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FORMULATION AND EVALUATION OF WOUND HEALING ACTIVITY OF *MACROPTILIUM ATROPURPUREUM* IN ALBINO WISTAR RATS

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ABSTRACT

Considering various adverse affects associated with synthetic medicines, researchers are shifting their thinking towards herbal based medicines which are safe to use. Throughout the world tribal's as well as the folklore traditions used a huge number of plant extracts for various elements including curing cuts, wounds, bruises and burns. Wounds are physical injuries that result in an opening or breaking of the skin. Whereas healing is a natural phenomenon by which body itself overcome the damaged to the tissue but the rate of healing is very slow and chance of microbial infection is high. Therefore proper healing of wounds is essential for the restoration of disrupted anatomical stability and disturbed functional status of the skin. The present attempt is to highlight wound healing potential of petroleum ether and aqueous extract of *Macroptilium atropurpureum* (200mg/kg/day) in wistar albino rat using excision and incision wound model in the form of ointment base. All experiments were conducted according to standard procedures. The parameters studied were breaking strength in case of incision wounds and epithelialisation and wound contraction in case of excision wound. The results obtained indicated that *Macroptilium atropurpureum* posses extreme significant ($P<0.0001$) of wound healing process when compared to control group and somewhat similar to standard (i.e, Silver sulfadiazine).

KEYWORDS: *Macroptilium atropurpureum*, Wound healing, Excision wound, Incision wound, Silver Sulfadiazine Ointment.

INTRODUCTION

Wound is the first medical problem faced by the human race[1] which is nothing but a common injury due to internal or external factors, which is subsequently associated with many stages such as coagulation, epithelization, granulation, collegenation and tissue remodeling.[2]

Where as wound healing begins from the time of injury and can continue for varying periods of time, depending on the degree of wound.

In the present situation it has been considered / assumed that if the drug is effects fast it will have side effects but advanced treatment through medicinal plants are fixing them faster then ever.

India is one of the biggest biodiversity reservoirs in the world with vast range of plant species.[3] *Macroptilium atropurpureum* is one among the Indian plant which is commonly referred as *purple bush-bean*, or *siratro*, and it is distributed in tropical and subtropical regions of South America, India, Brazil etc.[4] The plant is commonly used for hey fever and as anti- microbial agent. *Macroptilium atropurpureum* contain phyto chemicals like phenols, flavonoids, terpinoids, carbohydrates, saponins, proteins, indoles and glycoside.[5]

Based on the chemical constituents present in *Macroptilium atropurpureum* it has carried out to evaluate wound healing activity which is scientifically not proved till date. All experiments were conducted according to standard procedures.

MATERIAL AND METHODS

Collection of Plant Material: The plant *Macroptilium Atropurpureum* was collected from the local fields of Hyderabad washed with tap water to remove the dust and soil. The whole plant was dried under shade, powdered and made to pass through sieve No.40 and stored in closed vessel. The plant specimen was identified and authenticated by L. Rasingam, Scientist In-charge, Botanical Survey of India, Deccan Regional Centre, Plot No.366/1, Pillar No.162, Attapur (V), Hyderguda (P.O), Hyderabad – 500048. Telangana, State. India.

A voucher specimen no. BSI/DRC/2017-18/Tech/935.

Extraction of Plant Material[6]

Preparation of Petroleum ether extraction

Plant was collected and shade dried, powdered in a mechanical grinder and passed through a sieve no.40 to obtain powder of desired particle size. The powder was packed in Soxhlet apparatus and extracted with petroleum ether for 48 hours. The extraction was transferred into the previously weighed empty china dish and evaporated to a thick paste on water bath, maintained at 50^0C to get the petroleum extract. The marc was air dried throughly to remove the solvent used previously before it was taken for further extraction with next solvent.

Preparation of Aqueous Extract

1. About 500 gms of dried marc was taken in a 1000 ml of beaker and macerated with 500ml of distilled water to which 5ml of chloroform was added as a preservative and kept it for seven days with occasional shaking daily in a closed vessel.
2. The supernatant was decanted and the marc was pressed then the pooled extract was concentrated on water bath at 50ºc to get a dry solid mass. The percentage yield was calculated and tabulated in Table-01.

Preliminary Phytochemical screening[7-13]

The preliminary phytochemical screening of the petroleum ether and aqueous extract of *Macroptilium atropurpureum*. Was carried out according to the standard procedures. And the results are tabulated in Table-02.

Preparation of Ointment By Fusion Method For Topical Application[14]

(a) Preparation of Simple Ointment

Wool fat -5gm: Hard Paraffin-5gm; Cetostearyl alcohol -5gm; White Soft Paraffin-85gm. Each Ingredient was mixed and heated gently with stirring then cooled. The base was then packed in a wide mouth container.

(b) Preparation of 10% Ointments[15]

Formulation of 10% petroleum ether extract and 10% aqueous extract of *Macroptilium atropurpureum* were prepared. Both formulations were having simple ointment base.

Evaluation of Ointment[16]

- **Subjective properties**

Subjective properties such as consistency, texture and irritation are observed and shown in Table 03.

- **Physical Evaluation**

The color, appearance and the feel on application of the prepared herbal ointment formulations were noted and the results are shown in Table 04.

- **Centrifugation**

It is believed to be a unique tool for the evaluation of accelerated deterioration of ointments. It was determined by using Remi centrifuge in 10 ml-graduated cylinder at 10,000 rpm for 10 min. Table 05.

- **Viscosity**

By using Brookfield viscometer (Model RVTDV II) at 100 rpm using spindle no. 6, the viscosity of the prepared formulations was assessed. Table 05.

- **Spreadability**

Assessment of the spreadability of the prepared formulations were determined individually by measuring the spreading diameter of 1gm of ointment between two glass plates (20cm × 20cm) by having a standard weight of 125gm on the upper plate.

Table 05

- **Extrudability**

The formulations were filled in the standard collapsible aluminum tubes which were sealed at the end. The weight of each tube was determined and recorded. Then the tubes were placed in between two glass slides which were clamped by having standard weight of 0.5 kg over the glass plates. Then the cap made to remove and weigh the extruded ointment from the tube. The percentage of extruded ointment was calculated. Table 05.

- **PH Measurement**

The pH of the ointment was determined by using a digital pH meter (Systronics pH meter type 335) 5gm ointment dissolved in 50 ml water and pH was determined by dipping the glass electrode completely into ointment solution system so as to cover the electrode. Then

instrument reading in terms of pH are tabulated in the Table 4. The pH was studied for 30 days. Table 06.

- **Stability testing**

Since the period of stability testing can be as long as two years, it is time consuming and expensive. Therefore, it is essential to device a method that will help rapid prediction of long term stability of drug. The accelerated stability testing is defined as the validated method by which the product stability may be predicted by storage of the product under condition that accelerated the change in defined and predictable manner. The stability studies of formulated ointments were carried out at 4 ^{0}C, 25 ^{0}C, and 45 ^{0}C and at a room temperature for the period of one month. The effect of temperature, humidity and time on the physical characterization of the ointments was evaluated for assessing the stability of prepared formulation. The results were shown in Table 07.

Experimental Animals[17]

Albino Wistar rats of either sex weighing 100-150 gm were used for the study in different models. Animal house was well maintained under hygienic conditions. The animals were housed in standard environmental conditions of temperature (31 ±1^{o}C), humidity (60± 0.2%) and a 12 h light and 12 h dark cycle.

They were provided with rodent diet and tap water ad libitum. Cleaning and sanitation work were done on alternate days. Paddy husk was provided as bedding materials, which was changed every day. The cages were maintained clean and all experiments were conducted according to the guidelines laboratory animal care.

Ethical committee report was obtained from Shadan Institude of Medical Science, Peerancheru, Himayat Sagar Road, Hyderabad.
(With reference number: 1864/PO/RC/S/16/CPCSEA)

Acute Dermal Toxicity – Fixed Dose Procedure[18]

The acute dermal toxicity study was carried out in adult female albino rats by fix dose method of OECD Guideline No.434. Extract of the plant *Macroptilium Atropurpureum* was applied topically at dose level of 2000 mg/kg body weight. And the results are discussed in Table 08.

Grouping of Animals

Animals were divided in to three groups, each group consisting of 6 rats.

Selection of dose: For the assessment of Cutaneous wound healing activity, dose level was chosen in such a way that, dose was approximately one tenth of the maximum dose during acute toxicity studies (200 mg/kg/day).

Group I **:** Control group (did not receive any treatment)

Group II : Received application of standard drug ointment i.e. Silver sulphadiazine ointment (1 %w/w)

GroupIII : Received application of extracts of *macroptilium atropurpureum* (200mg/kg/day).

WOUND HEALING ACTIVITY

Excision and incision wound models were used to evaluate the wound-healing activity of Pertroleum ether and aqueous extract of *Macroptilium Atropurpureum.*

The study was approved by the Institutional Animal Ethical Committee of Shadan College of Pharmacy, Peerancheru, Himayat Sagar road, Hyderabad.

Excision wound model

The animals were anesthetized during creation of the wounds. The rats were inflicted with excision wounds as described by 'Morton and malon'. The dorsal fur of the animals was shaved with an electric clipper the anticipated area of the wound to be created was outlined on the back of the animal. A full thickness of the excision wound of 2.5cm (circular area=300mm^2) in length and 0.2cm in depth was created using toothed forceps, a surgical blade and pointed scissors. The entire wound was left open.[19-20]

The animals were randomly divided into 4 groups and each group containing 6 animals. The treatments of each ointment were applied topically twice a day.

Group I- Control animals: received injury for wound formation but did not receive any ointment or drug treatment.

Group II – standard treated animals: received injury for wound formation and treated with silver silfadiazine ointment. (1%w/w).

Group III- Drug treated animals: received injury for wound formation and treated with *Macroptilium atropurpureum* aqueous extract ointment (10%w/w).

Group IV-Drug treated animals: received injury for wound formation and treated with *Macroptilium atropurpureum* petroleum ether extract ointment (10%w/w).

Until complete healing takes place. The wound closure rate was assessed by tracing the wound on alternate days of post wounding using transparency papers and a permanent marker. The wound areas recorded were measured using millimeter scale, graph paper. The day of Escher falling off, after wounding, without any residual raw wound was considered as the time until complete epithelialization.

The results were analyzed by one – way Anova followed by dunnet's t test.

- **Measurement of wound area.**

The progressive changes in wound area were measured until complete healing takes place on predetermined days i.e.2, 4, 8, 12, 16 and 20. Later on, wound area was measured by tracing the wound on a millimeter scale graph paper.[21] Results are showed in Table 09.

- **Measurement of wound contraction.**

Wound contraction was calculated as percentage of the reduction in original wound area size. It was calculated by using following formula.[22] Results are showed in table 09.

$$\% \text{ wound reduction} = \frac{\text{Wound area day 0 - Wound area on respective day}}{\text{Wound area day 0}} \times 100$$

- **Determination of period of epithelization.**

Number of days required for wound healing or Falling of scab leaving no raw wound behind was taken as end point of complete epithelization and the days required for this was taken as period of epithelization.[23] Results are showed in Table 09.

Incision wound model

The rats were anesthetized prior to and during the creation of wound the dorsal fur of the animals was shaved with an electric clipper. A longitudinal paravertebral incision were made using sterile blade of three centimetres in length was made through the skin and cutaneous muscle on the back as described by Ehrlich and hunt *et al.*[24-25] After the incision, surgical sutures were applied to the parted skin at intervals of one centimetre. The wounds were left undressed.[26-27]

The animals were randomly divided into 4 groups and each group containing 6 animals. The treatments of each ointment were applied topically twice a day.

Group I – Control animals: receive surgery for wound formation and did not receive any ointment or drug treatment.

Group II –standard treated animals: receive surgery for wound formation and treatment with silver sulfadiazine ointment (1%w/w).

Group III – Drug treated animals: received surgery for wound formation and treated with *Macroptilium atropurpureum* aqueous extract ointment (10%w/w).

Group IV – Drug treated animals: received surgery for wound formation and treated with *Macrptilium atropurpureum* petroleum ether extract cream (10%w/w).

The sutures were removed on 8th post wounding day and the treatment was continued.[28] The wound breaking strength was measured on the 10th post wounding day.

Measurement of tensile strength

On the 10th day the animals were sacrificed and there tensile strength was measured as follows: After sacrificing the animals after anaesthesia, sutures were gently pulled out. Both wound areas from each animal were removed carefully. Wound stripes of equal size (width) were then cut using a knife in which two blades were fixed at a fixed distance. Both ends of each strip were fixed with the help of a pair of steel clips. One clip allowed hanging on a standand a polyethylene bottle was then allowed to fill with water gradually till the wound strip was broken at the site of wound. The amount of water required to break the wound was noted and expressed as tensile strength of wound in gm[29] Results are showed in Table 10.

Data Statistical analysis

The data is expressed as mean ± sem and subjected to ANNOVA, Dunnett multiple comparisons test and the level of significance was set at $p < 0.001$.

RESULTS AND DISCUSSION

Plant Extract

The amount of extract obtained after Soxhlet extraction process from solvents; Petroleum ether and distilled water were given as follows.

Table 01: Plant Extracts.

S.No	Weight Drug	Solvent Used	Nature Of Extract	Colour	Obtaining Weight (Gm)	Percentage Yield
1	500gm *Macroptilium atropurpureum*	Petroleum Ether	Semi-solid	Yellowish brown	50	10%
2	500gm *Macroptilium atropurpureum*	Aqueous	Semi-solid	Dark brown	85	17%

The obtained extract of the plant was formulated into a suitable ointment base for topical application.

Preliminary Phytochemical Screening

The preliminary phytochemical screening of petroleum ether and aqueous extract of *Macroptilium atropurpureum* indicates the presence of most of secondary metabolites are listed as in the following table.

Table 02: Preliminary Phytochemical Screening.

Sl.No:	Plant Secondary Metabolite	Petroleum Extract	Aqueous Extract
1	CARBOHYDRATES	POSITIVE	POSITIVE
2	PROTEINS	POSITIVE	POSITIVE
3	GLYCOSIDES	POSITIVE	POSITIVE
4	ALKALOIDS	NEGATIVE	NEGATIVE
5	TANNINS	NEGATIVE	NEGATIVE
6	SAPONINS	NEGATIVE	POSITIVE
7	FLAVONOIDS	POSITIVE	POSITIVE
8	PHENOLS	POSITIVE	POSITIVE
9	AMINO ACIDS	NEGATIVE	NEGATIVE
10	STEROIDS	NEGATIVE	POSITIVE
11	VITAMINS	NEGATIVE	NEGATIVE

Evaluation of Ointment

A. Subjective Property

Table 03: Subjective Properties

PARAMETER	RESULT
CONSISTENCY	
Petroleum Extract Ointment	Good
Aqueous Extract Ointment	Good
TEXTURE	
Petroleum Extract Ointment	Smooth
Aqueous Extract Ointment	Smooth
IRRITATION	
Petroleum Extract Ointment	Nil
Aqueous Extract Ointment	Nil

Observation of Subjective Properties

The subjective properties such as consistency were good and texture of prepared herbal ointment was found to be smooth. No skin irritation was there on application of ointment to the skin surface. So it can be used safely.

B. Physical Properties

Table 04: Physical Properties.

PARAMETER	RESULT
COLOUR	
Petroleum Extract Ointment	Brown
Aqueous Extract Ointment	Brown
APPERANCE	
Petroleum Extract Ointment	Translucent
Aqueous Extract Ointment	Translucent
FEEL ON APPLICATION	
Petroleum Extract Ointment	Smooth
Aqueous Extract Ointment	Smooth

C. Centrifugation, Viscosity, Spreadibility & Extraudability.

Table 05: Various Properties.

CENTRIFUGATION	
Petroleum Extract Ointment	NO PHASE SEPERATION
Aqueous Extract Ointment	NO PHASE SEPERATION
VISCOSITY	
Petroleum Extract Ointment	4800 cps
Aqueous Extract Ointment	4800cps
SPREADIBLITY	
Petroleum Extract Ointment	65mm
Aqueous Extract Ointment	65mm
EXTRAUDABLITY	
Petroleum Extract Ointment	68% (fair)
Aqueous Extract Ointment	68%(fair)

From the result of physical evaluation, the color of prepared herbal ointments was brown, appearance of ointment was translucent and it was smooth on application. So it shows significant physical evaluation parameters.

Formulations complied with parameters like centrifugation, viscosity; spreadability and extrudability were found to be acceptable. As the skin irritation studies on the animals didn't show any significant effects like erythema, edema, itching, etc., it was stated to be safer in clinical practice.

D. pH of the Ointment Formulation.

Table 06: Ph of Ointment.

PARAMETER	RESULT
0 DAY	
Petroleum Extract Ointment	6.45
Aqueous Extract Ointment	6.45
2 DAY	
Petroleum Extract Ointment	6.48
Aqueous Extract Ointment	6.48
7 DAY	
Petroleum Extract Ointment	6.47
Aqueous Extract Ointment	6.47
14 DAY	
Petroleum Extract Ointment	6.50
Aqueous Extract Ointment	6.50
22 DAY	
Petroleum Extract Ointment	6.48
Aqueous Extract Ointment	6.48
30 DAY	
Petroleum Extract Ointment	6.47
Aqueous Extract Ointment	6.47

The **pH value** of ointment formulation was studied at room temperature are change in pH is observed and shown in Table pH value of prepared herbal ointment incorporating the medicinal plant extract was studied by using digital pH meter Systronics. (pH meter type 335). The pH was studied for 30 days at room temperature. Both formulations were in range of 6.45 – 6.50 pH at initial phase.

As we go from epidermis to dermis, pH of the skin increases and attained near to the neutral value i.e. 7. So ointment formulation having pH range 6.2 to 7 are desirable to skin since they do not interfere with the physiology of skin. The pH value of all ointments formulation showed slight difference after 2, 7, 14, 22 and 30 days. The change in pH value after 30 days shows minute difference and pH values are in a range of 6.2-7 which is desirable to skin and do not interfere with physiology of skin. This is also an indication that the ointments were non-irritant.

E. Stability Testing

Table 07: Stability Testing.

PARAMETER	INITIAL COLOUR	4^0C, 25^0C, 45^0C, AND AT ROOM TEMPERATURE			
		Ist Week	IInd Week	IIIrd Week	IVth Week
Petroleum Extract Ointment	Brown	+	+	+	+
Aqueous Extract Ointment	Brown	+	+	+	+

The prepared herbal ointment formulations were subjected to accelerated stability testing**.** The prepared herbal ointment was store at 4^0C, 25^0C, 45^0C in refrigeration, room temperature and oven for a period of 30 days to study effect of temperature and at different humidity condition. The result of study indicates that preparations are physically stable at 4^0C, 25^0C and 45^0C.

Acute Toxicity Studies

Application on acute dermal toxicity study was conducted as per OECD test guideline 434 (fixed dose). From the study, it was found that no mortality & morbidity was produced by the extract at 2000 mg/kg body. wt. dose level tested. This confirmed the nontoxic nature of the extract obtained.

Table 08: Acute Toxicities Studies

S. No.	Response	Concentration of Plant Extract (2000 mg/kg)
1	Motor Activity	N
2	Grooming	Ab
3	Touch Response	N
4	Pain Response	N
5	Tremors	Ab
6	Convulsions	Ab
7	Lighting Reflux	N
8	Gripping Strength	N
9	Pinna Reflex	P
10	Corneal Reflex	P
11	Writhing	N
12	Pupils	N
13	Urinations	N
14	Salivation	N
15	Skin Color	N
16	Lacrimation	N
17	Defecation	N
18	Diarrhea	Ab
19	Coma	Ab

N = Normal, P = Present, Ab = Absent

Wound Healing Evaluation

A. Excision Model

Table 09: Excision Model.

	% WOUND CONTRACTION ON					
GROUPS	**4th DAY**	**8th DAY**	**12th DAY**	**16th DAY**	**20th DAY**	**EPITHELIZATION TIME (DAYS)**
Control	6.926 ± 1.39	17.05 ± 1.21	28.86 ± 1.73	39.57 ± 2.04	58.86 ± 1.18	26.66 ± 0.49
Petroleum ether extract 2%	16.70 ± 1.48	37.02±2.70	58.69 ± 2.82	79.27 ± 1.23	90.66 ± 0.70	20.5 ± 0.42
Aqueous extract 2%	16.53 ± 0.56	39.98±1.88	62.41 ± 3.33	79.47 ± 1.77	92.77 ± 1.04	19 ± 0.36
Silver sulfadiazine Standard	16.84 ± 0.83	38.68 ± 1.22	58.46 ± 0.81	80.93 ± 1.09	95.59 ± 0.13	17.83 ± 0.40

Vales are the Mean ± SEM of six rats / treatment. Followed by ANOVA, Dunnett Multiple Comparisons Test. P <0.0001 Extremely Significant, P <0.01 Moderately Significant, P <0.05 Significant, P >0.05 Not Significant (NS).

In excision wound model study, the tropical application of petroleum ether and aqueous extract of *Macroptilium atropurpureum* showed significantly greater wound healing activity when compared to control animals where as it was compared with silver sulfadiazine as standard.

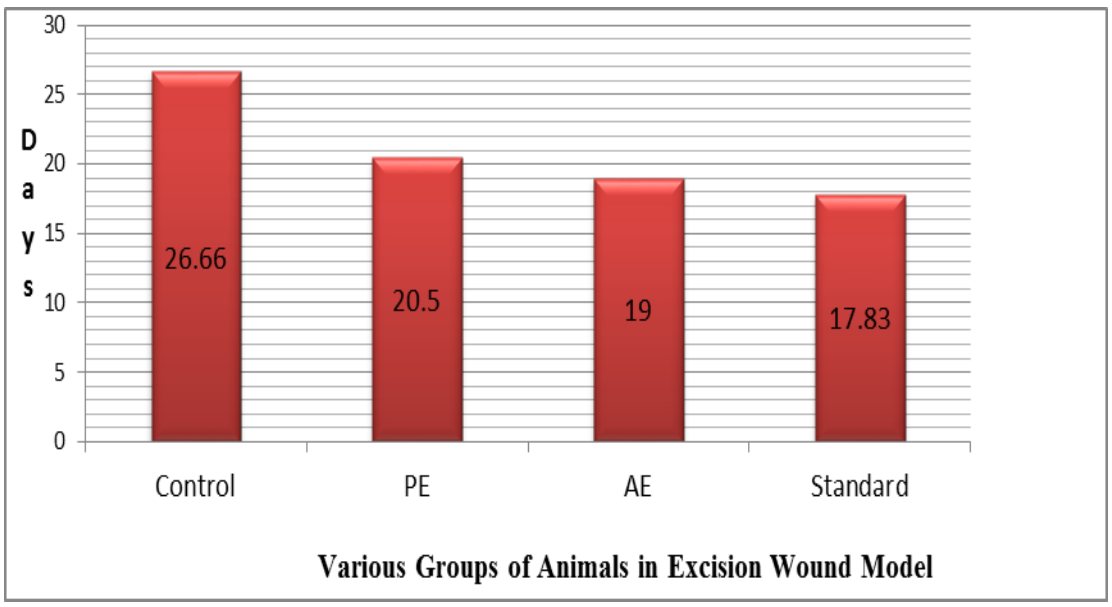

Figure 01: Effect of Petroleum Ether and Aqueous Extract in Excision Wound Model.

EPITHELIZATION TIME DAYS

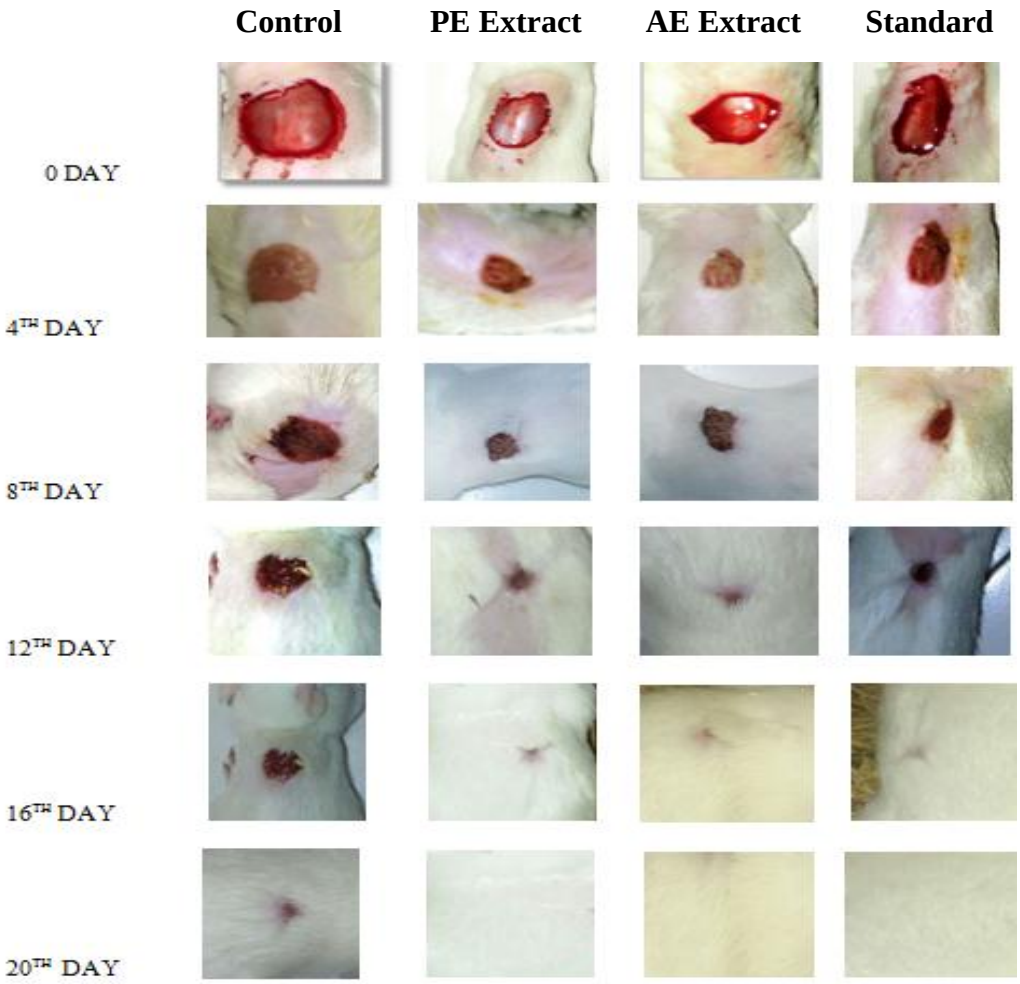

Figure: 02 Photograph Showing Day Wise Contraction of Wound.

Whereas

PE=PETROLEUM ETHER EXTRACT

AE=AQUEOUS EXTRACT

B. Incision Model

Table 10: Incision Model.

GROUPS	BREAKING STRENGTH
Control	125.55 ± 2.01
Petroleum Extract	286.58 ± 3.50
Aqueous Extract	311.45 ± 3.91
Silver sulfadiazine Standard	322.60 ± 4.00

Vales are the Mean ± SEM of six rats / treatment. Followed by ANOVA, Dunnett Multiple Comparisons Test. P <0.0001 Extremely Significant, P <0.01 Moderately Significant, P <0.05 Significant, P >0.05 Not Significant (NS).

In incision wound model study, significant increase was observed in the skin tensile strength of petroleum ether and aqueous extract of *Macroptilium atropurpureum* treated group on 10th post wounding day when compared to control.

The results of petroleum ether and aqueous extract of *Macroptilium atropurpureum* on both excision and incision wound model showed significant acceleration in the process of wound healing by decreasing the surface area of the wound and increasing the tensile strength.

Our present study emphasized the present need of medicinal plants against synthetic drugs on wound healing potentials.

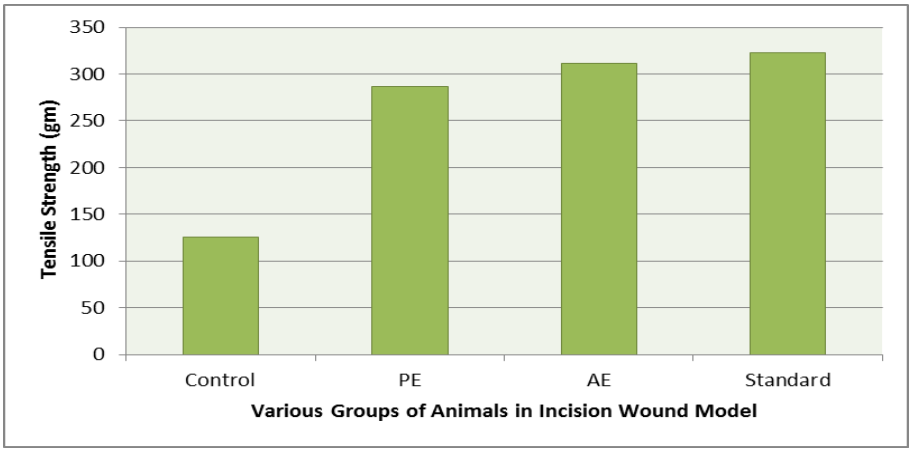

Figure 03: Effect of Petroleum Ether and Aqueous Extract In Incision Wound Model.

CONCLUSION

The wound healing activity of petroleum ether and aqueous extract of *Macroptilium atropurpureum* was studied by using excision and incision wound model and the extract showed significant wound healing activity when compared to control and similar to standard silver sulfadiazine Moreover the extract did not produce any adverse effect and because of this it can be strongly recommend in different wound healing models like burn wound, dead space wound, injury by X-ray radiation and ultraviolet light etc.

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