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WOUND HEALING EFFECTS OF *AGERATUM CONYZOIDES* LINN.

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ABSTRACT

The petroleum ether, chloroform, methanol and aqueous extracts of the leaves of *Ageratum conyzoides* Linn. (Asteraceae) were evaluated for their wound healing activity in rats using excision (normal and infected), incision and dead space wound models respectively. The effects of test samples on the rate of wound healing were assessed by the rate of wound closure, period of epithelialisation, wound breaking strength, weights of the granulation tissue, determination of hydroxyproline, super oxide dismutase (SOD), catalase and histopathology of the granulation tissues. Nitrofurazone (0.2% w/w) in Simple ointment I. P. was used as reference standard for the activity comparison. The results of the study revealed that the animals treated with methanol and aqueous extracts of *A. conyzoides* showed faster rate of wound healing compared to other extracts under study. The chloroform extract of the selected plants also produced promising results but the effects are seen to be of lesser extent than the corresponding methanol and aqueous extracts. The petroleum ether extract did not produce significant results. The present work justifies the use of the leaves of *A. conyzoides* for wound healing activity as claimed in the folklore literature.

KEY WORDS

Ageratum conyzoides Linn., Wound healing, Excision wound model, Incision wound model, Dead space wound model.

INTRODUCTION

Ageratum conyzoides Linn. (Asteraceae), is an annual erect half-hardy ornamental shrub of 30-90 cm height, found as one of the commonest weeds of the tropics, native to tropical America. It is naturalized as a weed throughout India in planes and hills and in forests as under growth[1-2]. Traditionally, the plant is reported to be used externally to cure wounds, leprosy and boils and as antihaemorrhagic[3-5], internally as diuretic and antipyretic[6]. The leaves are used externally as antiseptic and haemostatic[7-10]. The hot aqueous extract of the leaves is used orally for treating intestinal worms and as an antispasmodic, abortifacient and for treating diabetes[11, 12]. The tribes of Cuttack district of Orissa apply the leaf juice over fresh wounds and claim to be beneficial for quick healing.

Presence of a good number of pyrrolizidine alkaloids, chromenes and flavones have been reported from *A. conyzoides*[13-15]. The anti-inflammatory, analgesic[16-18], gastro protective[19], cytotoxic[20], smooth muscle relaxant[21] antibacterial[22], antifungal[23] and antimalerial[24] activities of the plant have been reported by different authors. To the best of our knowledge, there is no published article demonstrating scientifically wound healing effect of *A. conyzoides*. Therefore, this study was set to establish the pharmacological basis for the apparent wound healing activity of leaves of *A. conyzoides.* This knowledge could enable more rational exploitation of the plant both in traditional medicine and in the empirical development of new herbal therapy for wounds.

MATERIALS AND METHODS

(i) Plant material and extraction

Fresh leaves were collected from young matured trees and authenticated by the taxonomists of the Botanical Survey of India, Shibpur, Howrah. After authentication, the plant materials were collected in bulk, washed under running tap water to remove adhering dirt followed by rinsing with distilled water. The plant material was then shade dried and pulverized in a mechanical grinder followed by sieving (sieve no. 40) to obtain coarse powder. The powdered leaves (500 g) was successively extracted with petroleum ether (40-60^0 C), chloroform, methanol and water for 48 h in a soxhlet extractor. Following extraction, the liquid extracts were concentrated under vacuum to yield dry extracts. Standard methods[25-27] were used for preliminary phytochemical screening of the different extracts to know the nature of phytoconstituents present within them (Table 1).

Table 1

Preliminary phytochemical screening of different extracts of A. conyzoides leaves

Extract	Alkaloids	Carbohydrates	Glycosides	Gums and mucilages	Proteins and amino acids	Tannins and phenolic compounds	Steroids and sterols	Triterpenoids	Saponins	Flavonoids
Pet. Ether	-	-	-	-	-	-	+	+	-	-
Chloroform	+	-	-	-	-	-	+	+	-	-
Methanol	+	-	-	-	-	+	-	-	-	+
Aqueous	-	+	-	+	+	+	-	-	-	+

(+): Present; (-): Absent.

(ii) Animals

Healthy Wistar albino rats (150–250 g) of either sex and of approximately the same age were used for the study. They were individually housed, maintained in clean polypropylene cages and fed with commercially pellet diet (M/s Hindustan Lever Ltd., Mumbai) and water *ad libitum*. The experimental protocols were subjected to scrutiny of Institutional Animal Ethics Committee for experimental clearance (No. 1025/C/07/CPCSEA).

(iii) Wound healing activity

The selected extracts of *A. conyzoides* were separately evaluated for their wound healing activity in rats using excision (normal and infected), incision and dead space wound models. The effects of test samples on the rate of wound healing were assessed by the rate of wound closure, period of epithelialisation, wound breaking strength, weights of the granulation tissue, determination of hydroxyproline, super oxide dismutase (SOD), catalase and histopathology of the granulation tissue. Nitrofurazone (0.2% w/w) in Simple ointment I. P. was used as reference standard for the activity comparison. The test extracts were mixed with Simple ointment I. P. (10% w/w) and used in the excision and incision models. For the dead space wound model, the methanol extract was suspended in water and used.

(iv) Excision Wound Model (Normal wounds)

Animals were anesthetized prior to and during creation of the wounds, with 1 ml of intravenous ketamine hydrochloride (10 mg/kg). The rats were inflicted with excision wounds as described by Morton and Malone[28] and suggested by Kamath *et al.*[29]. An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear on the anaesthetized rat. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of circular area of 500 mm^2 and 2 mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open[30]. All surgical procedures were performed under aseptic conditions.

The control group animals (Group I) were treated with the vehicle (Simple ointment I. P.), the positive control (Group II) was applied with 0.2% w/w nitrofurazone in Simple ointment I. P.

Other groups of animals were treated with the following: petroleum ether, chloroform, methanol or aqueous extracts of *A. conyzoides* at a concentration of 10% w/w in Simple ointment I. P. in a similar manner. The wound closure rate was assessed by tracing the wound on days 1, 4, 6, 8, 11, 14 and 16 post wounding days using transparent paper and a permanent marker. The wound areas recorded were measured using graph paper[31]. The percentage of wound healing was calculated of original wound size for each animal of group on predetermined days i.e. 1, 4, 6, 8, 11, 14 and 16 days post-wounding for final analysis of results. Changes in wound area were calculated, giving an indication of the rate of wound contraction[32]. The period of epithelialisation was calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound. The results are tabulated in Table 2.

Table 2
Effect of various extracts of A. conyzoides leaves on percentage (%) wound closure (Excision Wound Model)

Group	Treatment	Concentration	Percentage (%) wound closure.						Period of epithelialization (No. of days)
			4th days	6th days	8th days	11th days	14th days	16th days	
I	Control	-	23.52±1.21	37.72±1.58	51.92±1.71	71.28±2.23	79.24±1.18	83.56±1.03	23.16±0.71
II	Nitrofurazone	0.2% w/w	48.53±2.87*	74.23±3.32*	84.8±1.26**	96.54±1.29**	100±00**	-	13.5±1.54**
III	Pet ether extract	10% w/w	28.36±1.65	44.45±2.88	58.25±3.08	72.56±1.23	81.79±1.36	83.26±1.4	21.16±0.99
IV	Chloroform extract	10% w/w	26.12±1.64	46.47±2.64	60.54±2.57	78.93±2.77*	88±1.77**	90.13±1.83**	19.5±1.25*
V	Methanol extract	10% w/w	29.88±1.86	55.29±1.62*	82.08±1.6**	92.96±1.14**	97.59±1.39**	100±00**	16.5±0.88**
VI	Aqueous Extract	10% w/w	29.16±2.66	48.48±3.35*	65.12±3.96*	82.35±2.22**	92.35±1.4**	94.9±1.09**	18.83±1.02*

Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA.
** P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.*

(v) Incision Wound Model

The rats were anaesthetized prior to and during creation of the wounds, with 1 ml of intravenous ketamine hydrochloride (10 mg/kg). The dorsal fur of the animals was shaved with an electric clipper. A longitudinal paravertebral incision of 6 cm long was made through the skin and cutaneous tissue on the back as described by Ehrlich and Hunt[33]. After the incision, the parted skin was sutured 1 cm apart using a surgical thread and curved needle. The wounds were left undressed[34]. Extracts were topically applied to the wound once a day. The sutures were removed on 8th post wound day and continued the application of the extract. The wound breaking strength[35] was measured on the 10th day evening after the last application. The results are tabulated in Table 3.

Table 3

Effect of various extracts of A. conyzoides leaves on wound breaking strength (Incision Wound Model)

Group	Treatment	Breaking strength (g)
I	Control	327.5±16.58
II	Nitrofurazone (0.2% w/w)	491.21±16.26**
III	Pet ether extract	337.16±11.43
IV	Chloroform extract	347±14.63
V	Methanol extract	487.2±10.42**
VI	Aqueous extract	389.5±20.84*

Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA.
** P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.*

(vi) Excision Wound Model (Infected wounds)

The results of the excision and incision wound models revealed that the methanol extract of *A. conyzoides* possess comparatively better wound healing activity compared to other test extracts under study. Therefore, infected wound model was separately performed on the methanol extract of *A. conyzoides* taking *Staphylococcus aureus* and *Pseudomonas aeruginosa* as the infecting bacteria.

The methods of *Abo et al*., 2004[36] was followed. The selected rats were divided into three groups, each containing 6 animals. A round seal of 20 mm diameter was impressed on the two sides of the central trunk depilated and sterilized with ethanol. Excision wound was inflicted on the rats as described earlier. Full skin thickness was excised from the marked area to get a wound measuring about 314 mm^2. After achieving complete haemostasis by blotting the wound with cotton swab soaked in warm saline, the wound of each animal was inoculated separately with an overnight (18 h old) *S. aureus* and *P. aeruginosa* cultures. The animals were placed singly in individual cages. The infected wounds on each animal of the control group were treated topically with Simple ointment I. P. Other groups of animals were treated separately with one of the following: 0.2% w/w nitrofurazone or 10% w/w methanol extract of *A. conyzoides* in Simple ointment I. P. in a similar manner.

Treatments of the infected wounds commenced on the 3rd day to allow for the establishment of the infection on the wound. The wound area was measured with a transparent graph paper on 1, 4, 6, 8, 11, 14 and 16 day. Wound contraction was calculated as a percentage of the original wound size. The results are presented in Table 4 and 5.

Table 4

Screening for wound healing activity of the methanol extract of A. conyzoides (Excision Wound inoculated with Stapphylococcus aureus)

Group	Treatment	Dose	Percentage (%) wound closure.						Period of epithelialization (No. of days)
			4th days	6th days	8th days	11th days	14th days	16th days	
I	Control	ml/kg	10±1.29	19.5±2.34	34±3.83	44.5±5.75	52±4.53	58.4±5.85	29.12±2.65
II	Nitrofurazone (0.2% w/w)	mg/kg	15.83±6.21	36.33±2.98**	59.5±4.48**	76.33±4.91**	90.5±3.39**	97.33±1.97**	18.03±1.23**
III	Methanol extract (AC)	mg/kg	13.16±2.35	22.66±2.82	48.35±3.12*	61.83±3.01*	71±2.63**	88.33±2.59**	22.05±0.67*

*Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA. * P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.*
AC - A. conyzoides.

Table 5

Screening for wound healing activity of the methanol extract of A. conyzoides (Excision Wound inoculated with Pseudomonas aeruginosa)

Group	Treatment	Dose	Percentage (%) wound closure.						Period of epithelialization (No. of days)
			4th days	6th days	8th days	11th days	14th days	16th days	
I	Control	ml/kg	8.22±1.81	15.6±1.22	21.08±2.71	32.41±3.33	46.61±3.85	51.13±2.15	30.33±2.23
II	Nitrofurazone (0.2% w/w)	mg/kg	14.21±1.09	25.73±3.81*	39.43±2.22**	57.63±3.11**	78.56±2.48**	92.53±3.25**	18.73±1.81**
III	Methanol extract (AC)	mg/kg	13.86±1.85	21.06±2.41*	34.35±3.87*	52.62±3.24**	76.41±2.57**	86.73±3.21**	19.21±0.81**

*Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA. * P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.*
AC - A. conyzoides

(vii) Acute oral toxicity studies

Acute oral toxicity studies of the extracts were carried out as per the OECD guidelines, draft guidelines 423[37]. Different groups of animals each containing three female rats (180–210 g) received *A. conyzoides* methanol extract suspended in water separately at doses of 300, 600 and 2000 mg/kg orally by gavage. Animals were observed individually after dosing once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. Observations included changes in skin and fur, eyes and mucous membranes, respiratory and behaviour pattern. A special attention was directed

to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. The change in body weight, food and water intake was recorded at two days interval.

There was no mortality or morbidity observed in animals through the 14-day period following single oral administration at all selected dose levels of the methanol extract of *A. conyzoides*. Morphological characteristics (fur, skin, eyes and nose) appeared normal. No tremors, convulsion, salivation, diarrhea, lethargy or unusual behaviours such as self mutilation, walking backward and so forth were observed; gait and posture, reactivity to handling or sensory stimuli, grip strength were all normal. There was no significant difference in body weights between control and treatment groups.

(viii) Dead space Wound Model

Dead space wounds were created by implanting two pre-weighed sterilized polypropylene tube (2.5 length x 0.25 cm diameter) beneath the dorsal para-vertebral skin of the anaesthetized rats[38]. The animals were randomly divided into two groups of six each. The control group animals were provided with plain drinking water and the other group rats were separately administered with the methanol extract of *A. conyzoides* at a dose of 100 mg/kg daily. On the 10th post wounding day, the granulation tissue formed on the implanted tubes was carefully detached from surfaces of the tubes. The wet weight of the granulation tissue collected was noted. The tissue samples were dried at 60° C for 12 h and weighed to determine the dry granulation tissue weight. The results are depicted in Table 6.

Table 6

Wound healing effects of the methanol extracts of A. conyzoides in Dead Space Wound Model, Hydroxyproline content in granulation tissues and the level of antioxidant enzymes in granuloma tissue

Treatment	Wet tissue weight (mg)	Dry tissue weight (mg)	Concentration of hydroxyproline (mg/100 g dry tissue)	Superoxide dismutase (units/mg)	Catalase (unit/mg)
Control	91.36 ± 2.37	42.22±2.07	2933.33±326.60	0.117±0.011	0.08±0.013
Methanol extract (AC)	114.16±4.98**	63.0±4.21**	6066.66±467.62**	0.178±0.016*	0.46±0.012*

*Values are expressed as mean ± S.E. (n = 6). * P<0.05, ** P<0.01 when compared to control; Dunnet's t-test. (AC – A. conyzoides).*

The dried tissue (50 mg) was added to 1 ml 6 M HCl and kept at 110^{o} C for 24 h. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxyproline[39]. Part of the granulation tissue was collected in phosphate-buffered saline for the estimation of antioxidant enzymes superoxide dismutase (SOD)[40] and catalase[41].

(ix) Histological Studies

For histological studies, pieces of granulation tissues from dead space wound model were fixed in 10% neutral formalin solution for 24 h and dehydrated with a sequence of ethanol-xylene series of solutions. The materials were filtered

and embedded with paraffin (40-60 °C). Microtome sections were taken at 10 µ thickness. The sections were processed in alcohol-xylene series and stained with hemotoxylin-eosin dye. The histological changes were observed under a microscope. Photographs were taken from each slide and presented in Fig. 1.

(x) Statistical Analysis

The data obtained in the studies were subjected to one way of analysis of variance (ANOVA) for determining the significant difference. The inter group significance was analyzed using Dunnet's t-test. A p-value <0.05 was considered to be significant. All the values were expressed as Mean ± SEM.

RESULTS

The preliminary phytochemical screening of *A. conyzoides* leaf extracts showed presence of steroids and sterols, triterpenoids, alkaloids, flavonoids, saponins, tannins and phenolic substances, gums and mucilages, carbohydrates and proteins respectively in different extracts (Table 1).

The studies on excision wound healing model (without infection) showed that there is almost complete healing (100% wound closure) on the 16th post wounding day with the methanol extract (Fig. 7.1.1). The chloroform and aqueous extracts also showed significant wound healing activity (90.13 ± 1.83 and 94.9 ± 1.09 percentage wound closure respectively). However, the petroleum ether extract 83.26 ± 1.4 did not show significant percentage wound closure till 16 day of the study when compared with the control group of animals. The nitrofurazone treated group demonstrated 100% wound closure on 14th day of the study. The epithelialization period was also reduced in the methanol extract treated groups when compared with other extract treated groups, as evidenced from Table 2.

In the incision wound model (Table 3), the breaking strength of the methanol extract was found to be more significant ($p<0.01$) than the aqueous extract ($p<0.05$). The methanol and aqueous extracts showed greater wound breaking strength (487.2 ± 10.42 and 389.5± 20.84 g respectively) compared to the other extracts. The petroleum ether and chloroform extract treated group of animals failed to produce significant wound breaking strength. The nitrofurazone treated group of animals represented significant wound breaking strength (491.21 ± 16.26).

The results of the excision and incision wound models revealed that the methanol extract possesses comparatively better wound healing activity compared to other test extracts under study and therefore, investigation was further carried out using the methanol extract for the infection wound model. In the infection wound model (Table 4), the methanol extract exhibited 88.33 ± 2.59 percentage wound closure on 16th day of the study as against 97.33 ± 1.97 of the reference drug nitrofurazone against the wounds inoculated with *S. aureus*. The methanol extract exhibited significant activity right from 8th day of the study. The control group however, registered only 58.4 ± 5.85 % wound closure in the infected excision wound model. The epithelialization period was reduced in the extract treated group and this is equally reflected by the percentage of wound contraction in both normal and *S. aureus* inoculated wounds. Further, with the wounds inoculated with *P. aeruginosa* (Table 5), the methanol extract showed significant activity from 6th day similar to that of the reference control nitrofurazone. The period of epithelialization was the smallest when compared to the methanol extract treated groups of other plants under study.

In the dead space wound model (Table 6), significant increase in dry granulation tissue weight indicated the presence of higher protein content. Increase in the granulation tissue dry weight and

increased epithelialization could be attributed to the increased hydroxyproline content in the wound tissue. The hydroxyproline content of the granulation tissue also revealed significant rise in the figure compared to the control group of animals. SOD activity in granulation tissue was significantly increased in the case of rats treated with the methanolic extract ($P < 0.01$) when compared with control. Catalase level in granulation tissue was also significantly increased.

The histological profile of granulation tissue in control group of animals revealed clumping of macrophages with poor collagenation [Fig.-1 (a)]. Similar results were also observed with petroleum ether extract treated group of animals [Fig.-1 (c)], while in chloroform extract treated animals moderate collagen deposition with scattered macrophages have been noticed [Fig.-1 (d)]. However in the animals treated with methanol and aqueous extract revealed increased collagen fibers with few macrophages [Fig.-1 (e) & (f)] indicating their effect of on collagen maturation. The nirofurazone treated group of animals also revealed increased collagen fibers with few macrophages [Fig.-1 (b)].

Histology of granulation tissue of A. conyzoides .

Fig- 1 (a)

Granulation tissue of group I animal (control) showing with less collagen and more macrophages.

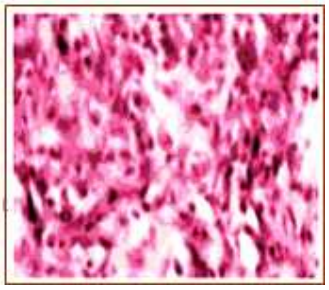


Fig- 1 (b)

Granulation tissue of group II (standard) animal showing significant collagenation, lesser fibroblasts and capillaries.

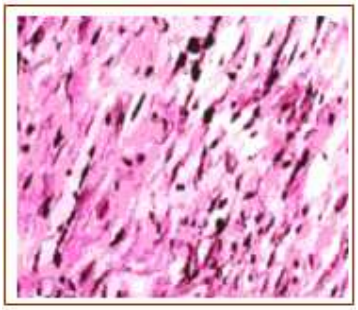


Fig- 1 (c)

Section of granulation tissue of group III animal (pet-ether extract) showing with less collagenation with less monocytes and fibroblasts and capillaries.

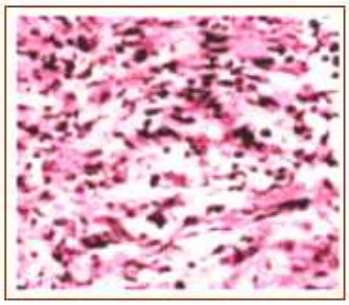


Fig- 1 (d)
Granulation tissue of group IV (chloroform extract) animal showing with less collagen and moderate macrophages.

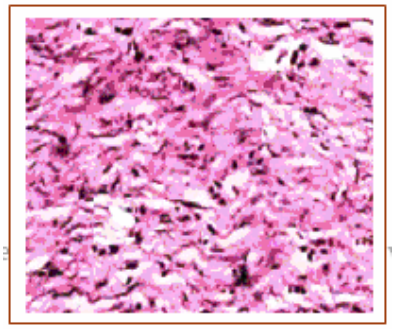


Fig- 1 (e)
Histological section of granulation tissue of group V animal treated with methanol extract showing significant increased collagenation, few macrophages and capillaries.

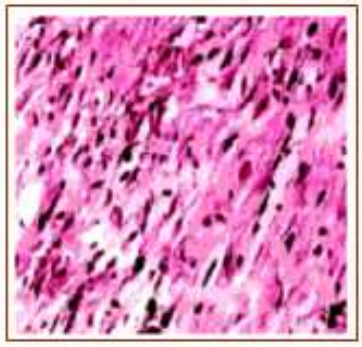
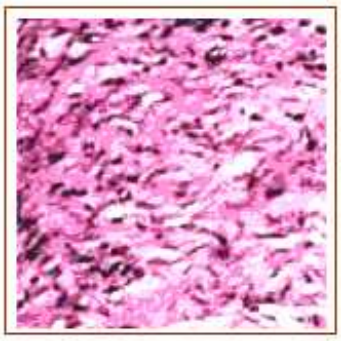


Fig- 1 (f)
Section of granulation tissue of group VI (aqueous extract) animal showing moderate collagenation with less macrophages and fibroblasts, and capillaries.



DISCUSSION

The results of the present study revealed that, animals treated with methanol and aqueous extracts of *A. conyzoides* showed faster rate of epithelialization in excision wound model compared to other extracts under study. The chloroform extract of the selected plants also produced promising results but the effects are seen to be of lesser extent than the corresponding methanol and aqueous extracts. The petroleum ether extract of all the plant materials did not produce significant results. The wound healing effects of the chloroform, methanol and aqueous extracts may be attributed to the presence of phytoconstituents like alkaloids, triterpenoids, tannins and flavonoids in the extracts which are known to promote the wound healing process mainly due to their antimicrobial property. Flavonoids and triterpenoids are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialisation[42-44]. In the present laboratory all the surgical interventions were carried out under sterile conditions and animals were closely observed for any infection; those which showed signs of infection were separated and excluded from the study. This is very important and researchers proved that the control microbial infection is necessary for better wound healing and its management[45, 46].

Increase in skin breaking strength and tissue breaking strength in incision and dead space wound model respectively indicated enhanced collagen maturation. Increase in the granulation tissue dry weight and hydroxyproline content indicated the high collagen turnover which may be due to the activity of some phytoconstituents like flavonoids which are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibers, by increasing the circulation, by preventing the cell damage and by promoting the DNA synthesis[47]. Hence, the wound healing promoting activity of *A. conyzoides* may also be attributed to the antioxidant and antibacterial potency of the active constituents present in them.

Thus, wound-healing property of the methanol and aqueous extracts may be attributed to the phytoconstituents they contain, which may be either due to their individual or additive effect that fastens the process of wound healing. The methanol extracts of each selected plant materials were found to possess better wound-healing property over other extracts. At this stage, it is difficult to say which component(s) of the extracts are responsible for the wound healing activity. However, further phytochemical studies are needed to isolate

the active compound(s) responsible for these pharmacological activities.

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