IJBPAS, December, 2015, 4(12): 6467-6480

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ANTIOXIDANT AND WOUND HEALING POTENTIAL OF STANDARDIZED ETHYL ACETATE FRACTION (AS-3) OF *ALLIUM STRACHEYI* IN RATS

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ABSTRACT

The present study reveals the antioxidant and wound healing activity of *Allium stracheyi* Baker, an ethno botanically important medicinal and aromatic plant endemic to western Himalayas. It is widely used ethnomedicinally for treating skin diseases. To validate the ethnotherapeutic claims of the plant wound healing activity was studied, besides antioxidant activity to understand the mechanism of wound healing activity. The antioxidant activity of the ethyl acetate fraction (AS-3)of *Allium stracheyi*was evaluated by using DPPH free radical scavenging activity and total phenolic content was also calculated. The AS-3 showed variable degrees of antioxidant activity. TheAS-3 was studied for wound healing activity at different concentration (1%, 2.5% and 5%) by incorporating the samples in simple ointment base. The rates of wound healing were calculated at 4, 8, 12, and 16 days after the wounding by excision model and wound strength was measured on 10th day after incision wound model in rats. The effects of formulations on wound healing were assessed by the rate of wound closure, period of epithelialization, tensile strength and hydroxyproline content in scab. Treatment of wound with ointment containing 5% (w/w)

AS-3 exhibited significant ($P < 0.001$) wound healing activity. The level of hydroxyproline and tensile strength were also correlative with the observed healing pattern. The present study provides scientific evidence for the traditional use of *Allium stracheyi*in the management of wound healing and protection of the cells from hydrogen peroxide-induced injury, all of which could play some role in its effect on tissue repair.

Keywords: Antioxidant, wound healing, hydroxyproline, *Allium stracheyi*, excision wound, incision wound

INTRODUCTION

A large number of medicinal plants have been used in the traditional system of medicine for wound healing. These herbal base remedies are used since ancient times; however the mechanism of action of very few of them has been evaluated scientifically [1]. India is a vast repository of medicinal plants having immense potential for the management and treatment of wounds. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. These phyto-medicines are not only cheap and affordable but are also safe.A wide range of antibiotics is being used at present for treating wound infections, but they are now proved to have adverse effects in the human body, also these pathogens developed resistance to the antibiotics targeted against them [2]. In view of this, so much recent attention has been paid to biologically active compounds isolated from plant species for wound healing activity [3].

Wound healing is a process that results in the restoration of a functional barrier and contraction or closure of the wound.Repair of injured tissues occurs as a sequence of events, which includes inflammation, proliferation and migration of different cell types [4].Wounds can be categorized broadly as having either a chronic or an acute etiology which includes surgical wound abrasion, burn, laceration, bites or acute inflammatory phase followed by synthesis of collagen and the extracellular macromolecules which remolded later and form, scar [5].It is well known that reactive oxygen species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues. Free-radical-scavenging enzymes are a group of cytoprotective enzymes that has an essential role in the removal of ROS as well as regulating the wound healing process[6]. Topical applications of compounds with free-radical-scavenging properties protect tissues

from oxidative damage and significantly improve wound healing [7].

The present study deals with evaluation of the antioxidant andwound healing activity of *Allium stracheyi (A. stracheyi)*.It islocally known as Jambu or Pharan, is a multipurpose ethnobotanically important aromatic and medicinal herb endemic to western Himalaya. Traditionally the plant has been reported for its uses as medicine for jaundice, cold, cough, inflammation,pain, wound healing and other stomach problems[8-10]. The major chemical components reported from the *A. strycheyi* are alkaloids, Tanins, saponins, fixed oils, phytosterols, phenolics and flavonoids. They contain sulphur rich compounds with antioxidant, anti-inflammatory, and antimicrobial properties[11]. The plant has been scientifically proved to have anti-inflammatory, analgesic and hemolyticactivities[12].Ayurveda reports utility of *A. stracheyi*for wound healing process, however, the literature survey revealed that no systematic study had been carried out on wound healing activity. Hence, in the present study, an effort was made to establish wound healing potential of the plant using different models.

MATERIALS AND METHODS

Sample collection and authentication

Air-dried leaves of *A.stracheyi*Baker were procured from local areas of Munsyari (Pithoragarh) in the month of August, 2014 and identified by Dr. H.B. Singh, Chief Scientist & Head, Raw Materials Herbarium & Museum, National Institute of Science Communication And Information Resources, New Delhi, India and a specimen of the plant material was also deposited at NISCAIR. A specimen no of the plant material is NISCAIR/RHMD/Consult/-2014-13/2020/28.

Extraction, fractionation and ointment preparation

The powdered plant material (1 Kg) was subjected to percolation process with 90 % methanol at room temperature. After exhaustive extraction, the methanolic extract was concentrated under reduced pressure at 55 °C. Extract was adsorbed with silica and subjected to fractionation with solvents like Hexane, chloroform and ethyl acetate[13]. Forpharmacological evaluation, ointment of ethyl acetate fraction (AS-3) was prepared in various concentrations (1%, 2.5% and 5%).

Determination of total phenols

The total phenols content of AS-3 was determined by Folin-Ciocalteau method, referring to calibration curve of gallic acid, phenol compound used as a standard. 250 µl of Folin-Ciocalteu's phenol reagent was

mixed with 50 µl of the samples, and 500 µl of 20% water solution of Na_2CO_3 was added to the mixture. Mixtures were vortexed and completed with waterto 5 ml. As control, reagent without adding AS-3 was used. Afterincubation of the samples at room temperature for 30 min, theirabsorbance was measured at 765 nm (Systonics-2202). Thetotal polyphenols were estimated as gallic acid equivalent (GAE)and expressed in mg GAE/g test drug (dw)±standard deviation (S.D.)[14].The data were obtained from the average of three determinations.

Determination of the total flavonoid content

The aluminum chloride colorimetric method was used for determination of total flavonoids. AS-3 in a concentration of 1mg/mL was used in the analysis. The reaction mixture was prepared by mixing 0.5 mL of the AS-3, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The absorbance was measured at 415 nm after 30 min incubation at room temperature. Quantification was done on the basis of a standard curve of quercetin. The results were expressed as quercetin equivalents (mg of quercetin/g of AS-3)[15].

HPLC fingerprinting of AS-3

HPLC fingerprinting of the AS-3 was developed as described earlier [13]. AS-3 wasdissolvedinof 80%methanolandfilteredthrough 0.22 micron nylon syringefilter. Inject 2 µl intothe HPLCsystem for 20 min.The separation was carried outon HPLCsystem of Agilent technologies 1200 series composed with EZ-Chrom system controller, auto sampler, LC-binary pump,diodearray detector andC18(4.6mm×250mm)column.Asolventsystemof water (A) and methanol (B)wasusedas follows: 20(A):80(B) at flowrateof1.0mL/min.The chromatogram was obtained at wavelengthof 262 nm. For making a standard chromatogram of quercitin was dilutedin 80% methanolandinjectedintotheHPLCtogive chromatograms.

DPPH radical scavenging assay

The radical scavenging activities of the AS-3against 2, 2-Diphenyl-1-picryl hydrazyl radical (Sigma-Aldrich) were determined by UV spectrophotometry at 517 nm. The following concentrations of the AS-3were prepared, 250, 500, 1000 and 2000 µg/ml in methanol. Vitamin C was used as the antioxidant standard at the same concentrations. 1 ml of the AS-3was placed in a test tube, and 3 ml of methanol was added followed by 0.5 ml of 1 mM DPPH in

methanol. A blank solution was prepared containing the same amount of methanol and DPPH[16]. Inhibition of DPPH in percent (I%) was calculated as given below: where $A_{control}$ is the absorbance of the control reaction (containingall reagents except the test sample), and A_{sample} is the absorbance ofthe test drug/reference

$$I\% = \frac{(A\ control - A\ sample)}{A\ control} \times 100$$

Animals

The study was conducted on male wistar rats (180-200 g). The ethical committee of the Shoolini University instituted for animal handling approved all protocols. The animals were bred and maintained under standard laboratory conditions: temperature (25±2 °C) and photoperiod of 12 h. Commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water were given *ad libitum.* According to ethical regulations on animal research, all animals used in experimental work received human care.

Preparation of formulations

The ointments of AS-3were prepared to evaluate its efficacy, in comparison with povidone–iodine ointment, USP. Ointment base was prepared by mixing the ingredients (woolfat5g, hardparaffin 5g, cetostearylalcohol 5g, soft white paraffin 85g) as per British Pharmacopoeia (1980) in a beaker at 65º C waterbath. After cooling, the mixture was homogenized by a homogenizerat1500rpm for10-15min.The most stable ointment base was selected for the preparation of four formulations (three test groups and one control).The stability was further evaluated at accelerated conditions to obtain the most stable formulations. The drug formulations were freshly prepared on every fifth day.

Acute dermal toxicity

The acute dermal toxicity study was carried out to determine the therapeutic dose ofthe ointment of AS-3. The acute dermal toxicitytesting was done by applying the ointments containing AS-3of the highest concentrations of 5% (w/w)on the shaved back of the rats. The OECD guidelines no. 402 were followed for the study[17].

Evaluation of wound-healing activity

The incision, excision and biochemical estimation were used to evaluate the wound-healing property of ointment of AS-3. The animals (wistar rats) were divided into five groups, containing six animals in each group, for excision and incision wound models. The formulated ointments were applied topically to each animal once a day. The animals of group I received ointment base (control), while group II were treated with a 5%w/w povidone-iodine ointment. The animals of

groups III to V were treated with 1%, 2.5% and 5% w/w ointments of AS-3 respectively. All the animals were observed closely for any kind of infection, so that the infected one can be excluded from the study.

Excision wound

The animals were anaesthetized with ketamine hydrochloride(60 mg/kg body wt.) prior to and during the creation of experimental wounds. Rats are then inflicted with excision wound as described by Morton and Malone (1972). The dorsal fur of the animals was shaved with electric clipper and full thickness of excision wound of 500mm^2 was created along the marking using toothed forceps, a surgical blade and pointed scissor and the wound was left open (Figure 3). All animal groups were treated in the similar manner as mentioned above. The wounds were monitored and the area of wound was measured on0, 4, 8 12 and 16thpost-wounding days usingtransparencypaperandamarker. The recorded wound areas were measured graphically and the rate of wound contraction and epitheliazation time were calculated [18, 19].

$$\text{The Percentage (\%) wound contraction} = \frac{\text{(Wound area on day 0} \times \text{wound area on day n)}}{\text{Wound area on day 0}} \times 100$$

Estimation of biochemical marker

Circular wound with approximate area of 500mm^2 was createdusing the procedure described in excision wound model.The wounds were treated with topical application of ointmentsas described above for 10 days. On day 11 the scab was removedand dried in oven at 110 ◦C. The content of hydroxyprolinein dried scab was determined by extracting hydroxyproline from scab using concentrated HCl followed by reaction between amino groups of hydroxyproline with p-dimetylaminobenzaldeyde to develop red colour. The intensity of red colour was thus measured on spectrophotometer at 558 nm [20].

Incision wound

The Incision wounds of about 6 cm in length and2mm in depth were made in anesthetized rats with sterile scalpel on the shaved backof the rats 30 min later the administration of ketamine injection.The skin was kept together and stitched with black silk at0.5cm intervals (Figure 4). A curved needle and surgical thread were used for stitching. Thewounds of animals in the different groupswere treated with topicalapplication of the ointments as described above, for the10

days. The wounding day was considered as day 0.

The sutures were removed on the 8thpost-wounding day, when wounds were cured thoroughly and the tensilestrength of the skin was measured by tensiometer on the 10thday [21].

Statistical analysis

Data were expressed as mean ± SEM, and statistical analysis was carried out using one-way ANOVA (Bonferroni correction multiple comparison test). Dunnett's test was used to analyze the different variables in the same subject, and p values <0.05 were being taken as statistically significant.

RESULTS

Total phenolic and flavonoid content

The total phenolic content and total flavonoids in the AS-3were33.15±2.8 mg/g gallic acid equivalent and 23.7 (µg/ml) respectively.

HPLC fingerprinting of AS-3

The HPLC chromatogram of AS-3 is shown in Figure 1. Standard quercetin showed peak at 3.113 and AS-3 showed various peaks along with one peak at 3.153 which showed the presence of quercetin.

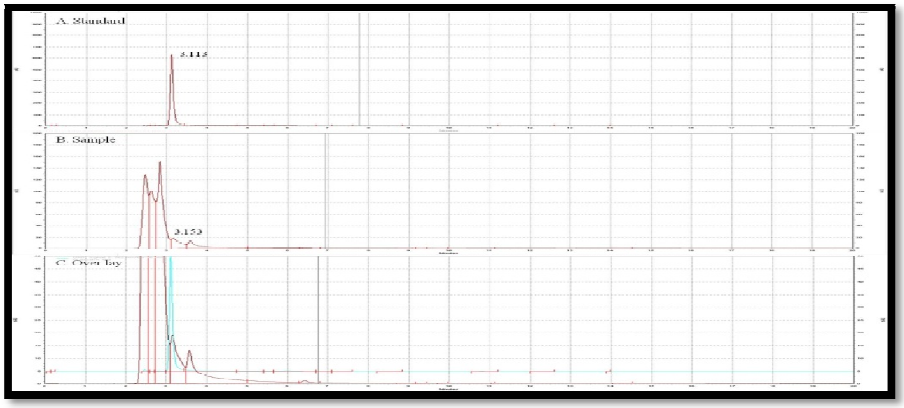

Figure 1: A. HPLC fingerprinting of AS-3 showing chromatogram of standard quercetin (3.113); B. Chromatogram of sample (AS-3); C. Overlay chromatogram of sample and standard quercitin. The separation was carried out C18 (4.6mm×250mm) column, 5 µm particle size, and the temperature was maintained at 25 °C.

DPPH scavenging assay

The antioxidant reacts with stable free radical, DPPH and converts it to 1, 1-diphenyl-2-picryl hydrazine. The ability to scavenge the free radical, DPPH, was measured at an absorbance of 517 nm. The AS-3showed maximum 85% scavenging activity. Ascorbic acid was taken as referencewhich showed 93% scavenging activity. These results showed that the AS-3possess significant antioxidant activity. The overall results of percentage inhibition as shown in Figure 2.

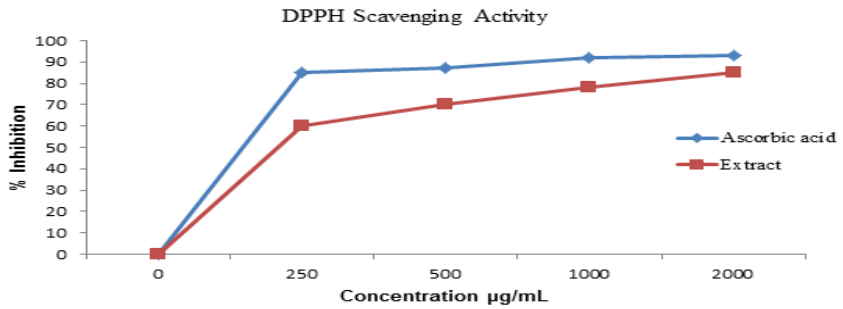

Figure 2: Effect of AS-3on free radicle scavenging activity. Free radicle scavenging activity of AS-3was tested using DPPH method and ascorbic acid was used as standard antioxidant. Values are means ± SE; *p<0.05 and **p<0.01 (control vs. AS-3, treated groups; one-way ANOVA followed by Bonferroni multiple comparison test). For experimental details, refer to Materials and methods section.

Excision wound model

The ointment of AS-3showed significant wound contracting ability than that of the control (p<0.001) and presented in table 1.Theointment of AS-3showed significant wound healing from the 8thday onwards which was comparable to that of the standard drug, povidine iodine ointment. The wound closure time was less, as well as the percentage of wound contractions was much higher in the AS-3 (5%) treated group.

Biochemical marker estimation

The biochemical marker such as hydroxyproline content in the scab of excision wound created in the animals treated with stated AS-3 was determined on the 11th day and presented in Table 2. The animals treated with ointment containing 5% (w/w) AS-3indicated significantly high

Table 1: Effect of topical application of ointment of AS-3 on wound contraction of excision wound

Group	4th Day	8th Day	12th Day	16th Day	Epithelisation Day
Control	13 ± 2.4	28 ± 2.4	44 ± 1.8	63 ± 1.8	28
Standard	21 ± 2.4*	41 ± 1.8**	68 ± 2.4***	92 ± 1.8***	20
1%	18 ± 2.4	37 ± 2.4	59 ± 1.4*	74 ± 1.8*	24
2.5%	20 ± 2.4	44 ± 2.4*	61 ± 3.1**	86 ± 1.8**	21
5%	23 ± 2.2*	49 ± 2.4**	72 ± 2.4***	97 ± 2.4***	18

n* = 6 animals in each group.The treated groups are compared by Student t test with the control group. *p*< 0.001,** *p*< 0.01, * *p*< 0.05.**

(*p*<0.001) levels of hydroxyproline (20.24µg/500mg) as compared to control (9.24µg/500mg)

Table 2: Effect of topical application of ointment of AS-3on hydroxyproline content in the scab of excision wound

Group	Hydroxyproline (µg/500 mg)
Control	9.24 ± 0.48
Standard	18.22 ± 0.54***
1%	13.33 ± 0.56*
2.5%	17.42 ± 0.62**
5%	20.24 ± 0.36***

n* = 6 animals in each group.The treated groups are compared by Student t test with the control group. *p*< 0.001, ** *p*< 0.01, * *p*< 0.05**

Measurement of tensile strength or wound breaking strength (incision wound)

Incision wound model was evaluated by determining the tensile strength of the wound of different groups viz. control treated with simple ointment, standard group treated with drug povidone iodine and the test group treated with the different concentrations of ointment of AS-3. The results are presented as mean weight in gram±SEM required for open the re-sutured wound in Table 3. The animals treated with ointment containing 5% (w/w) AS-3indicated significantly high ($p< 0.001$) tensile strength (648g) as compared to the control group (475g).

Table 3: Effect of topical application of gel containing ethyl acetate fraction of *Allium stracheyi*on tensile strength of the skin having incision wound.

Group	Tensile Strength in gram (Mean± SEM)
Control	475.24 ± 8.2
Standard	655.64 ± 8.29***
1%	576.36 ± 6.24**
2.5%	612.83 ± 9.34**
5%	648.21 ± 8.62***

n* = 6 animals in each group.The treated groups are compared by Student t test with the control group. *p*< 0.001, ** *p*< 0.01, * *p*< 0.05.**

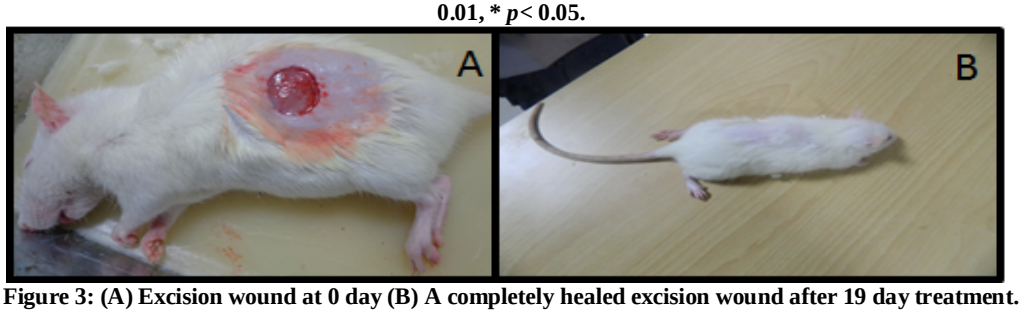

Figure 3: (A) Excision wound at 0 day (B) A completely healed excision wound after 19 day treatment.

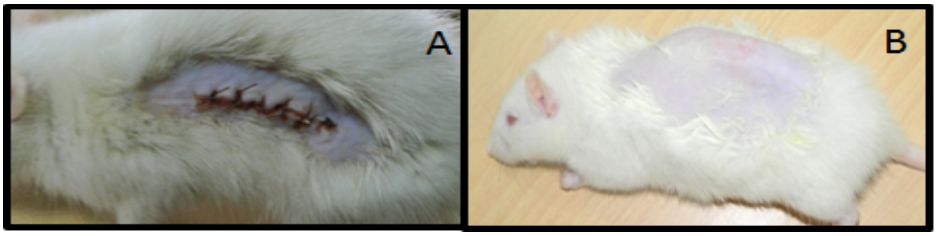

Figure 4: (A)Incision wound on the day 0, (B)A completely healed incision wound after 8 day treatment.

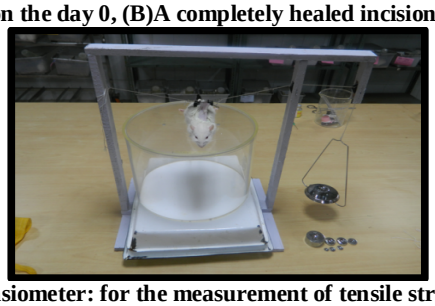

Figure 5: Tensiometer: for the measurement of tensile strength of skin.

DISCUSSION

Wound healing process is characterized by homeostasis, re-epithelialization, granulation tissue formation, and remodeling of the extracellular matrix. The principle behind wound healing is to minimizetissue damage and provide an adequate tissue oxygenation and perfusion,proper nutrition and moist wound healing environmentto restore the anatomical continuity and function of the affectedpart[22]. In this study, the antioxidant and wound healing activities of the AS-3at various concentrations were evaluated. Furthermore, the total phenolic contents and HPLC finger printing profile of the AS-3weredetermined. For the evaluation of the woundhealing activity linear incision and circular excision wound modelswere employed.

Topical application of *A.stracheyi* at the wound site produced significant wound healing activity, which may be due to its angiogenic and mitogenic potential. A healing tissue synthesizes collagen, which is a constituent of growing cell. Collagen not only confers strength and integrity to the tissue matrix but also plays an important role in homeostasis and in epithelialization at the latter phase of healing[23].

Reactive oxygen species i.e. oxidants, are vital parts of healing and serve as cellular messengers thatdrive numerous aspects of molecular and cell biology. At high concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation. The antioxidant activity may be due to potent-radical-scavenging activity of the phenolics present in the AS-3. The activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metal chelating potential [24]. Hence the synergistic effect of antioxidant activity accelerated the wound healing process.

The result of excision wound model showed that on day 4 there was no significant increase in the contraction of wound in all the groups as compared to the control group. But, on day 8, 12, and 16 there was significant increase ($p< 0.01$, 0.001 and 0.001 respectively) in the percentage wound contraction in the group treated with standard drug that is povidone iodine and 5% (w/w) ointment of AS-3, shown that it has ability to induce cellular proliferation.The content of hydroxyproline was increased significantly in the woundtreated with topical applications of ointment of AS-3, which indicatesincreased per cell collagen synthesis and increase in the synthesis of collagenprovides necessary tensile strength to repaired tissue[25].Besides

this, there is also increase in tensile strength of excised wound treatedwith ointment of the AS-3in a dose dependent manner, it alsogives an indication of increased collagen synthesis;remodeling of collagen and the formation of stable intra- andintermolecular crosslinks increase the tensile strength of newlyformed tissue [26].

The wound healing studies on the leaves of *A. stracheyi* shown, that the phenolic constituents and flavonoids play an important role inwound healing process [27]. The various recent studies have shown that phytochemical constituents likeflavonoids [28] and triterpenoids [29] are known to promote the wound healing processmainly due to their astringent and antimicrobial properties, whichappear to be responsible for wound contraction and increased rateof epithelialization. The HPLC profile of the AS-3showed the presence of quercetin which is already reported for its wound healing potential [30]. Therefore, it can be proposed that, the high content of flavonoids and triterpenoids in the leaves of *A. Stracheyi* may be responsible for wound healing activity. Thus, the folklore claim for the use of*A. stracheyi* leaves wound healing can bejustified by the present study. Therefore, it could bea drug of choice, effective in treating wounds.

CONCLUSION

Results of the present study have clearly demonstrated that the *A. stracheyi* possess wound healing potential which supports its traditional utilization in various folk medicines. The wound healing studies on leaves of *A. stracheyi* indicate that the phenols constituents and flavonoids play an important role in wound healing process. It can be proposed that, the high content of phenols and antioxidants in the leaves of *A. stracheyi*may be responsible for wound healing activity, probably due to astringent property of tannins. In a reference survey, no reports relating to the wound healing activityof *A. stracheyi* extracts have been found so far. To the best of ourknowledge, we herein report for the first time about wound healingactivity of *A. stracheyi* as topical application by excision and incision models.Additionally, merit studies are needed to distinguish its potential for clinical use in clinical trials

ACKNOWLEDGEMENTS

The authors are grateful to our vice-chancellor Dr. PK Khosla and Dean Dr. Neeraj Mahindroo for providing the necessary facilities.

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