



Wound healing activity of *Aponogeton natans* (Linn.) Engl. & Krause - An important folklore medicine

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Abstract

The present investigation was carried out to find the effect of *Aponogeton natans* Linn. leaf with leafstalks extracts for its wound healing activity in different experimental animal models. The wound healing activity was evaluated using excision wound healing, incision wound healing, dead space wound and hydroxyproline content models in rats. In excision wound model the parameters studied were percentage wound closure and mean epithelialization time. From the study it was noticed that methanol extract treated group showed significant wound healing activity than pet ether, benzene and chloroform extract treated groups when compared to control untreated group.

The wound closure and percentage wound contraction as observed on 18th day in framycetin sulphate and methanolic extract treated group were found to be 0.00 mm² 100% ($p \leq 0.01$) respectively, followed by chloroform extract (55.78±12.77 mm² and 81.55%) when compared to the control group. The mean epithelialization time of framycetin sulphate, methanol and chloroform was found to be 16.67±0.52 ($p \leq 0.01$), 17.50±1.05 ($p \leq 0.01$) and 21.50±1.87 ($p \leq 0.05$) were also comparatively less in these groups when compared to control respectively. In case of incision method the animals treated with methanol extract exhibited significant tensile strength 495.91±3.768, 434.38±5.087g ($p \leq 0.01$) respectively on the post wounding day 10th day followed by chloroform extract treated group 306.26±3.314 g when compared to control group 242.33±4.177g. The weight of the wet granulation tissue was significant more in the animals treated with methanol extract were found to be 309.58±3.155 mg/100gm rat ($p \leq 0.01$) followed by chloroform extract 192.66±2.028 mg/100gm rat ($p \leq 0.05$) in comparison to control 178.66±2.848 mg/100gm rat. The weight of the dry granulation tissue was also significantly more in the animals treated with methanol extract 60.26±3.570mg/100g rat ($p \leq 0.01$) respectively and chloroform extract 47.71±2.574 mg/100g rat ($p \leq 0.05$) when compared to control untreated group 36.93±2.480. The tensile breaking strength of the granulation tissue in methanol extract treated animals was significant 395.20±3.386g ($p \leq 0.01$) respectively followed by chloroform extract treated animals 298.33±3.801g ($p \leq 0.05$) when compared to that of control group 282.81±2.138. The hydroxyproline content of the granulation tissue followed the same pattern as that of tensile strength. Hydroxyproline content of granulation tissue obtained from animals treated with methanolic extract was comparatively more significant 57.61±3.269mg/g tissue ($p \leq 0.01$) followed by animals treated with chloroform extract 28.68±1.356mg/g tissue ($p \leq 0.05$) when compared to that of control group 19.43±1.296 mg/g.

KEY WORDS: APONOGETON NATANS (LINN.), EXCISION WOUND, INCISION WOUND, DEAD SPACE WOUND, HYDROXYPROLINE, METHANOL EXTRACT

Introduction

Aponogeton natans (Linn.) Engl. & Krause belongs to aponogetonaceae family. The plant occurs in plains, in the ponds and marshy places in Asia, Australia, India and Srilanka. Leaf pastes are consumed with hot water to treat cuts & wounds (1). Fresh tuber are ground into a paste and boiled with 200 ml of coconut oil and applied on hair before bath for three days to get rid of fungal infection (2). *Aponogeton natans* (Linn.) Engl. & Krause is an important ingredient in preparation Useerasava. This asava is useful for raktapitta (Haemothermia), anaemia, impurity of blood and diabetes (3). A perusal of existing reports reveals that the no detailed wound healing study had been done earlier. Therefore, the present study has been planned to investigate the wound healing activity of pet ether, benzene, chloroform and methanol extracts of *Aponogeton natans* (Linn.) Engl. & Krause. leaf with leafstalks in different experimental animal models.

Material And Methods

Plant material

Fresh parts of *Aponogeton natans* (Linn.) Engl. & Krause were collected from Salipur, Cuttack, Odisha, India which was identified and authenticated by Prof.P.Jayaraman, PARC, Chennai. The voucher specimen was given the No. PARC/2009/398. The air dried powdered leaves with leafstalks was loaded into Soxhlet apparatus and was subjected to extraction for about 72 hours with petroleum ether (60-80°C), benzene, chloroform and methanol successively. After extraction the solvent was distilled off and the extract was concentrated under reduced pressure using rotary evaporator. The extracts were stored in a closed bottle and kept in refrigerator until tested.

Drugs and Chemicals

Framycetin sulphate skin cream I.P. (FSC) sold as Soframycin skin cream (1%w/w) was obtained from

Aventis Pharma Ltd. and used as standard reference for the wound healing activity. All other chemicals used were of analytical grade, purchased from s.d. fine, Pvt. Ltd. Mumbai.

Animals for wound healing activity

Swiss wistar strain rats of either sex weighing 150-200 g were procured from Institute of Pharmacy and Technology, Salipur and were maintained at standard housing conditions. The animals were fed with commercial food and water *ad libitum* during the experiment. The animal study was permitted by the Institutional Animal Ethical Committee, 19/IAEC-IPT/13.

Evaluation of wound Healing Activity

The wound healing efficiency of *Aponogeton natans* Linn. leaf with leafstalk extracts were evaluated employing three animal models viz., excision wound model, incision wound model and dead space wound model.

Drug formulations

Drug formulations were prepared for evaluation of wound healing activity. For topical application 5g of each extract was separately incorporated with 100g of simple ointment IP97. The formula for simple ointment I.P. is:

Sl no	Ingredients	Quantity(gm)
1	White bees wax	20
2	Hard paraffin	30
3	Cetosteryl alcohol	50
4	White soft paraffin	900

For oral administration, suspensions of 200mg/ml different extracts were incorporated with Tween 80 (3% w/v).

Excision wound model

The screening for excision wound healing activity was followed as per standard procedures (4, 5).

Under light ether anaesthesia a circular wound of about 300 mm² was made on depilated ethane sterilized dorsal thoracic region of rats.

The animals were divided into six groups of six animals each. The animals of Group I were left untreated (control group), animals of Group II, Group III, Group IV and Group V were treated with 50mg of 5% ointment prepared from pet-ether, benzene, chloroform and methanol leaf with leafstalks extracts of *Aponogeton natans* Linn. Group VI served as reference standard and treated with 1% (w/w) Framycetin sulphate cream (FSC) IP. The ointment was topically applied once a day, starting from the day of operation, till complete epithelialization. The animals were housed individually. The wound were traced on mm² graph paper on the day of 6th, 12th and 18th post wounding days and thereafter on alternate days until healing was complete. The percentage of wound closure (% contraction) and period of epithelialization (number of days required for falling of the dead tissue remnants of the wound without any residual raw wound) were calculated.

Determination of Percentage Wound Contraction

Incision wound model

The screening for excision wound healing activity was followed as per standard procedures.(6). Under light ether anaesthesia, 6cm long Para vertebral incisions were made through the full thickness of the skin on either side of the vertebral column. The wounds were closed with interrupted sutures of 1cm apart. The animals were divided into six groups of six animals each. The animals were left undressed and housed separately. The animals of Group I were left untreated (control group), animals of Group II, Group III, group IV and Group V were treated with 50mg of 5% ointment prepared from pet-ether, benzene, chloroform and methanol leaf with leafstalks extracts of *Aponogeton natans* Linn. Group VI served as reference standard and treated with 1% (w/w) Framycetin sulphate cream (FSC) IP.

The ointments were applied to the wound topically once a day from the day of operation till complete healing. The sutures were removed on 8th post wounding day and the skin breaking strength of the wounds were measured on the 10th day according to the continuous constant water flow technique of Lee et al (7) as follows:

$$P_n = 100 - \frac{W_n}{W_1} \times 100$$

where

P_n: percentage wound contraction on Nth day

W_n: wound area on Nth day

W₁: wound area on 1st day

The anaesthetized rat was placed on operation table. The Allis forceps were firmly applied on the lines, facing each other. The forcep on one side was hooked to a metal rod, fixed firmly to the operation table, while the other to a light polythene container through a string runs over a pulley. Water was allowed to flow at a constant rate into the polythene container so as to build a gradual pulling force necessary to disrupt the wound. The flow of water was regulated by means of an occlusion clamp on rubber tubing connected to a reservoir, kept at a suitable height. As soon as the gaping of the wound was observed, the water flow was stopped. The volume of water in the polythene container was measured and converted to the corresponding weight assuming the density to be equal to 'one'. The tensile strength was expressed as the minimum weight of water necessary to bring about the gaping of the wound.

Dead space wound model

Under light ether anaesthesia, the dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5cm x 0.3cm) in the region of groin on both the sides and then the wounds were sutured. The animals were divided into five groups of six animals each. The group I animals were left untreated and served as control group, Group II, Group III, Group IV and Group V animals received oral suspensions of pet-ether, benzene, chloroform and methanol extracts (200 mg/kg. b.w., p.o.) of *Aponogeton natans* Linn.

respectively from 0 to 9th post wounding day. The granulation tissues formed around the piths were carefully harvested on the 10th post wounding day. The wet weight of the granulation tissue was noted. The breaking strength of the granulation tissue was measured as described already under incision wound model. The granulation tissue was dried at 60°C for 24 hrs and weighed and the dry weight of the granulation tissue was noted. The dried tissue was added 5 ml of 6N Hydrochloric acid and kept at 110°C for 24hrs the acid hydrolysate of dry tissue was used for estimation of hydroxyproline content (8).

Collagen estimation (Hydroxyproline content)

For the preparation of protein hydrolysate, 50 mg of tissue sample in 1.0 ml of 6.0 N Hydrochloric acid was weighed and sealed in screw-capped glass tube. The tubes were autoclaved at 151.056 kilograms per cm² for 3 hrs. The hydrolysate was neutralized to pH 7.0 and brought to the appropriate volume. Test tubes marked as sample, standard and blank were taken. 1.0 ml of test sample was added to test tubes marked as sample, 1.0 ml of demineralized water to test tubes marked as blank and 1.0 ml standard solutions to test tubes marked as standard. 1.0 ml of 0.01 M Copper sulphate solution was added to all the test tubes followed by the addition of 1.0 ml of 2.5 N Sodium hydroxide and 1.0 ml of 6% Hydrogen peroxide.

The solutions were occasionally mixed for 5 min and then kept for 5 min in a water bath at 80°C. Tubes were chilled in ice-cold water bath and 4.0 ml of 3.0 N Sulphuric acid was added with agitation. 2.0 ml of *p*-(dimethylamino) benzaldehyde was then added and heated in water bath at temperature 70°C for 15 min. The absorbance was measured at 540 nm using UV spectrophotometer. The hydroxyproline content of the samples were determined by interpolating the O.D. values on the standard graph. (8)

Statistical analysis

The data obtained from each experiment were

subjected to one way ANOVA followed by Dunnet's t test.

Results

Evaluation of wound healing activity

The results of the excision wound model are given in **Table 1, Figure 1 and 2**. The wound healing activities of leaf with leafstalk extracts of *Aponogeton natans* Linn. was evaluated employing three different animal models viz., excision, incision and dead space wound models. In all the models studied, significant wound healing activity was observed with the methanol extract of *Aponogeton natans* Linn. followed by chloroform extract when compared to control group. In excision wound model the parameters studied were percentage wound closure and mean epithelialization time. Significant wound healing activity was observed in the animals treated with methanol extract compared to control group. The wound closure and percentage wound contraction as observed on 18th day in framycetin sulphate (Group VI) and methanol extract (Group V) treated group were 0.00 mm² 100% ($p \leq 0.01$) respectively followed by chloroform extract (Group IV) 55.78±12.77 mm² and 84.34% when compared to the control untreated group (Group I). The mean epithelialization time of farmycetin sulfate, methanol and chloroform was found to be 16.67±0.52 ($p \leq 0.01$), 17.50±1.05 ($p \leq 0.01$) and 21.50±1.87 ($p \leq 0.05$) also comparatively less in these groups when compared to control untreated group respectively. The data obtained from pet ether and benzene extract treated group (Group II and III) was not appreciable and that of pet-ether extract treated group was very poor and hence less significant compared to control untreated group.

In the incision wound model the parameter studied was wound breaking or tensile strength of wounds treated with different extracts of *Aponogeton natans* Linn. The results of the incision wound model are given in **Table 2 and Figure 3**. The animals treated with farmycetin sulphate (Group VI) and methanol (Group V) extract exhibited signifi-

cant tensile strength 495.91 ± 3.768 , $434.38 \pm 5.087g$ ($p \leq 0.01$) respectively on the post wounding day 10th post wound day compared to control untreated group (Group I) followed by chloroform extract treated group (Group IV) $306.26 \pm 3.314g$ ($p \leq 0.05$). However the tensile strength of animals treated with the pet ether extract and that benzene extract treated group (Group II and III) were not appreciable.

The results of the various parameters studied under dead space wound model such as wet and dry weight of granulation tissue, tensile strength/breaking strength of granulation tissue, and hydroxyproline content of the granulation tissue are shown in **Table 3 and Figure 4**.

The weight of the wet granulation tissue was significant more in the animals treated with methanolic extract (Group V) 309.58 ± 3.155 mg/100gm rat ($p \leq 0.01$) respectively followed by chloroform extract (Group IV) 192.66 ± 2.028 mg/100gm rat ($p \leq 0.05$) in comparison to control untreated group (Group I) 178.66 ± 2.848 mg/100gm rat. The weight of the dry granulation tissue was also significantly more in the animals treated with methanolic extract (Group V) 60.26 ± 3.570 mg/100g rat ($p \leq 0.01$) respectively and chloroform extract (Group IV) 47.71 ± 2.574 mg/100g rat ($p \leq 0.05$) when compared to control (Group I) 36.93 ± 2.480 mg/100g rat.

The tensile breaking strength of the granulation tissue in methanol extract (Group V) treated animals was significant $395.20 \pm 3.386g$ ($p \leq 0.01$) respectively followed by chloroform extract (Group IV) treated animals $298.33 \pm 3.801g$ ($p \leq 0.05$) when compared to that of control group (Group I) $282.81 \pm 2.138g$, while that of pet-ether and benzene extract treated groups (Group II and III) were not significant shown in **Table 3 and Fig.5**.

The hydroxyproline content of the granulation tissue followed the same pattern as that of tensile strength. Hydroxyproline content of granulation tissue obtained from animals treated with methanolic extract (Group V) was comparatively more significant 57.61 ± 3.269 mg/g tissue ($p \leq 0.01$) respectively, followed by animals treated with chloroform

extract (Group IV) 28.68 ± 1.356 mg/g tissue ($p \leq 0.05$) when compared to that of control (Group I) 19.43 ± 1.296 mg/g tissue. The pet ether and benzene extract treated groups (Group II and III) yielded less significant results **Table 3 and Fig.6**.

Discussion

The results of the present study revealed that the methanol leaf with leafstalk extract of *Aponogeton natans* Linn. possesses a definite pro-healing action, which was demonstrated by a significant increase in the rate of wound contraction and by enhanced epithelialization.

Significant increase was also observed in skin breaking strength and hydroxyproline content of granulation tissue in methanol extract treated groups which were a reflection of increased collagen levels and gain in granulation tissue breaking strength. This indicated improved collagen maturation by increased cross linking while an increase in dry granulation tissue weight indicated higher protein content. The animals treated with chloroform extract also shown significant wound healing activity in all the three models, while the results were insignificant with pet-ether and benzene extracts.

The wound healing property of *Aponogeton natans* Linn. leaf with leafstalks may be attributed to the phytochemicals present in the plant, and the quicker process of wound healing would be a function of either the individual or the additive effect of the phytoconstituents.

Conclusion

Hence in the present investigation the wound healing activity of methanolic extract of *Aponogeton natans* Linn. may be attributed to chiefly polyphenolics, flavonoids, tannins and triterpenoids. The results of this investigation provide pharmacologic evidence on the folkore use of *Aponogeton natans* Linn. leaf with leafstalks for wound healing activity.

Acknowledgement

The authors sincerely thank Prof. P. Jayaraman (PARC) Chennai, for identifying and providing the information about plant

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Groups	Treatment	0 th day	6 th day	12 th day	18 th day	Period of epithelization
Group I	Control (Untreated)	299.25 ±14.0	277.69±18.85 (7.20)	248.64 ±20.50 (16.91)	190.39±11.55 (36.37)	32.17 ± 3.06
Group II	ANPE	308.09 ±17.72	256.63 ±22.58 (16.70)	207.69 ±23.82 (32.58)	144.53 ±41.77 (53.08)	27.83 ± 1.47
Group III	ANBE	306.25±10.49	251.45±20.86 (17.89)	205.83±10.02 (32.79)	136.15±27.88 (55.54)	25.66*± 2.58
Group IV	ANCE	302.41±16.27	199.98*±15.33 (33.87)	115.94*±7.96 (61.33)	55.78*±12.77 (81.55)	21.50*±1.87
Group V	ANME	294.89±17.74	180.93**±11.21 (38.64)	67.39**±17.22 (77.14)	00**±0.00 (100)	17.50**±1.05
Group VI	Farmycetin sulphate	296.23±12.47	166.23**±12.78 (43.88)	57.20**±18.05 (80.69)	00**±00 (100)	16.67**±0.52

Table 1. Effect of topical application *Aponogeton natans* Linn. leaf with leaf stalks extracts on percentage closure of excision wound area (mm² ± S.E.)

ANPE: *Aponogeton natans* pet ether extract; ANBE: *Aponogeton natans* benzene extract; ANCE: *Aponogeton natans* chloroform extract; ANME: *Aponogeton natans* methanolic extract

Results expressed as mean ± S.E.M. (n=6). Significant at *P<0.05, **P<0.01 as compared with control group (One way, ANOVA followed by Dunnet's t-test). Figures in parenthesis denote percentage of wound contraction

Groups	Treatment	Wound breaking strength (g)
Group I	Control (Untreated)	242.33±4.177
Group II	ANPE	251.53±3.421
Group III	ANBE	254.05±5.322
Group IV	ANCE	306.26±3.314*
Group V	ANME	434.38±5.087**
Group VI	Farmycetin sulphate	495.91±3.768**

Table 2. Effect of topical application of *Aponogeton natans* Linn. leaf with leaf stalks extracts on incision wound model

ANPE: *Aponogeton natans* pet ether extract; ANBE: *Aponogeton natans* benzene extract; ANCE: *Aponogeton natans* chloroform extract; ANME: *Aponogeton natans* methanolic extract

Results expressed as mean ± S.E.M. (n=6). Significant at *P<0.05, **P<0.01 as compared with control group (One way, ANOVA followed by Dunnet's t-test)

Groups	Treatment 200mg/kg	Wet weight granulation tissue (mg/100gm rat)	Dry weight granulation tissue (mg/100gm rat)	Breaking strength of granulation (g)	Hydroxyproline (mg/g tissue)
Group I	Control (2ml vehicle)	178.66±2.848	36.93±2.480	282.81±2.138	19.43±1.296
Group II	ANPE	182.16±2.088	37.56±1.893	286.46±1.984	23.20±1.083
Group III	ANBE	185.83±2.786	38.43±1.685	287.16±3.458	25.41±2.722
Group IV	ANCE	192.66±2.028*	47.71±2.574*	298.33±3.801*	28.68±1.356*
Group V	ANME	309.58±3.155**	60.26±3.570**	395.20±3.386**	57.61±3.269**

Table 3. Effect of oral administration of *Aponogeton natans* Linn. leaf with leafstalks extracts on Dead space wound model and hydroxyproline content.

ANPE: *Aponogeton natans* pet ether extract;

ANBE: *Aponogeton natans* benzene extract;

ANCE: *Aponogeton natans* chloroform extract;

ANME: *Aponogeton natans* methanolic extract

Results expressed as mean ± S.E.M. (n=6). Significant at *P<0.05, **P<0.01 as compared with control group (One way, ANOVA followed by Dunnet's t-test).

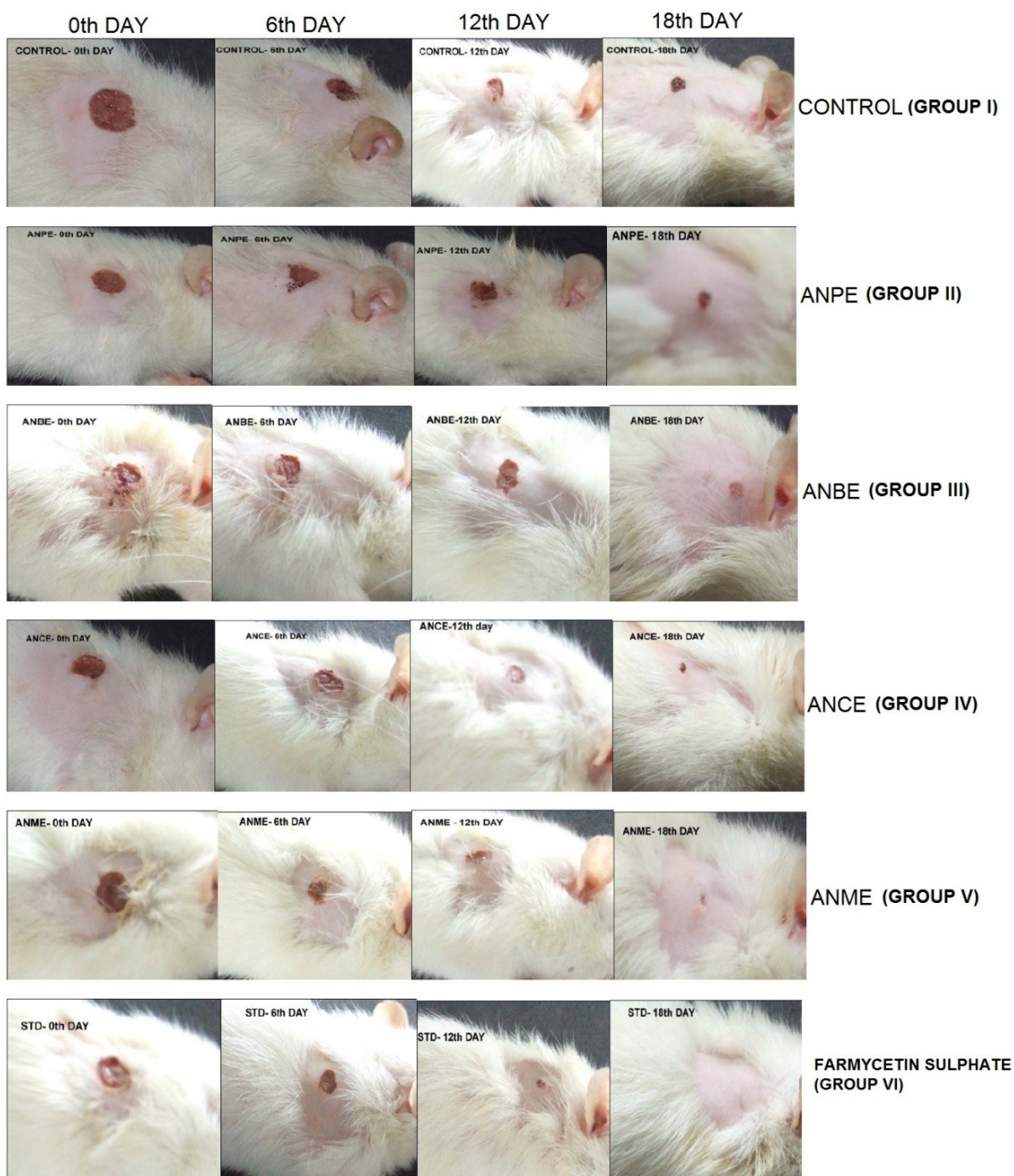


Figure1. Wound areas of different groups in excision wound model

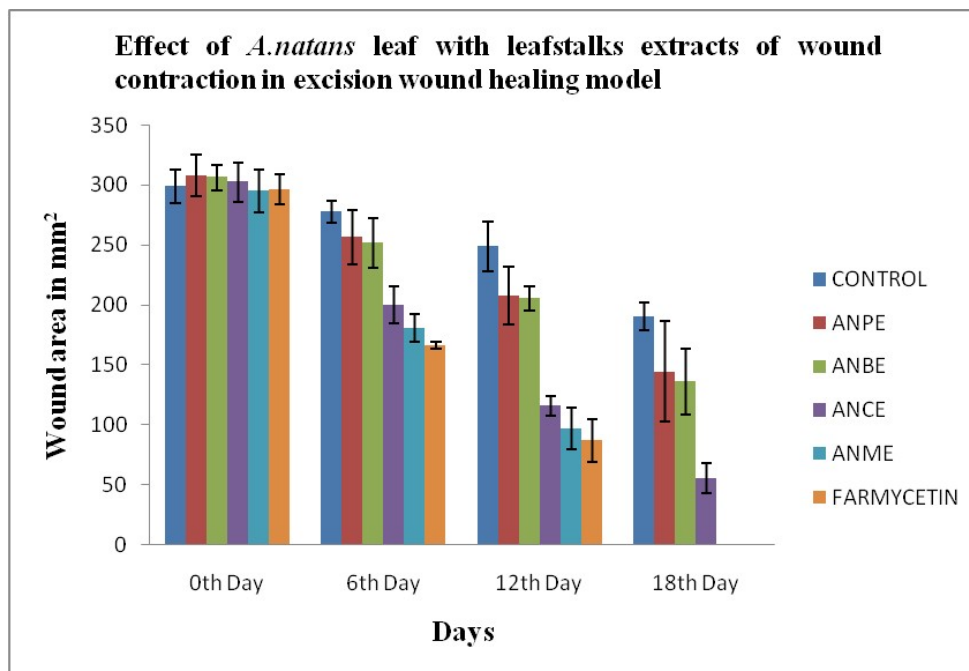


Figure 2

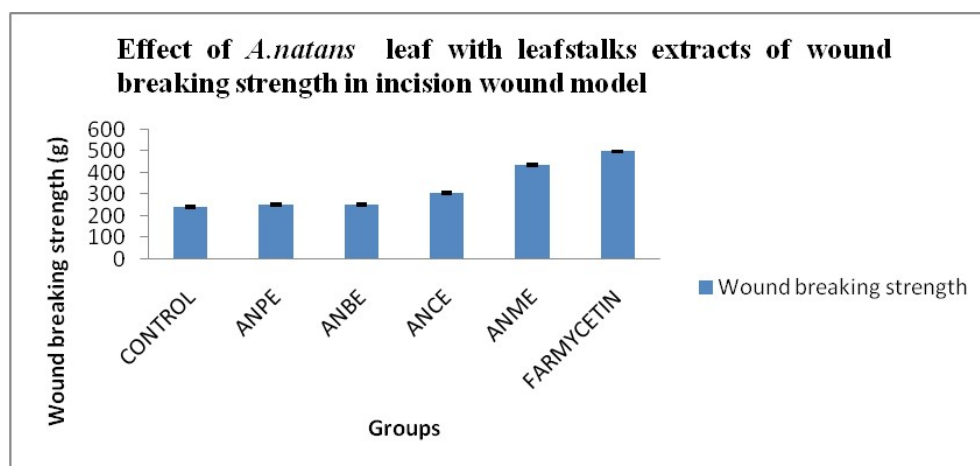


Figure 3

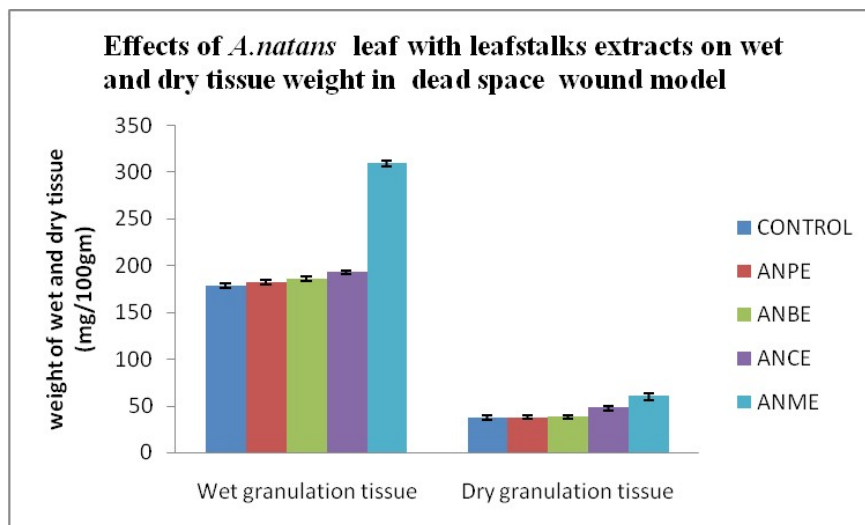


Figure 4

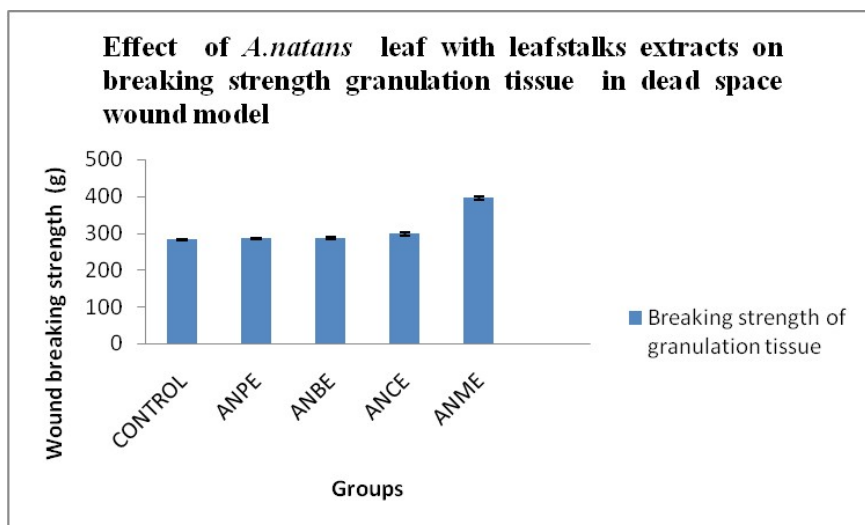


Figure 5

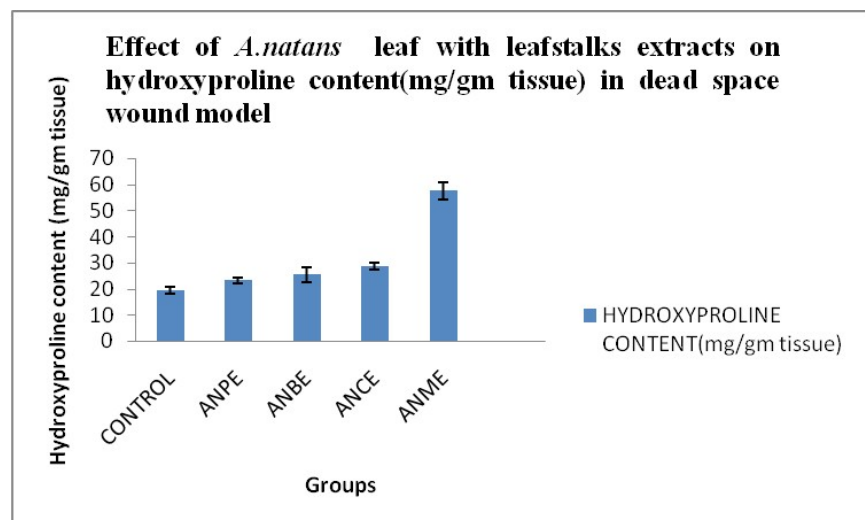


Figure 6