Pharmacogn J. 2020; 12(2): 335-341
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Wound Healing Activity of Ethanolic Extract of *Selaginella Bryopteris* on Rats

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ABSTRACT

The objective of the present study was to determine wound healing activity of ethanolic extract of *Selaginella bryopteris* on rats. The whole plant of *S. bryopteris* Linn. was collected from Andhra Pradesh, India and extraction was done using ethanol. GC-MS analysis was performed to determine active metabolites present in the extract followed by determination of total phenolic and flavonoid contents. *In vivo* wound healing activity of ethanolic extract was evaluated using excision wound model. The extract was applied topically on animals by preparing ointment in two concentrations (5% and 10%) where soframycin (10%) was taken as positive control. Antioxidant activity of *S. bryopteris* extract was observed by measuring oxidative enzymatic levels i.e. Superoxide dismutase (SOD), Catalase (CAT), reduced Glutathione (GSH) and lipid peroxidation (LPO) in animal tissues. Histopathological studies of excised skin were carried out after the experimental period. The contraction rate of the wound was higher and dose-dependent in rats treated with 5% and 10 % ointment of extract in comparison to untreated control group. The drug treated groups showed recovery phase and the percentage of healing was more in 10% at the end of experimental period. Results exhibited sufficient insights on the healing process with normal recovery stages and restored oxidative enzymatic levels. Histopathological findings provided additional positive results; the dermis with proliferating capillaries and skeletal muscle were replaced by cellular fibrous tissue and collagen fibers. Overall, the results showed that ethanolic extract of *S.bryopteris* was an interesting traditional agent that possess significant wound healing activity.

Key words: *Selaginellabryopteris*, Total Phenolic Content, Flavonoid Content, Oxidative Stress, Wound Excision Model.

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History

- Submission Date: 26-05-2019;
- Review completed: 10-06-2019;
- Accepted Date: 20-06-2019.

DOI : 10.5530/pj.2020.12.53

Article Available online

http://www.phcogj.com/v12/i2

INTRODUCTION

Wound can be defined as an injury of living tissue or damage in the epithelial integrity of the upper layer of skin, which may further lead to disturbance in skin anatomy, physiology and their function.[1] Wounds are the major cause of morbidity and mortality throughout the world. Wound is the result of accidental damage, which may be susceptible to bacterial and other pathogens that provides an entry point for systemic infections.[2] Chronic wounds affects the quality of life especially in older adult population.[3] Wound healing is a systematic process leading to the restoration of injured tissues.[4] It can be described as regeneration of the injured connective tissue of wounds followed by proliferation and migration of dermal and epidermal cells, and matrix synthesis.[5] Healing of wounds depends on factors such as repairing ability of the tissue, type and extent of damage and general state of the health of the tissue.[3] However, wound healing drugs are still unsatisfactory due to their high cost, low availability, and various side effects.[6] Medicinal plants have been used in the treatments of several diseases and such traditional medicines are still widely practiced today.[7] Therefore, herbal medicine would be useful as therapeutic or even in the prevention of chronic wounds due to common belief that they are safe, reliable, clinically effective, low cost and better tolerated by patients.[8]

Selaginellabryopteris Linn. Bak is commonly known as 'Sanjeevani'. The Indian tribal communities mainly use it as a strength tonic in improving fitness and to extend lifespan[9,10] and used as a major ingredient in local pills for the treatment of patient with spermatorrhoea, venereal diseases, constipation, colitis, indigestion and urinary problems (diuretic). It is also treat patients who are unconscious, and to lower the body temperature in patient with fever.[11,12] All parts of this plant are considered to be a good source of large number of bioactive substances. The important natural compounds in this plant are characteristic flavonoid-dimers, bioflavonoids.[13] Earlier phytochemical analysis of *S. bryopteris* showed the presence of 2,3-dihydrohinokiflavone, tetrahydro-hinokiflavon, 2,3-dihydroamentoflavone, tetrahydroamento flavone, lanaroflavone, sciadopitysin, sequoiaflavone, hinokiflavone, 2″, 3″dihydroamento flavone and tetra-o-methyl-hinokiflavone.[14] The herb also possesses chemo-preventive and anti-carcinogenic property[14,15] anti-stress,[16] memory enhancement,[17] anti-diabetic activity,[18] anti-depressant activity[19] and antimicrobial.[20] Many plants are used in tribal and folklore medicine across many countries for the treatment of wound and burns. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. The presence of various life-sustaining constituents or potential bioactive principle(s) in plants has urged scientific community

Cite this article: Paswan SK, Srivastava S, Rao CV. Wound healing activity of ethanolic extract of *Selaginella Bryopteris* on rats. Pharmacog J. 2020;12(2):335-41.

to examine these plants for their potential wound healing properties.[13] Therefore, the findings in this study could provide baseline information and accumulating evidence suggest that ethanolic extract of *S. bryopteris* have an effective wound healing and antioxidant activities by promoting the cellular proliferation, migration of fibroblast cells and restoration of oxidative enzymes. In the present study we investigated the effects of *S. bryopteris* extract in excision wound healing model and presents data that warrants further clinical studies.

MATERIALS AND METHODS

Chemicals and reagents

Soframycin, Soft paraffin, Cetostearyl alcohol, Polysorbate 60, Butylated hydroxyl anisole, Formalin, Xylene, Hematoxylin, Eosin Polypropylene cages were purchased from Sigma Chemical Co. USA and from Indian firms.

Plant material and extraction of metabolites

The whole plant of *S. bryopteris* Linn. was collected from Andhra Pradesh, India and authenticated by K. Madhava Chetty, Venkateswara University, Tirupati, India. The voucher specimen (voucher no. 1098) was deposited in the institute. The collected plant material was dried at room temperature and powdered using grinder then 100 g of powdered material was weighed and allowed to dipin 70% ethanol for 48 h. The extraction of metabolites was carried out till the transparent color was obtained. The ethanolic extract was collected and concentrated through vacuum in evaporator Christ RCV 2-18 plus. The concentrated extract was stored at 20°C for the further analysis.

GC-MS analysis

The non-targeted qualitative and quantitative analysis of ethanolic extract was performed through Gas Chromatography-Mass Spectrometry (GC-MS) with three replicates. For the GC-MS analysis of the metabolites, a volatile trimethylsilyl (TMS) derivative of the sample was prepared. Sample (5 mg) was dissolved in 50 µl of methoxyamine hydrochloride solution in pyridine (20 mg/ml). The mixture was shaken for 2 hr at 40°C using thermomixer comfort (Eppendorf India Ltd.). After this 70 µl of *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was added followed by shaking for another 30 min at 40°C. GC–MS analysis was performed using Thermo Trace GC Ultra coupled with Thermo Fisher DSQ II mass spectrometers. Chromatographic separations of metabolites were carried out using thermo TR50 column (polysiloxane column coated with 50% methyl and 50% phenyl groups). Xcalibur software was used to process the chromatographic and mass spectrometric data. The oven temperature of GC was maintained at 70°C for 5 min, then gradually raised at the rate of 5°C min^{-1}; 70°C to 310°C and maintained for 5 min. The sample was injected in the split mode (splitting ratio of 1:16). Helium was used as a carrier gas and set at a constant flow rate of 1 ml min^{-1}. The mass selective detector was run in the electron impact (EI) mode, with electron energy of 70 eV and mass range between 50-800 amu. The characterization of individual metabolites was carried out using WILLY and NIST mass spectral library[21]

Assay of total phenolic contents

The amount of total phenolic content of ethanolic extract of *S.bryopteris* was determined according to Folin-Ciocalteu procedure and the content was expressed as gallic acid equivalents (GAE) in mg/g of extract. In this method, *S. bryopteris*extract (200 µL of 1 mg/mL) was mixed with Folin-Ciocalteu (0.5 mL) for 3 min. In this mixture, 2 mL of sodium carbonate (20%, w/v) was added then the mixture was placed in dark condition and absorbance measured at 550 nm.[22]

Assay of total flavonoid contents

Total flavonoids were also estimated according to aluminum chloride colorimetric method. In this method, *S. bryopteris*extract (50 µL of 1 mg/mL) was mixed distilled water (4 mL) and $NaNO_2$ solution (5% of 0.3 mL) followed by addition of 10% $AlCl_3$ solution after 5 min incubation. This mixture was stand for 6 min followed by addition of 1 mol/L NaOH solution and final volume was make up with distilled water (10 mL). The absorbance was measure at 420 nm. A yellow color indicated the presence of flavonoids. Total flavonoid contents were calculated as rutin (mg/g) [22].

In vivo wound healing activity

Experimental animals

Wistar rats weighing between 180-200 g with no prior treatment were used for the study. Animals were housed in polypropylene cages maintained at 22 ± 2°C temperature with 12 h light and 12 h dark cycle. The animals were fed on a standard pelleted diet and had free access to water throughout the experiment. Animals that are described as fasting were deprived of food for at least 16 h but allowed free access to drinking water. The animals study was approved by the Institutional Animal Ethics Committee, CSIR-National Botanical Research Institute (Approval No. 1732/GO/RE/CPCSEA), Lucknow, as stated by prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Preparation of ointments

Ointments of ethanolic extract of *S.bryopteris*in two concentration of 5% and 10 % (w/w) were prepared by mixing 5 and 10 mg of extract, respectively and each with soft paraffin (25 mg), glycerin (12 mg), polysorbate 60 (5 mg), cetostearyl alcohol (4 mg) andbutylated hydroxyl anisole (0.02 mg)[23].

Experimental design

A total of 24 rats were divided into four groups each containing six rats with identical wounds. Group – 1 was control group containing untreated rats; Group – 2 and 3 were treated groups containing 5% and 10% prepared ointments, respectively; Group – 4 was positive control group containing standard drug Soframycin.

Excision wound model

Excision wound was made on the depilated region of the skin. A standardized wound area (8 mm^2) was marked on the dorsal surface of the anaesthetized rats and skin in full thickness was excised to obtain a wound area of about 8 mm^2. Homeostasis was attained by blotting the ulcerated area with cotton swab dipped in normal saline. The wound diameter was measured using calipers on alternate days and the epithelialization period recorded at the end of the study.[15] Number of days required for falling of the eschar without any residual raw wound gave the period of epithelization. It was measured in days from wounding day (day zero) till the full Epithelialization. The wound area was measured with a translucent paper and traced on every 3rdday. The contraction of wound was expressed as percentage of the reduction in wound size. Percentage of wound contraction was measured using the formula given below.

$$\text{Percentage of Wound contraction} = \frac{(\text{Initial wound area} - \text{Specific day wound area})}{\text{Initial wound area}} \times 100$$

Antioxidant activity

Antioxidant activity was determined by measuring oxidative stress enzymes in wound tissue homogenate i.e. Superoxide dismutase (SOD), Catalase (CAT), reduced Glutathione (GSH) and lipid peroxidation (LPO) were estimated after day 12 post wounding. Tissue homogenate was prepared by excision of wound tissue using the same punch (8 mm^2 diameter), which excised wounded area without contaminating it with normal skin then tissue was collected in Phosphate buffered saline (PBS, pH 7) and sample preparation was done according to *Shukla et al.*[24] SOD activity was analyzed and expressed in µmoles/min/mg of protein according to the method of *Kakkar et al.,*[25] Colour intensity of the chromogen was measured at 560 nm. For determination of CAT enzyme level, 900 mg tissue was homogenized in 3.0 ml M/150 phosphate buffer in ice and centrifuged at 30,000 rpm for 1 h at 4°C. The supernatant was taken to determine catalase activity. For determination of GSH level tissue homogenate (30% w/v) was prepared in 0.15 M Tris-HCl buffer (pH 7.4) then trichloroacetic acid was added to precipitate proteins. Samples were centrifuged at 15000 rpm at 4°C for 1 h. The supernatant was analyzed for contents of reduced glutathione and expressed in terms of µg/g of liver tissue.[26] Lipid peroxidation assay was performed by taking tissue homogenate in 3.0 ml 0.15 M Tris-HCl buffer (pH 7.4) and centrifuged at 3000 rpm at 4°C for 1 h. The supernatant was collected and estimated for lipid peroxidation which was expressed in expressed as nmol per mg of proteins.[27]

Histopathological study

Skin tissues were dipped into 10% formalin solution and preserved for 24 h at 4°C. These tissues were dehydrated using alcohol and then embedded in paraffin wax at 58 - 60°C. Thin sectioning of the skin tissues (5-7 µm) were fixed onto a slide and stained with haematoxylin, and later counterstained with eosin [16].

Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc.). One-way ANOVA followed by't' test was used to analyses the significance in wound contraction rate. Two-way ANOVA followed by Bonferroni post-hoc test was used for testing groups with different time points with respect to percentage of wound healing. $^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$ were considered statistically significant. Data are expressed as mean ± SD.

RESULTS

GC-MS analysis

GC-MS analysis of hydroethanolic extract of *S.bryopteris* exhibited presence of sugar, sugar alcohol, glucosides steroids, Saturated and unsaturated fatty acids and vitamin as shown in Figure 1. The relative concentration of major compounds were Sucrose (27.95%), maltose (16.31%), oleic acid (9.25%), palmitic acid (6.01%), glucopyranosides (13.40%) and glycerol (3.39%). Several other compounds were also exhibited in S. bryopteris extract as shown in Table 1.

Assay of total phenolic and flavonoid contents

Total phenolic content was expressed as mg gallic acid equivalent per gram extract dry weight. Phenolic content of the extract was calculated and the value was found to be 364.2 ± 1.92 mg/g. Similarly, total flavonoid content was expressed as mg rutin equivalent per gram extract dry weight and value was found to be 50.30 ± 2.01 mg/g.

Excision wound model

Application of *S. bryopteris* ointment on marked area showed significant wound healing activity when compared to control group. Rats showed normal healing process with signs of improvement at weekly intervals and this was determined by their contraction rate as shown in Table 1. A dose dependent effect was observed in rats treated with ointment. These groups showed faster wound contraction when compared to normal and positive control group. On day 14,

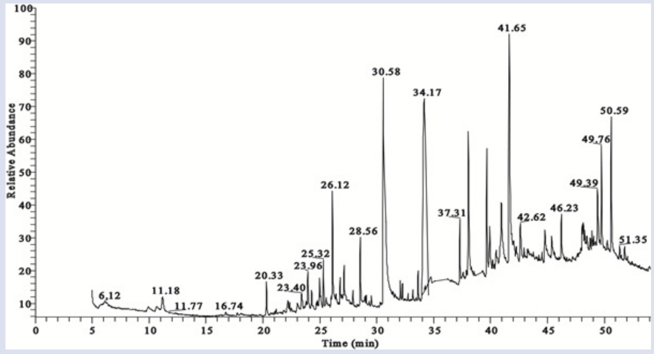

Figure 1: GC-MS image of *S. Bryopteris* extract.

Table 1: Compounds present in GC-MS analysis of *S. Bryopteris* extract.

Rt	Metabolites	Chemical formula	Relative concentration(%)	Class of compounds
11.2	Glycerol	C12H32O3Si3	3.39	Polyol
25	Glucitol	C24H62O6Si6	1.3	Sugar alcohol
25.34	Glucose,	C22H55NO6Si5	1.12	Sugar
30.6	Palmitic acid	C19H40O2Si	6.01	Saturated fatty acid
34.17	Oleic acid,	C21H42O2Si	9.25	Unsaturated fatty acid
37.29	Glucopyranoside	C36H86O11Si8	13.4	Glucosides
37.59	Turanose	C33H78O11Si7	1.86	Sugar
38.79	Sucrose	C36H86O11Si8	27.95	Sugar
39.71	Maltose	C36H86O11Si8	16.31	Sugar
46.23	Tocopherol	C31H56O2Si	0.44	Vitamin
48.07	Cholesterol	C30H54OSi	0.41	Steroid
49.41	Campesterol	C31H56OSi	0.74	Steroid
49.76	Stigmasterol	C32H56OSi	1.14	Steroid
50.62	β-Sitosterol	C32H58OSi	1.34	Steroid

5% ointment treated group showed 52.66% wound contraction while 61% wound healing activity was observed at 10% ointment, which was higher than standard drug soframycin (56%) as shown Figure 2. The mean time taken for complete epithelialization of the excision wound in 10% ointment treated group was less in comparison to positive control group (Figure 3). However, untreated control group animals showed very less improvement in the contraction rate with less than 50% as the wound was visible on day 28. Whereas, rats treated with ethanolic extract and soframycin showed full recovery at the end of the day 28.

Histopathological changes of skin in rats

Histopathology of skin showed improvement in the acute wound healing process with normal architecture, granulation tissue formation having fibroblasts and inflammatory cells in untreated control group animals. The deep vascular layer of the skin showed proliferative tissues consisting of lymphocytes, collagen fibers and blood vessels (Figure 4A). The rats treated with 5% ointment exhibited dermal fibrosis with collagen fibers. The wound is covered with one or more layers of cells and there is renewal of the dermis with proliferating capillaries (Figure 4B). However, rats treated with 10% ointment showed normal restoration of the skin with focal area showing only spindle cell stroma and proliferating capillaries and more neovascularization. The dermis and skeletal muscle has been replaced by cellular fibrous tissue and collagen fibers (Figure 4C). Positive control showed focal ulceration

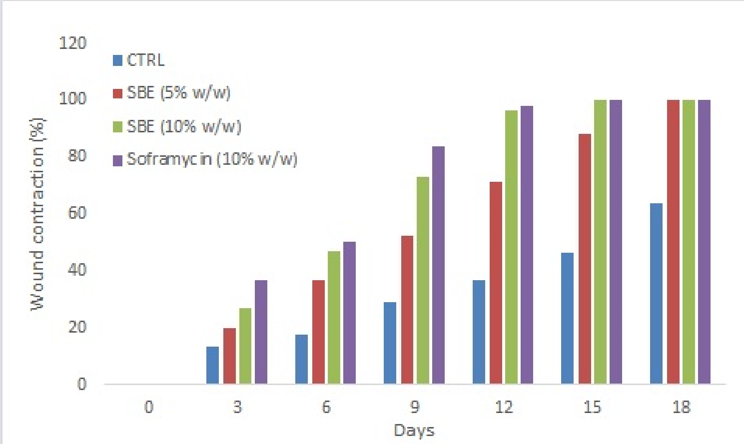

Figure 2: Percentage of wound contraction treated with *S. Bryopteris* extract. Values are represented as mean ± SD (n=6), statistically significant at *$P< 0.05$; **$P< 0.01$; ***$P< 0.001$. CTRL, Control; SBE, *S. bryopteris* extract.

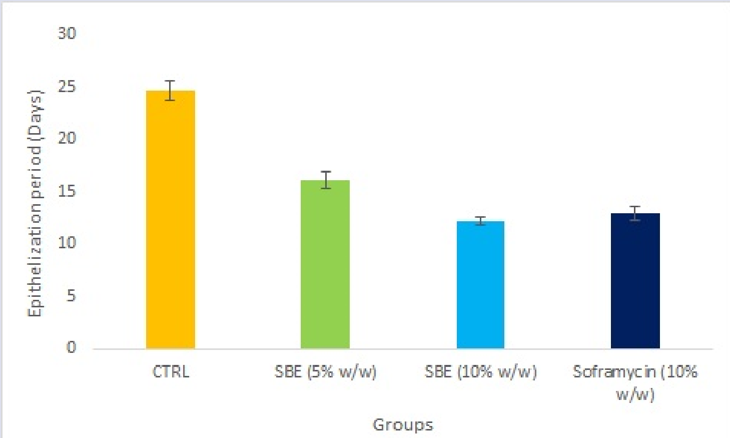

Figure 3: Epithelization of wounds treated with *S. Bryopteris* extract. Values are represented as mean ± SD (n=6), statistically significant at *$P< 0.05$; **$P< 0.01$; ***$P< 0.001$. CTRL, Control; SBE, S. bryopteris extract.

and replacement of skeletal muscle along with the capillaries. The wound was covered by the epithelium consisting of one or more layers of cells (Figure 4D).

Photomicrograph of sections of skin from rats with H & E skin microscopic image of (A) Untreated control group (B) *S. Bryopteris* extract ointment (5% w/w), (C) *S. Bryopteris* extract ointment (10 % w/w), and (D) Soframycin (10% w/w) treated group.

Antioxidant activity of *S. bryopteris* extract

Treatment with extract of *S. bryopteris* was significantly restored antioxidant enzymatic levels in the rats. SOD, CAT and GSH in wound tissues were significantly increased in the case of rats treated with 10% (26.0 nmol/mg, 0.8 IU/mg and 180.2 μmol/mg, respectively) and 5% (26.8 nmol/mg, 0.8 IU/mg and 175.9 μmol/mg, respectively) w/w ointment in the excision wound model in comparison to the untreated control group animals (22.1 nmol/mg, 0.5 IU/mg and 49.2 μmol/mg) as shown in Table 2. However, LPO level was reduced with the treatment of plant extract i.e. 4.3 nmol/mg and 2.9 nmol/mg for 5% and 10%, respectively compared to control group (7.3 nmol/mg). The enzymatic levels of soframycin treated group was near to extract treated group. The values were 25.9 nmol/mg, 0.7 IU/mg, 209.0 μmol/mg and 1.7 nmol/mg for SOD, CAT, GSH and LPO, respectively (Table 2).

DISCUSSION

The antimicrobial results obtained from our previous work[14] further provides supporting information on the wound healing properties of ethanolic extract, of *S. bryopteris*. We evaluated the wound healing process by measuring the progressive contraction size of the wound. Wound contraction, a process that occurs throughout the healing process, commencing in the fibroblastic stage (area of the wound undergoes shrinkage), enters a proliferative phase, characterized by inflammation, angiogenesis, collagen deposition, granulation tissue formation and epithelialization finally leads to wound contraction resulting in a smaller amount of apparent scar tissue.[28] Inflammation, the preliminary part of the wound healing process plays a significant role in the removal of infective microorganisms. In the absence of effective treatment, however, inflammation may be prolonged and the microbial load may increase. Thus, the bacterial load and the endotoxins may lead to the prolonged elevation of pro-inflammatory cytokines and the wound may enter a chronic state and fail to heal due to the prolonged inflammatory phase.[17,18] The results of the present study showed significant acceleration of the wound healing process in animals treated with 10%ethanolic extract of *S.bryopteris* (Table 3), which could be due to the shortened inflammatory phase and antimicrobial effect of the bioactive compound. Our results are in agreement with other

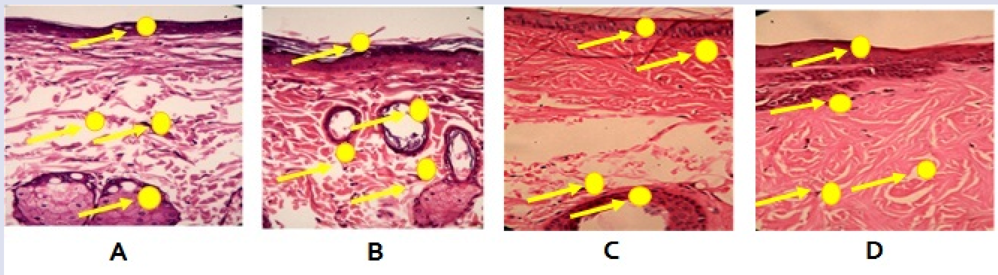

Figure 4: Histopathology of skin excised from rats treated with extract of *S. Bryopteris.*

Table 2: Effect of *S. bryopteris*ointment on the uninfected excised wound in rats.

Groups	Wound contraction (mm²) Days						
	0	3	6	9	12	15	18
CTRL	8.41 ± 0.30	7.37 ± 0.21	6.96 ± 0.34	5.94 ± 0.23	5.31 ± 0.11	4.57 ± 0.13	3.13 ± 0.19
SBE (5% w/w)	8.74 ± 0.37a	7.14 ± 0.30b	5.68 ± 0.38	4.03 ± 0.27c	2.59 ± 0.24b	0.99 ± 0.32c	0.00
SBE (10% w/w)	8.53 ± 0.40b	6.29 ± 0.23b	4.54 ± 0.28c	2.37 ± 0.33c	0.39 ± 0.32c	0.00	0.00
Soframycin (10% w/w)	8.67 ± 0.37c	5.98 ± 0.30b	4.37 ± 0.34c	1.94 ± 0.40c	0.17 ± 0.27c	0.00	0.00

Values are expressed as mean ± SEM; n=6, One Way ANOVA followed by Student-Newman-Keuls t-test; significantly different at a: *P<0.05*; b: *P<0.01*; c: *P<0.001*, respectively.

Table 3: Antioxidant activity of *S. bryopteris*ointment on the uninfected excised wound in rats.

Oral treatment (mg/kg, od×10 day)	GSH (nmol/mg)	SOD (IU/mg)	CAT (mol/mg)	LPO (nmol/mg)
CTRL	22.1 ± 1.09	0.5 ± 0.09	49.2 ± 1.96	7.3 ± 0.42
SBE (5% w/w)	26.8 ± 1.55b	0.8 ± 0.06b	175.9 ± 0.4c	4.3 ± 0.22b
SBE (10%w/w)	26.0 ± 1.07c	0.8 ± 0.07b	180.2 ± 0.52c	2.9 ± 0.35c
Soframycin (10% w/w)	25.9 ± 0.89c	0.7 ± 0.09a	209.0 ± 0.70c	1.7 ± 0.19c

Values are expressed as mean ± SEM (n=6), One Way ANOVA followed by Student-Newman-Keuls t-test; significantly different at a: *P<0.05*; b: *P<0.01*; c: *P<0.001*, respectively.

studies[19,29] that showed less inflammatory cells, more collagen and increased angiogenesis in wounds treated with plant extracts.

A significant amount of total phenolics (364.2 ± 1.92 mg/g) and total flavonoids (50.30 ± 2.01 mg/g) may promoted the wound-healing process mainly due to antioxidant, antibacterial activity, wound contraction and increased rate of epithelialization.[20] Flavonoids reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis, but also by improving vascularity. The pathways involved in the mechanism of wound healing process could contribute to stimulate the production of antioxidants in wound site, providing a favorable environment for tissue healing.[30] It is possible that ethanolic extract could have regulated one such pathway. A more recent investigation on humans revealed the potential benefits of herbal ointments, wherein polyherbal formulations in comparison to soframycin showed effective wound healing. The early revival of the inner vascular layers of the skin in the treated groups confirmed that the ointment containing ethanolic extract had significant wound healing property. The healing was found to be accelerated due to the early growth of the epithelium, cellular proliferation and granulation tissue formation. Studies on phenolics and flavonoids have shown significant increase in the cell proliferation and thus can be used to treat wounds. These compounds have been shown to increase collagen synthesis, promote the cross-linking of collagen, decrease the degradation of soluble collagen, accelerate the conversion of soluble collagen to insoluble collagen, and inhibit the catabolism of soluble collagen.[31] From a clinical point of view, deposition and increased synthesis of collagen, diminishing overproduction of free radicals, facilitating oxygen diffusion and increase in lymphatic drainage are important events that occurs to improve healing.[32] *Rocha et al.* showed a large amount of fibroblast proliferation, collagen synthesis, and neovascularization in treated wound, resulting in increased tensile strength and acceleration of healing process.[33] The results obtained in our study also showed marked epithelialization, collagen fibres and a stratified squamous epithelium with new blood vessel formation in treated rats.

CONCLUSION

This research provides evidence for the wound healing activity of ethanolic extract of *S.bryopteris* in rats and exhibited potential dose-dependent wound healing effect. Thus *S.bryopteris* ointment can become an attractive alternative of the common drugs to provide necessary re-epithelialization in excision wound healing.

ETHICAL APPROVAL

The authors declare that experiments were performed on animals in this study (Approval No. 1732/GO/RE/CPCSEA), Lucknow, India.

CONSENT FOR PUBLICATION

We certify this manuscript has not been published elsewhere and is not submitted to another Journal.

AVAILABILITY OF DATA AND MATERIAL

It can be made available on demand.

COMPETING INTERESTS

The author(s) declare that they have no competing interests.

FUNDING

University Grant CommissionRGNSRF, New Delhi, India,Grant Number F1-17.1/ 2014-15-RGNF-2014-15-SC-UTT-60684.

ACKNOWLEDGEMENT

The authors are thankful to our Honorable Director, CSIR-NBRI, Lucknow, for their assistance and providing laboratory facilities. I am so much thankful to my colleague Mrs. PrittVerma (SRF) and Satish Chandra Gupta for their constant support during the experiment and animal work. Further, we extend our gratitude to University Grant CommissionRGNSRF, New Delhi, India, for providing a grant to perform this study.

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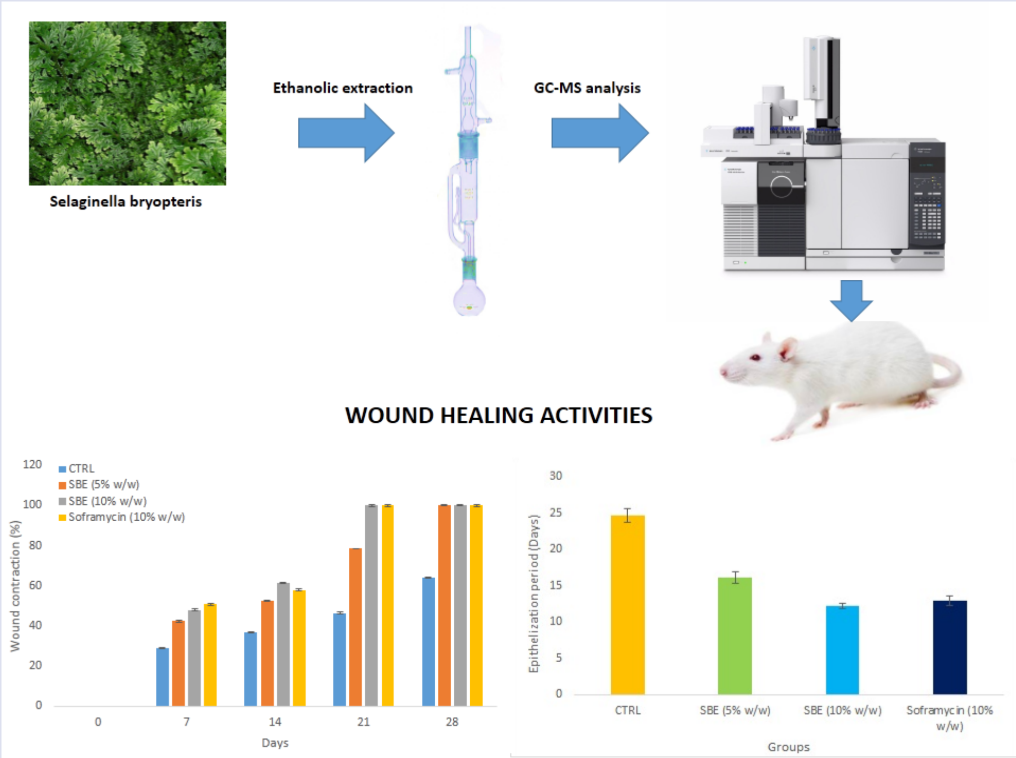
GRAPHICAL ABSTRACT



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Cite this article: Paswan SK, Srivastava S, Rao CV. Wound healing activity of ethanolic extract of *Selaginella Bryopteris* on rats. Pharmacog J. 2020;12(2):335-41.