that the antioxidant activity of methanol extract of *S. persica* may contribute to wound healing by reducing oxidative stress. Polymicrobial wounds contain several potential pathogens, any one of which can cause infection. The development of a wound infection negatively impacts the wound healing process, delaying healing. External application of topical formulation prevents microbes from invading through wound and protects the wound against infection. Microorganisms such as *Staphylococcus aureus, Streptococcus pyogenes, Clostridium perfringens, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Klebsiella aerogenes, Candida albicans* and *Aspergillus niger* are reported as wound pathogens [32]. Hence, the agar diffusion method was employed to screen inhibitory potential of the extract against these wound pathogens. For all the test microorganisms, methanol extract of *S. persica* twigs (100 mg/well) showed pronounced antibacterial activity against *K. pneumoniae (*12.33 ± 0.58 mm), moderate activity against *S. aureus (*8.33 ± 1.53 mm) and *P. aeruginosa (*7.00 ± 0.00 mm), low activity against *K. aerogens (*6.66 ± 1.53 mm), *S. pyogenes (*6.33 ± 1.53 mm), *Clostridium perfringens (*6.00 ± 1.73 mm) and *E. coli (*5.33 ± 1.53 mm) as compared to standard chloramphenicol It also showed antifungal activity against *C. albicans* (3.67 ± 0.58 mm) and *A. niger* (1.33 ± 0.58) as compared to fluconazole. (Table 2). This study observed a moderate to effective antimicrobial activity of the extract against all tested wound pathogens. Antimicrobial agents promote wound healing by forming barrier against microbial infection in the wound.[33] Hence, the antibacterial activity of ME-SPT against common wound-infecting pathogens might contribute remarkably to the faster wound healing rate.

To make sure that effect of extracts treatment on migration was not interfering by any toxicity; impact of different concentrations (100, 500, 1000 and 2000 µg/mL) of ME-SPT on 3T3L1 cells over the course of 24 hours was determined (Table 3). The non-toxic concentration of ME-SPT was identified by performing MTT viability assay. Negative sign indicates more than 100% viability of cells. It was observed that ME-SPT, showed maximum viability of 3T3L1 cells at a concentration of 100 µg/mL treatment as compared to other concentrations. Hence, a concentration 100 µg/mL of ME-SPT was used for further testing in scratch assay.

Fibroblast cell cultures have been proposed as a method for investigation of wound healing activity. Dermal fibroblast is the first line of defense which responds to injury and is essential for cutaneous wound repair by proliferation and migration process into the wound site.[34] The effect of ME-SPT at 100 µg/mL concentration on percent migration of fibroblast cells was determined. The images of scratch assays on 3T3L1cells at 0, 24, 48 and 72 hours post-injury time show progression of wound closure The percent migration of fibroblast cells was found to be 100% with ME-SPT at 72 hours. Whereas wound area at 72 hours post-injury remained open for negative control and vehicle control groups on scratch wounded 3T3L1 cells (Table 4 and Fig. 1). proliferation and migration of the cells are important events in wound healing process; therefore

Table 4: Effect of *S. persica* extract on % cell migration by scratch assay

Extracts/samples	*% cell migration at 100 µg/uL*		
	24 hours	*48 hours*	*72 hours*
Negative control	28.95 ± 2.31	65.17 ± 1.09	84.54 ± 1.88
Vehicle control	23.38 ± 2.73	61.21 ± 1.38	90.95 ± 1.88
ME-SPT	32.23 ± 2.09	63.24 ± 0.86	100 ± 0.00

Values are expressed as Mean ± SD (n=4)

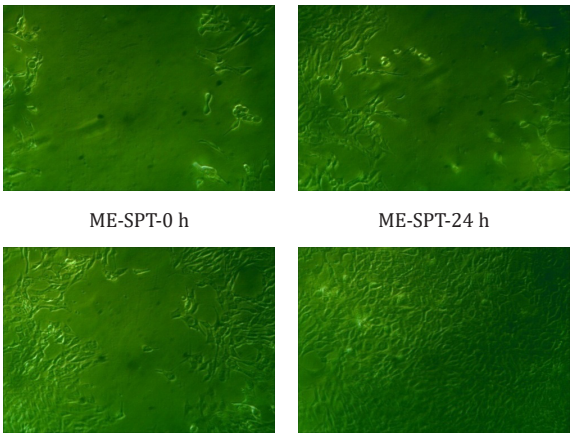

Fig. 1: Measurement of fibroblast cell migration on 24, 48 and 72 hours after

increased percent migration of fibroblast observed with ME-SPT, may contribute in improving their cutaneous wound healing progression.

Treatment with ME-SPT

An essential feature of normal wound repair is the formation of granulation tissue, i.e., fibrovascular tissue containing fibroblasts, collagen, and blood vessels, which is the hallmark of an established healing response. Inhibition of angiogenesis impairs wound healing.[35,36] Hence, ME-SPT was evaluated for angiogenic activity by CAM model in fertilized eggs. ME-SPT at concentration of 2000 µg/mL showed marked increase in number of blood vessels as compared to negative control (Table 5 and Fig. 2). The angionenic effect of ME-SPT extract may accelerate the angiogenesis process which is required to proceed into proliferative phase of wound healing process.

Herbal formulations containing plant extracts have been reported to be useful in wound care, facilitating wound healing, minimizing pain and discomfort of the patient.[37] Carbopol gel formulation is more preferred for topical application because of their advantages such as good bioadhesive properties, high viscosity at low concentration and good patient acceptability.

In evaluation of *in-vivo* wound healing activity, no single method is adequate to represent the various components of wound healing process.[38] Determination of various individual components of the phases of wound healing can

Table 5: Angiogenesis activity of S. persica extract by CAM assay

Extracts/Samples	*No. of blood vessels*
Negative control	46.67 ± 13.01
Vehicle control (DMSO)	36.90 ± 17.14
Positive control (Erythropoeitin-10 IU/mL)	67.43 ± 27.42
ME-SPT (2000 µg/mL)	51.56 ± 20.96

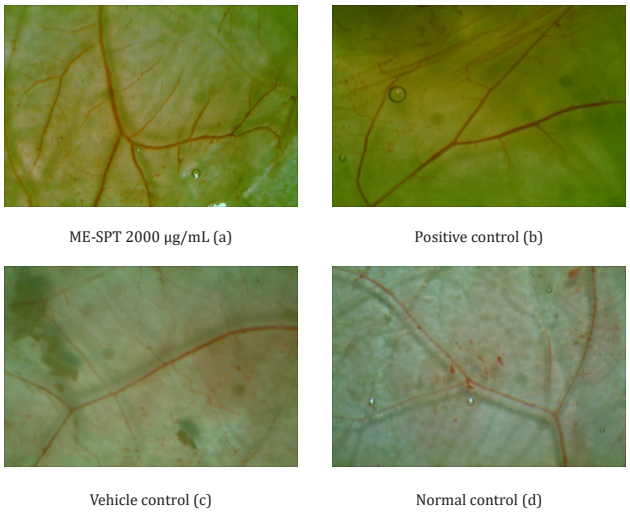

Fig. 2: Images of angiogenesis in 10th day old chick eggs after treatment with: ME-SPT (a), positive control (b), vehicle control (c) and Normal control (d)

provide important insights about events operative during repair. Hence, three different models (incision, excision and dead space wound models) in diabetic rats were used to assess the effect of the developed gel formulation containing methanol extract of *S. persica* twig (F2-ME-SPT) on various phases of wound healing, which run concurrently but independent of each other.

Rats treated with F2-ME-SPT accelerated wound healing in diabetic rats by influencing different aspects of the healing process. All the parameters observed for wound healing activity were significantly affected.

Wound contraction indicates the rate of reduction of the unhealed area during the healing process. In excision wound model, the enhanced rate of wound contraction was observed in rats treated with F2-ME-SPT. On 24th day, the wounds treated with F2-ME-SPT healed 100%. It was also observed that duration of healing was extended upto 29 d with wound contraction of 97.26 ± 0.69% in diabetic negative control rats and 28 d with 100% wound contraction in diabetic vehicle control rats. Rats treated with F2-ME-SPT and standard Betadine exhibited statistically significant increase in wound contraction on day 20 (p<0.01) as compared to diabetic negative control group (Fig. 3).

In excision wound model, the epithelialization period indicates the formation of new epithelial tissue to

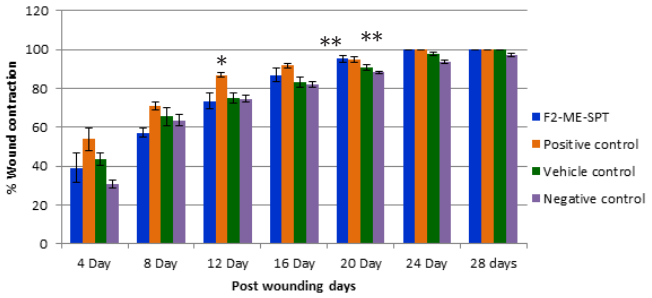

Fig. 3: Effect of gel formulation on % wound contraction of excision wound in diabetic rats. Values are expressed as Mean ± SEM (n=6) *p< 0.05; **p<0.01, versus negative control; (One-way ANOVA, followed by Bonferroni test)

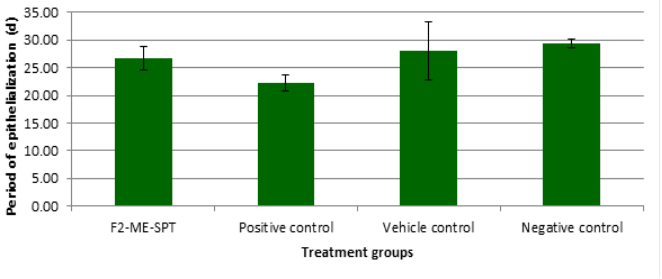

Fig. 4: Effect of gel formulation on epitheliazation period of excision wound in diabetic rats. Values are expressed as Mean ± SEM (n=6) (One-way ANOVA, followed by Bonferroni test)

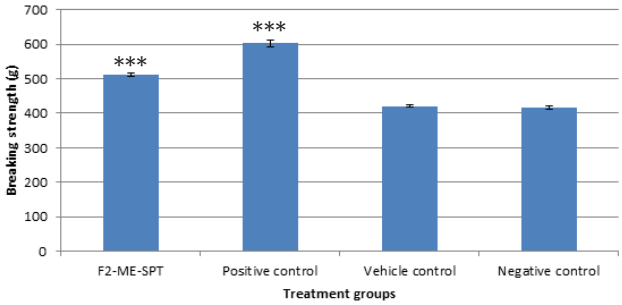

Fig. 5: Effect of gel formulation on breaking strength of incision wound in diabetic rats Values are expressed as Mean ± SEM (n=6) ***p<0.001 versus negative control, (One-way ANOVA, followed by Bonferroni test)

cover the wound. The standard Betadine healed the wound with an epithelialization period of 22.24 ± 3.56 d. Complete epithelialization was observed on 26.75 ± 5.15 d in rats treated with F2-ME-SPT. The time taken for complete epithelialization of excision wound in diabetic negative control group was 29.33 ± 1.96 d (Fig. 4). The epithelialization time was also shorter in animals treated with F2-ME-SPT and standard betadine when compared to a negative control group. Epithelialization involves the proliferation and migration of epithelial cells across the wound bed.[39] Therefore, the shorter epithelialization time in the rats treated with F2-ME-SPT and standard betadine might be due to facilitated proliferation of epithelial cells and/or increased epithelial cell viability.[40] Thus, shorter epithelialization periods in F2-ME-SPT and standard Betadine treated group indicate its potential application as a wound healing agent.

In the incision wound model, the breaking strength is the strength of a healing wound and is measured experimentally by force required to disrupt it. The breaking strength increases rapidly as collagen deposition increases, and cross-linkages are formed between the collagen fibres. The rats treated with F2-ME-SPT (511.67 ± 4.22 g) and standard betadine (602.50 ± 9.47 g) showed statistically significant increase (p < 0.001) in breaking strength of wound as compared to the diabetic negative control group (416.17 ± 4.48 g) (Fig. 5). Increase in breaking strength of incised wound treated with F2-ME-SPT and standard betadine is an indication of increased collagen synthesis; remodeling of collagen and the formation of stable intra- and intermolecular crosslinks increase the breaking strength of newly formed tissue and thereby, strength of repaired wound tissue.[41]

Dead space wound model provides to assess wound collagen accumulation by measuring hydroxyproline and hexosamine content in granulation tissue. It was observed that F2-ME-SPT and standard betadine showed higher hydroxyproline content in granulation tissues when compared with diabetic negative control group and vehicle control group (Fig. 6). The increased hydroxyproline content of the dead space wounds indicated faster collagen turnover leading to rapid healing with concurrent increase in the tensile strength of the treated wounds.[42] Hence, based on the amount of hydroxyproline detected in granulation tissue of dead space wound model, it could be assumed that the methanol extract of *S. persica* twigs has enhanced the strength of the incision wound by increasing the collagen levels, which could stick the wound edges together at the repaired site.

Hexosamine is a matrix molecule, which act as ground substratum for the synthesis of new extracellular matrix and secreted during tissue repair.[43] It was observed that hexosamine content was increased in granulation tissue of F2-ME-SPT treatment group compared to diabetic negative control group (Fig. 6). Increased hexosamine content indicates more stabilization of collagen molecules via enhanced electrostatic and ionic interactions, thereby enhancing the wound healing process.

Thus, it can be concluded that developed gel formulation containing methanol extract of *S. persica* twigs exhibited significant healing effects when applied topically on the wounds of diabetic rats by affecting various stages of the healing process. The present study demonstrated for the first time that the topical application of formulation containing methanol extract of *S. persica* twig promoted wound healing activity in diabetic rats, probably due to its free radical scavenging properties, xanthine oxidase inhibitory and protein glycation inhibitory activity, antimicrobial, cell proliferating properties

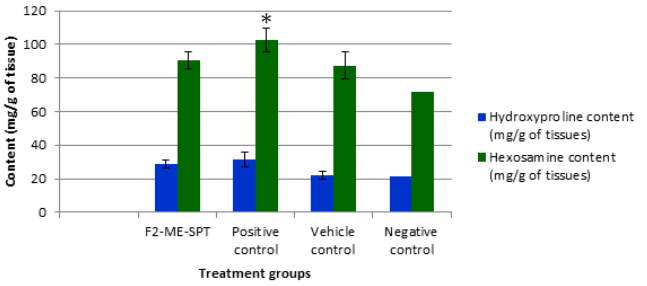

Fig. 6: Effect of gel formulations on hydroxyproline and hexosamine content of granulation tissues in diabetic rats. Values are expressed as Mean ± SEM (n=6) *p<0.05 versus negative control. (One-way ANOVA, followed by Bonferroni test)

and angiogenesis. Hence, *S. persica* twig extract with multimodal actions such as antioxidant, antimicrobial, and proangiogenic activities may be helpful in enhanced healing of diabetic wounds.

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