

Phytochemical and Wound Healing Effects of Methanolic Extract of *Salvia multicaulis* Vahl. in Rat

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Abstract

Background: Plants and their phytochemicals have immense potential for the management of different types of wound. *Salvia multicaulis* Vahl. is an evergreen shrub species, which traditionally used as a wound healing agent.

Objective: The aim of the present study is to evaluate phytochemicals and wound healing activity of *S. multicaulis* in an excision wound model.

Methods: Preliminary phytochemical screening tests were performed on the methanol (80%) extract. Total phenolic content was measured using Folin-Ciocalteu reagent. Antioxidant activity of the extract was assessed by (DPPH) free radical scavenging test. 2x2 excision wounds were induced in the dorsal area of 30 Wistar rats. Animals were received topical eucerine, 1% phenytoin as well as 5%, 10% and 20% ointments of the plant extract in eucerine base. Wound areas were photographed and wound contraction was calculated as the reduction percentage of wound size compared with zero days. Last day skin samples of wounded area were used for histological evaluations.

Results: Collagen deposition for positive control group and A20% were low fibrils and A10% high fibrils and A5% highest fibrils. Total phenolic content was 262.083 ± 47.15 mg GAE/g extract. IC50 was 54.02 μ g/mL. All concentrations of the extract ointments were significantly effective in the animal model of excision wound ($P < 0.05$) which was also confirmed in histological study.

Conclusion: It is concluded that *S. multicaulis* extract could act as a wound healing agent. This activity is possibly due the high concentrations of phenolic compounds.

Keywords: Wound, *Salvia multicaulis* Vahl, Antioxidant, Total phenol, Exision



Introduction

Various plant species have served as a source of medicine for people all over the world [1]. For year, plants were of the most intense areas of natural product research, yet the field is far from being exhausted. Plants and their extracts have immense potential for the management and treatment of wound [2, 3]. These natural agents induce healing and regeneration of the tissue by multiple mechanisms however, there is need for scientific validation, standardization and safety evaluation of plants of traditional medicine before they could be recommended for human subjects [4]. Wound healing is a complex, multifactor cascade of events involving several cellular and biochemical processes to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin that occurs in four stages. The first phase is coagulation which controls excessive blood loss from the damaged vessels. The next stage is inflammation followed by re-epithelialization which includes proliferation, migration and differentiation of epithelial cells. In the final stage of the healing process collagen deposition and remodeling occurs within the dermis [5].

There are 58 *Salvia* species growing in Iran among which 17 are endemic. *Salvia multicaulis* Vahl. is an evergreen shrub growing to 0.3m × 0.25 m. It is growing wildly in Eastern and Central Asia [6]. Several phytochemicals are identified in *S. multicaulis* including diterpenoids, norditerpenoids, triterpenoids and salvimultine [7, 8]. The essential oil of *S. multicaulis* from Iran has also been previously analyzed. Bornyl acetate,

β -caryophyllene and α -pinene were the major constituents of the essential oil of flowering shoots [9], while the main constituents of the oil from leaves and flowers were α -pinene, 1,8- cineole, limonene and camphor [10]. Many pharmacological and biological effects of *S. multicaulis* have been reported including Anti-Inflammatory, Antimicrobial and analgesic effect [11, 12]. *S. multicaulis* is traditionally used as a wound healing agent in different parts of Iran and turkey [13]. The aim of the present study is to assess wound healing activity of *S. multicaulis* in an animal model of excision wound.

Materials and Methods

Chemicals

All reagents and solvents were of analytical grade or pure quality which was purchased from Merck Company (Germany) with the exception of phenytoin sodium 1% which was purchased from Medipharm Company (Iran).

Preparation of extract

The flowered aerial parts of *S. multicaulis* were collected from Kermanshah in spring 2013 and were identified by Dr. Iraj Salimikia. A voucher specimen (NO. 240 TEH) was deposited at Herbal Museum, Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences. Dried aerial parts (2 kg) of *S. multicaulis* were crushed then extracted with methanol (80%) by maceration for 48 h at room temperature (thrice). The extract was concentrated using a rotary vacuum evaporator and then freeze dried (68 g). Preliminary phytochemical screening tests were performed based on standard



methods for the semi-quantitative assessment of the presence of major plant metabolites in the extract.

1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

The DPPH scavenging activity was measured using the method described by Sanchez-Moreno *et al.* with some modifications [14]. Briefly, 2 ml of a methanolic solution of DPPH (0.004%) was mixed with 1 ml of serial dilutions (10, 25, 50 and 100 µg/ml) of *S. multicaulis* extracts. 1 ml of butylated hydroxyl toluene (BHA) solution (0.001%) and 1 ml of methanol was mixed and used as reference standard. The absorbance was immediately recorded at 517 nm and its decline was measured every 5 min up to 30 min, using a UV spectro-photometer (optizem 2120 UV plus Mecasys, Korea). All solutions were kept in darkness and at room temperature during this period. Methanol was used as blank. All tests were carried out in triplicate. Finally the radical scavenging activity was calculated as percentage of DPPH discoloration using the equation below:

$$\text{Scavenging DPPH free radical or Inhibition (\%)} = 100 \times [1 - ((\text{AE} - \text{AC}) / \text{AD})]$$

Where AE is the absorbance of the DPPH solution, when extract has been added at a particular level, AD is the absorbance of the DPPH solution with methanol (without extract) and AC is the absorbance of the control solution.

Total phenolic content (TPC)

Total phenolic content of *S. multicaulis* extract was determined according to the method of Miliauskas G, *et al.* [15]. In brief, extract (10 mg) was dissolved in methanol (1 mL). Out of these aliquots, 0.5 mL of each were transferred to separate flasks, Folin-Ciocalteu reagent (2.5 mL) was added to each, followed by addition of sodium carbonate solution (2 mL; 7.5%). The mixtures were stirred well and incubated at 40 °C for 30 min to ensure the completion of reaction. The absorbance of final mixtures was read at 765 nm using spectrophotometer. Total phenolic content was expressed as gallic acid equivalent per g of extract (mg GAE/µl g extract).

Determination of minimum inhibitory concentration (MIC)

Macrodilution method was used to evaluate *in vitro* antimicrobial activity of the extract against predominant wound pathogens. *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027) were culture overnight at 35°C in Mueller-Hinton agar (MHA) and *Candida albicans* (ATCC 10231) was cultured 48 h at 25°C in Sabouraud dextrose agar (SDA). Dilution series of the plant extract using double strength Mueller-Hinton broth (MHB) for bacteria and double strength Sabouraud dextrose broth (SDB) for *C. albicans* were prepared from 25 to 2.5 mg/ml. The bacterial and fungal inocula were prepared by suspending colonies from MHA and SDA in 0.9% saline, respectively. The inocula were

adjusted photometrically at 600 nm to a cell density equivalent to 0.5 McFarland standards (1.5×10^8 CFU/ml). The suspensions were diluted in sterile saline to give 10^6 CFU/mL. Then 1 ml of each microbial suspension was added to each sequential dilution to reach the final inoculum size of about 5×10^5 CFU/ml. The tubes containing bacteria were incubated at 30-35°C for 24 h and those containing fungi were incubated at 20-25°C for 48 h. After incubation, the tubes were tested for the absence or presence of visible growth in comparison with that of the growth in extract-free control tubes. The endpoint MIC is the lowest concentration of the compound at which the test strain does not demonstrate visible growth.

Ointment preparation for topical application

An alcohol free extract of *S. multicaulis* was used for the preparation of the ointment for topical application. Three concentrations of the extract (5%, 10% and 20% w/w) was formulated using eucerine as base [16].

Animal study

30 male Wistar rats of 250±30g body weight were used in study. All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27 °C and humidity 60-65% with 12:12 light: dark cycles). Food was provided in the form of dry pellets and water *ad libitum*. All animal experiments comply with the ethical standards of animal handling and approved by institutional animal ethics committee. Excision wound model was used

for the evaluation of *in vivo* wound healing activity. Rats were shaved at dorsal parts under ketamine: xylazine (80-100 mg/kg: 5-10mg/kg i.p.) and prepared for operation. Wound of 2x2 areas was produced in each rat by excising the skin. The wounded animals were kept individually (one in each cage) and were randomly divided into 5 groups (6 per each) receiving eucerine (negative control), 1% w/w phenytoin sodium ointment (positive control) as well as 5%, 10% and 20% ointments of the plant extract. Treatments began 24 h after wound induction and were applied topically each 24 h for 14 days. Wounded areas were everyday photographed by a digital camera and a paper ruler was used as a scale. Wound contraction rate was calculated using the formula bellow:

$$\text{Wound closure (\%)} = 100 \times [(\text{first day wound size} - \text{specific wound size}) / \text{first day wound size}]$$

Histological evaluation

Animals were sacrificed on the last day and granulated tissue was collected and preserved in 10% buffered formalin. Series of 3-4 µm thickness sections were prepared and stained with hematoxylin and eosin and photographed under ×400 magnification. Tissue histological changes were evaluated by a blind histologist.

Statistical analysis

All values were expressed as mean ± S.D. Data were analyzed by one-way ANOVA, followed by Tukey's post hoc test. The results were considered significantly different at $P < 0.05$.



Results

Extraction and preliminary phytochemical screening tests

The extraction yield was 30% and the results of preliminary phytochemical screening tests are shown in Table 1. This extract contained flavonoids and tannins.

DPPH free radical scavenging activity

This extract exhibited a good and notable dose dependent inhibition of the DPPH activity. BHA inhibition activity at the end of 30 min was significantly lower than extract in 300 and 500 µg/ml ($P < 0.05$). The inhibitory concentrations 54.02 µg /ml (IC₅₀) for plant extract defined as the concentration causing 50% inhibition in DPPH absorbance were measured from the concentration inhibition.

Total phenolic content

The TPC value was 262.083±47.15 mg GAE/g extract.

Rate of wound contraction

Table 2 shows the percentage of wound contraction for each group. The wound area was found to decrease significantly in all treated groups when compared to control on the day 14 ($P < 0.05$).

Histological study

Collagen deposition for negative control was lowest fibrils and positive control group and A20% were low fibrils and A10% high fibrils and A5% highest fibrils. Macrophages and lymphocytes infiltration for negative control was highest but positive control and A10% and A20% were high but for A5% was high lower than others groups. Polymorph nuclear (PMN) for negative control was highest and for positive control and A10% and A20% were high but for A5% was high lower than others groups. Blood vessels for negative

Table 1- Preliminary phytochemical screening of extracts of *S. multicaulis*

Extract	Antraquinone	Alkaloid	Saponin	Flavenoid	Tannin
Hydromethanolic	-	-	-	++	++

(-, absent; +, Present; ++, abundant; +++, most abundant).

The intensity of the coloration determined the abundance of the compound with the exception of the tannin and flavenoid test

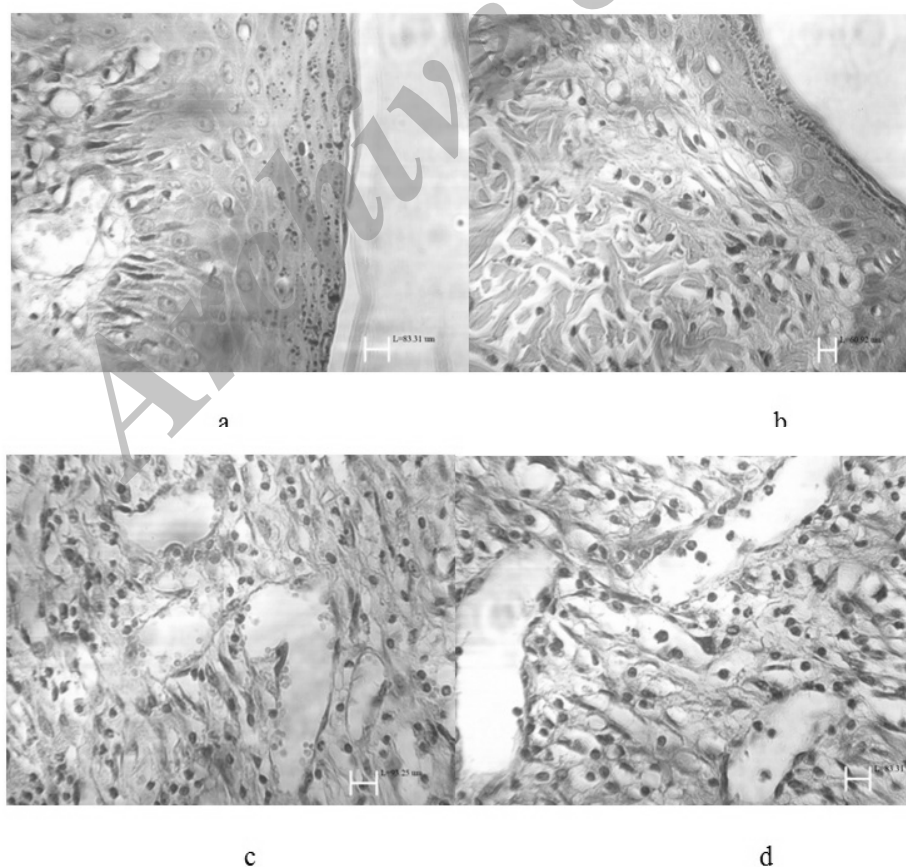
Table 2- Percentage of wound contraction for each group on day 14

Contraction rate	14 th
Negative control	89.61±6.59
Positive control	96.35±1.97
A5%	99.38±1.97*a
A10%	97.38±2.78*a
A20%	97.54±1.54*a

Values are expressed as mean ± standard deviation for each group significant differences: * $P < 0.05$ in comparison to positive control. A: aerial parts extract

control was highest but for positive control and A10% and A20% were high but for A5% high lower than others groups. Skin appendage formation for all groups was negative. Ridge and papilla for all groups were also negative. On day 14 epidermal regeneration for negative control in large part of wound not complete and for positive control and A10% not reach normal level and stratum corneum seen. A5% almost reaches normal level and stratum corneum seen. A20% epidermal formation in unrepaired parts of wound not complete. Collagen deposition for negative control lowest irregular bundels and many fibrils seen for positive control low irregular bundels and many fibrils seen for A5% and A10% almost well organized arrangement of bundle for A20% irregular bundles in unrepaired parts of wound fibrils. Macrophages and lymphocytes

infiltration for negative control was high but for positive control and A10% and A20% were low and A5% was lowest. PMN for negative control was high and for positive control lower than negative control for A10% and A20% were low but for A5% was lowest. Blood vessels for negative control was high and for positive control was lower than negative control and for A10 % and A20% were low and for A5% was lowest. Skin appendage formation for negative and positive control not form and for A5% begin to form and A10% better formation and A20% not form in unrepaired parts wound ridge and papilla for negative control was lowest and positive control not seen and A5% was highest and A10% not seen and A20% was also low (Figure 1).



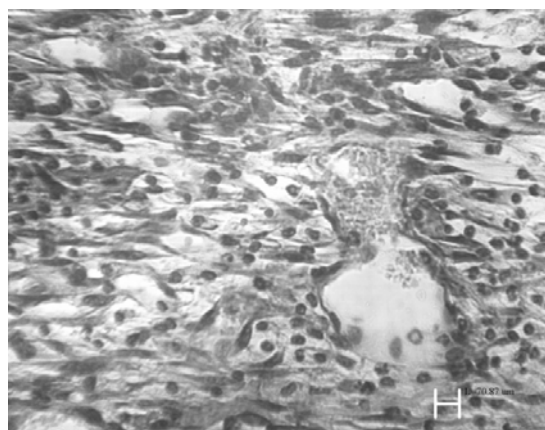


Figure 1- Microscopic panel of wound on the 14th day of treatment in rats: a) A5% treated wound, b) A10% treated wound, c) A20% treated wound, d) Phenytoin1% treated wound as reference standard, e) eucerin treated wound as control.
A: aerial parts extract, 400× magnification

Antimicrobial activity

In MIC determination test, no microbial inhibitory activity was observed for the extract at any of the tested concentrations. Therefore, the results of MIC determination for hydromethanolic extract of *S. multicaulis* aerial parts indicated the MIC values of higher than 25 mg/ml against *S. aureus*, *P. aeruginosa* and *C. albicans*.

Discussion

Wounds are disruption of normal anatomic structure and function of tissue. Skin wounds could happen due to physical or chemical injuries resulting in skin damage [17]. The most common symptoms of wounds are bleeding, loss of feeling or function below the wound site, heat and redness around the wound, painful or throbbing sensation, swelling of tissue in the area and pus like drainage [18]. Wound healing process is an orchestrated cascade of events which lead to skin repair and comprises four stages including coagulation which controls blood loss,

inflammation and re-epithelialization, and the final stage which is collagen deposition and tissue remodeling [19]. A high percentage of patients with burn injuries die because of shock, infection, respiratory failure and central nervous system damages [20]. An epidemiological study in Kohkiluyeh va Boyer-Ahmad province (Iran) showed that burned patients have a period of 8 to 43 days of hospitalization with a rate of 2.1 to 59.5 % of fatality [21]. Due to numerous complications of skin injuries, scientists are keen on researching new options to treat different types of wound. Since ancient times, medicinal plants could demonstrate beneficial effects in different diseases and disorders including skin damages [2]. Current study evaluated the wound healing activity of *S. multicaulis* as a traditional and folk medicinal plant used by people around the world. Animal study showed the beneficial effects of *S. multicaulis* ointments which were almost similar to that of the phenytoin. Histological evaluations also demonstrated the

wound healing activity of *S. multicaulis* extract. DPPH radical scavenging IC₅₀ was 54.02 µg /ml which could be considered as high antioxidant capacity of the extract. Total phenolic content of the plant was 262.083±47.15 mg GAE/g extract. Semi-quantitative phytochemical screening tests also demonstrated the presence of high amounts of flavonoids and tannins which confirms polyphenolic compounds. Considering antioxidant, anti-inflammatory and astringent properties of polyphenols, wound healing activity of *S. multicaulis* extract could be attributed to the high amount of phenolic compounds, especially flavonoids and tannins.

Conclusion

Hydromethanolic extract of *S. multicaulis* aerial parts showed acceptable wound healing activity in animal model of excision wound. This effect, at least in part, is due to the antioxidant, anti-inflammatory and astringent effects of polyphenolic components as major active constituents of the extract. Further assessments on the wound healing activity of the plant in other animal models of wound as well as more phytochemical evaluations can lead to better understanding the pharmacological properties of *S. multicaulis*.

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