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Evaluation of Wound Healing Activity of Some Flavonoidal Drugs and Their Polyherbal Formulation on Wound Healing Models

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Abstract: *Tephrosia purpurea commonly known as Sarphank belongs to Fabaceae and its Aerial part used in the study. Asteracantha Longifolia which is commonly known as Kokilaksha, belongs to Acanthaceae Family and its Aerial part used. Trichopus zeylanicus commonly known as Arogyapacha belongs to Dioscoreaceae Family. Flavonoids are low molecular weight bioactive polyphenols which play a vital role in photosynthesising cells Flavonoids are secondary metabolites characterized by flavan nucleus and C6-C8-C6 carbon-skeletonAll These three plants Extract and their polyherbal formulation used for evaluation of wound healing activity by using Excision, Incision, Dead space and burn wound model. polyherbal formulation of all three selected plant material shows very strong wound healing potential as compare to Control and selected drug individually which was evidenced by Incresing wound contraction, Epithelization time, Increasing tensile strength and increasing wet, dry granuloam weight and hydroxiproline estimation in various wound models.*

Key Words: *Aerial part, Flavonoids, polyherbal, Epithelization*

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I. Introduction

The skin is the most important organ of body which covers total area of about 20 square feet. The skin protects human body from microbes and the elements; helps regulate body temperature [1,] A wound is basically a break in the skin. Any Wound are usually caused by cuts and scalps and various symptoms associated with a wound are swelling, stiffness, tenderness, change the color of skin. Skin tightness, scabbing, itching and scar formation tissue[2] Flavonoids are low molecular weight bioactive polyphones which play a important role in photosynthesis. Flavonoids showing various pharmacological activities like anti-inflammatory, antibacterial, antiviral, anti allergic antitumor, treatment of neurodegenerative diseases, wound healing activity of selected plant was performed by using various wound healing models like Incision, Excision, Dead space and burn wound model[3]. All three selected plants possess anti-inflammatory, analgesic, antioxidant, anti microbial and anti rheumatic property[4]

II. Materials and Methods

Plant Material

Aerial parts of *Asteracantha longifolia, Tephrosia purpurea* and leaves of *Trichophus zeylanicus* were collected during the June to August and late September from the surrounding area of Bhopal (MP). The aerial parts and leaves collected were shade dried using tray under controlled temperature at 35 ^{0}C. Plants were then drug converted in powder by the help of pulverizer and powder was store in polythene bags which free from microbes. The plant material was identified from Department of Botany, SAFIA College Bhopal (**Voucher No. 281/bot.1/SAF/12)** and a specimen deposit in the institute. The crude drug was then shade dry and crushed in small pieces for extraction and extractive values. ..

Preparation of the extract

All Extraction were done by using successive solvent extraction method i.e. sox elation. The crude drugs were first converted in to powder and then by using pet ether they are defatted. successive extraction were performed by using different solvent as increasing their polarity .Ethyl alcohol and Methyl alcohol was selected for final extraction as their obtained extractive value for all three plants. The extracts obtained were evaporated till dryness at 40 ^{0}C and stored the dried extract at 4 ^{0}C in the refrigerator until further use.

Formulation preparation

Formulation of Ointment: Ointments was prepared of test drug extracts by using simple ointment base BP. All the doses for the test extract were fixed from the acute toxicity studies two types of drug formulations were prepared from the extracts. Topical application was made in the case of excision, incision and burn wound model whereas, dead space wound model receives oral treatment. For topical administration, 5% w/w of extract ointments was prepared using simple ointment base BP[9] Suspension of test drug extracts was prepared by mixing 2 gm of drug with 20 ml of Tragacanth mucilage. Mucilage was prepared by using formula given below. In which purified water added to make it 100gm[5] (Table 1)

Procedure: Glycerin 18gm, water 75ml were mixed in a tarred vessel and heated. The mixture was heated to boil then add Tragacanth 6gm and Benzoic acid 0.2gm, macerated the mixture occasionally then added enough purified water, stirred actively until uniform consistency and strained forcibly through muslin (Table 2)

Animals

After taking permission for animal studies from Institutional Animals Ethics Committee **(TIT/IAEC/831/P'COLOGY/2015/54**,) rats of wistar strain were procured and rats of either sex weighing 150-200 gm were selected, maintained at 24-28^{0}C, housed independently with free access to food and water. The animals were left free for 48 hr. to maintain them in the animal room conditions. Standard pellet diet was given to them. To perform the experiment, the rats were divided into SIX groups (n=6)**[6]** the results were analyzed by one-way ANOVA and a P-value less than 0.01 was considered significant.

Selection of model

Excision, Incision, Dead space, Burn wound model using Wistar Albino rats was selected for performing the wound healing activity. The various parameter for the evaluation of wound healing activity are rate of wound contraction, time required for full epithelization, tensile strength, granuloma weight and hydroxyproline estimation. These parameters were successful in evaluation because of easy availability of Albino rat and simplicity in handling them

Preparation of Test sample

For evaluation of wound healing activity control group received ointment and suspension base and standard group received standard drug. The test ointment was prepared by mixing the extract with a mixture of ointment base consisting of poly ethylene glycol 400 and 600 in a mortar thoroughly. Treatment was start immediately after the wound creation

Wound healing activity

Excision wound model

In this particular model wistar rats were selected and their hairs were removed from dorsal thoracic region before wounds were created. Diethyl ether used was used as a anesthetic agent. A wound of about 2.5 cm diameter was made which was circular on dorsal thoracic region of rats under aseptic conditions and was observed throughout the study. immediately the areas of the wounds were measured (in mm^2) by placing a transparent polythene graph paper over the wound and then tracing the area of the wound on it (Approx. area 500 mm^2). This was taken as the initial wound area reading. The rats are categorized into six groups (n=6). The animal of group I treated as control and only ointment base applied topically. The animal of group II, III and IV, treated as TEST I, TEST II, TEST III and ointment of *T. purpurea, A.longofolia, T.zeylanicus* applied topically, Group V contain polyherbal formulation and Group VI contain standard drug. Povidone iodine ointment. Samples were applied once daily for 16 days, starting from the day of wounding. The observations of percentage wound closure were made on 4th, 8th, 12th and 16th, post wounding days. The wound area of each animal was measured by using tracing paper method. The percentage of wound contraction was calculated from the days of measurements of wound area[7]

Incision wound model

In this particular wound model wistar rats were selected and they shave by removing hairs at the dorsal thoracic region. diethyl ether used as anesthetic agent. Six centimeter long para vertebral incisions were made through full thickness of skin on either side of vertebral column of the rat. The wounds were closed with interrupted sutures of one centimeter apart. The rats are categorized into six groups (n=6). The animal of group II, III and IV, treated as TEST I, TEST II, TEST III and ointment of *T. purpurea, A.longofolia, T.zeylanicus* applied topically, Group V contain polyherbal formulation and Group VI contain standard drug. Povidone Iodine**.** All the samples were applied once daily for 16 days. The sutures were removed on 8th post wounding day. The tensile strength of wounds was measured on 10th day following continuous water flow technique[7]

Dead space wound model

In this particular wound model a grass pith(2.5 cm x 0.3 cm), is selected and sterilized after that this grass pith implant in a dead space wound model by using light ether anesthesia on either side of the dorsal paravertebral surface of rat. The rats are categorized into six groups (n=6). The animal of group I treated as control and received one ml of 2 % tragacanth solution, orally. The animal of group II, III and IV treated as TEST 1, TEST 2 and TEST 3 and received one ml of oral suspension of *T. purpurea, A.longofolia, T.zeylanicus* applied topically, Group V contain polyherbal formulation in the dose of 500 mg/kg body weight and Group VI contain standard drug. All the samples were given once daily for 10 days, starting from the day of wounding.

On 10^{th} day of wounding granuloma tissue which was formed on grass pith were excised. The weight of wet and dry granulation tissues was measured along with estimation of biochemical parameter like hydroxyproline estimated[7]

Burn wound model

Medium thick burn wound were inflicted on overnight starved animal under light ether anesthesia using a metal rod(1.5cm in diameter) heated to $80\text{-}85^0$ c and exposed for 20sec.after 24 hrs dead tissue were excised using sterile surgical blade. The rats are categorized into six groups (n=6). The animal of group II, III and IV, treated as TEST I, TEST II, TEST III and ointment of *T. purpurea, A.longofolia, T.zeylanicus* applied topically, Group V contain polyherbal formulation and Group VI contain standard drug. Povidone iodine ointment. Samples were applied once daily for 16 days, starting from the day of wounding. The observations of percentage wound closure were made on 4th, 8th, 12th and 16th, post wounding days. It was basically actual days needed for the complete recovery of burn wound surface without leaving a raw wound behind. Rate of wound contraction was measured as percentage reduction in wound size at every 4 days interval. Granulation tissue was collected at regular interval of 03days and preserved in 10% buffered formalin .granulation tissue were collected on 11^{th} day[8]

Hydroxiproline Estimation

The granuloma tissue was collected for the estimation of hydroxyproline. Calculated quantities of tissue sample were immersed in 2 mL of 6 M-HCl, and the tubes were sealed without evacuation. Hydrolysis was done for 3 hr at 105º C. After hydrolysis of tissue, 50 µl of sample was taken and 0.4 mL isopropanol was mixed to it. Then, 0.2 mL of solution A was mixed and incubated at room temperature for 5 min. After incubation, 2.5 mL of solution B was mixed and incubated at 58º C for 25 min. Then this mixture was cooled in tap water and absorbance was taken at 558 nm within 30 min. The quantity of hydroxyproline was calculated with the help of standard curve

Statistical analysis

Data obtained from animal experiments were expressed as the mean standard error (SEM). Statistical difference between the treated and control groups were evaluated by ANOVA

III. Result

For the evaluation of wound healing activity of all three plant extract, six group were prepared. for Incision, Excision and Burn wound model which divided in control, Test I, Test II, Test III, polyherbal formulation, standard drug and for Dead space wound model six group were prepared which divided in control, Test I, Test II, Test III, polyherbal formulation, standard drug. Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on percentage wound contraction in Excision wound model on Initial, 4th, 8th, 12th, 16th day interval which is shown in **Table No 3**.It has been seen that faster wound healing took place in case of animals treated with Polyherbal Formulation which is 18 days and Test drug I took 19 days, Test drug II took 21 days, Test drug III took 22 days for complete wound healing. The least rate of wound healing was seen in control group which received no treatment and fastest rate of wound healing was seen in standard drug group where animals received standard drug which is Povidone iodine .Epithelization period (days) in excision wound healing model also shown in **Graph No 1**.

Measurement Tensile strength in Incision wound model

The tensile strength was calculated in incision wound model. On 10^{th} day the rats were again anesthetized and each rat is placed on a stack of paper towel on the middle of the board Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on Tensile strength in Incision wound model which is shown in **Table No4** which indicate that animals treated with polyherabl formulation seen highest Tensile strength after that Test I, Test II. Test III treated animal's shows their Tensile Strength. The least tensile strength seen in control where animal not received any treatment and highest Tensile Strength seen in standard drug group where animals received standard drug which shown in **Graph No 2.**

Tensile strength was measured by the Tensiometer

Wet Granuloma, Dry Granuloma Weight and Hydroxiproiline Measurement for Dead space wound model: Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on Dry, Wet granuloma weight and Hydroxyproline estimation in Dead space wound model which is shown in **Table No 5** which indicate that animals received Polyherbal formulation shown highest Wet granuloma weight (mg/100gm,Dry granuloma weight (mg/100gm), Hydroxiproline (mg/gm of tissue) Estimation after that Test drug I, Test drug II, test Drug III, treated animals shown their Wet granuloma weight (mg/100gm,Dry granuloma weight (mg/100gm), Hydroxiproline (mg/gm of tissue) Estimation, control group where animals not received any treatment shown least Wet, Dry granuloma weight and Hydroxiproline estimation and animals received standard drug (Povidone iodine) shown highest Wet, Dry granuloma weight and Hydroxiproline estimation. **Graph No 3, 4, 5** indicate effect of extract on wet granuloma weight, dry granuloma weight and on Hydroxiproline estimation

Wound Contraction and Epithelization time in Burn wound model

Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on percentage wound contraction in Burn wound model on Initial,4^{th},8^{th},12^{th},16^{th} day interval which is shown in **Table No6** which indicate that faster wound contraction took place in case of animals treated with Polyherbal Formulation which is 18 days and Test drug I took 19 days, Test drug II took 21 days, Test drug III took 22 days for complete wound healing. The least rate of wound healing was seen in control group which received no treatment and took 24 days for complete healing and fastest rate of wound healing was seen in standard drug group where animals received standard drug which is silve sulphadiazain which took 17 day for complete healing**.** percentage wound contraction in burn wound model shows in **Graph No 6.7**.

IV. Discussion

The skin is a very important organ of human body which covers approx 20 square feet of human body. The skin provides defensive mechanism for body against various microbes, infection and elements. It regulates body temperature. wound is break in the skin Wound are normally caused by cuts or scalps, symptoms at wound or injury include swelling, stiffness, tenderness, discoloration skin tightness, scabbing, itching and scar formation, two types of tissue injury

Wound healing involve with various complications like Infection, Implantation, Pigmentation, Deficient scar formation, Incisional hernia, Hypertrophied scars, keloid formation, Excessive contraction, Neoplasia. Some Local factors are affecting wound healing like Infection, Poor blood supply, foreign bodies, Movement, Exposure to ionizing radiation, Exposure to ultraviolet light, Type, size and location of injury. Some systemic factors like Age, Nutrition, Systemic infection, Administration of glucocorticoids, uncontrolled diabetes also affect wound healing process. Flavonoids show various biological activities. These includes: anti-inflammatory, antibacterial, antiviral, anti allergic, anti tumor, treatment of neurodegenerative diseases, vasodilatory action. Flavonoids are inhibiting lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation these are also reported to inhibit variety of enzymes like hydrolases, hyalouronidase, alkaline phosphatise, arylsulphatase, CAMP phosphodiesterase, lipase, α-glucosidase, kinase[9] Flavonoids Enhances wound healing activity which proves by various researches.

So the present investigation was aimed for the evaluation of wound healing activity of some flavonoidal drugs and their polyherbal formulation. All selected plants which contain flavonoids as a secondary metabolites Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on percentage wound contraction in Excision wound model on Initial, 4th, 8th, 12th, 16th day interval and It has been seen that highest wound healing took place in case of animals treated with Polyherbal Formulation which is 18 days and Test drug I took 19 days, Test drug II took 21 days, Test drug III took 22 days for complete wound healing. The slow rate of wound healing was seen in control group which received no treatment and fastest rate of wound healing was seen in standard drug group where animals received standard drug which is Povidone iodine

Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on Tensile strength in Incision wound model which indicate that animals treated with polyherabl formulation shown highest Tensile strength after that Test I, Test II. Test III treated animals shown their Tensile Strength. The minimum tensile strength seen in control where animal not received any treatment and maximum Tensile Strength seen in standard drug group where animals received standard drug**.** Measurement of Tensile strength was done by the Tensiometer. Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on Dry, Wet granuloma weight and Hydroxyproline estimation in Dead space wound model which indicate that animals received Polyherbal formulation shown highest Wet granuloma weight (mg/100gm,Dry granuloma weight (mg/100gm), Hydroxiproline (mg/gm of tissue)

Estimation after that Test drug I, Test drug II, test Drug III, treated animals shown their Wet granuloma weight (mg/100gm,Dry granuloma weight (mg/100gm), Hydroxiproline (mg/gm of tissue) Estimation, control group where animals not received any treatment shown least Wet, Dry granuloma weight and Hydroxiproline estimation and animals received standard drug (Povidone iodine) shown highest Wet, Dry granuloma weight and Hydroxiproline estimation.

Effect of control, Test I, Test II, Test III, polyherbal formulation, standard drug (Povidone Iodine) was observed on percentage wound contraction in Burn wound model on Initial, 4th, 8th, 12th, 16th day interval which indicate that highest wound contraction took place in case of animals treated with Polyherbal Formulation which is 18 days and Test drug I took 19 days, Test drug II took 21 days, Test drug III took 22 days for complete wound healing. The minimum rate of wound healing was seen in control group which received no treatment and took 24 days for complete healing and fastest rate of wound healing was seen in standard drug group where animals received standard drug which is silver sulphadiazain which took 17 day for complete healing. So of result obtained from the present investigation, it was well understood that polyherbal formulation of all three selected plant material shows very strong wound healing potential. It was also found that various formulations containing Flavonoids shows strong wound healing activity.

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Table1: Composition of Simple ointment base for control Group (100 gm)

S.No.	Constituents	Quantity
1.	Polyethylene Glycol 400	40 gm
2.	Polyethylene Glycol 600	60 gm

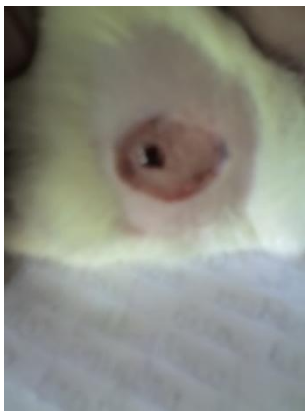
Table2: Composition of Tragacanth mucilage (100 gm)

S.No.	Constituents	Quantity
1.	Glycerin	18 gm
2.	Purified water	75 ml
3.	Tragacanth	2 gm
4.	Benzoic acid	0.2 gm

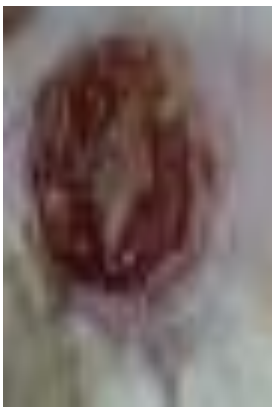
	Area of wound closure (sq mm ± S.E.M)					
Groups	**Initial**	**4th day**	**8th day**	**12th day**	**16th day**	**Epithelization period (Days)**
I (CONTROL)	10.82±0.68	18.82±0.68	38.12±1.80	48.21±1.80	68.69±2.60	24
II (TEST-I)	14.14±0.98*	35.14±0.54*	52.14±0.85*	75.12±0.79*	93.99±0.68*	19
III (TEST-II)	11.24±1,23*	24.22±1.42*	48.94±1.24*	66.92±0.13*	76.12±1.93*	21
IV (TEST-III)	13.26±0.29*	23.12±1.29*	47.94±1.10*	62.12±1.28*	74.19±0.95*	22
V (Polyherbal)	12.62±1.23*	39.24±0.64*	55.12±0.95*	79.98±1.08*	92.12±0175*	18
VI (Standard)	15.26±1.25*	42.24±1.07*	65.34±1.70*	90.12±1.08*	100±0.75*	16

Table 3: Mean Percentage wound contraction in Excision wound model

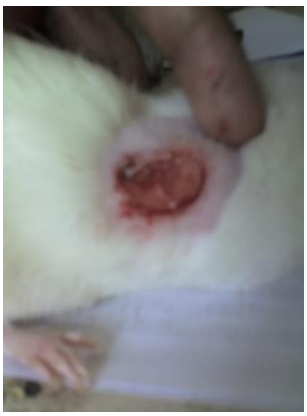
Initial wound area approx. 500 sq mm,
≈ n = 6 animals in each groups.,
≠ Result expressed as Mean Area ± S.E.M. (Standard Error Mean),
* P≤ 0.01 indicates significant when compared with control/ ,
Ψ Figure in parenthesis indicate percent wound contraction



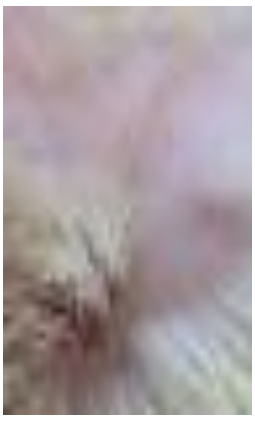
Day 01(Group I)



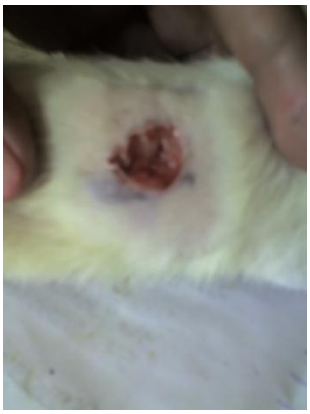
Group I (16th day)



Day 01(Group II)



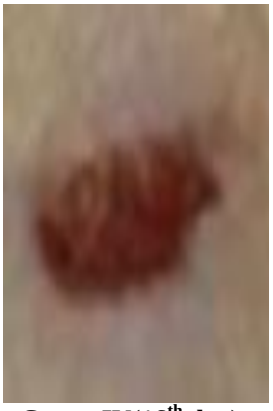
Group II (16th day)



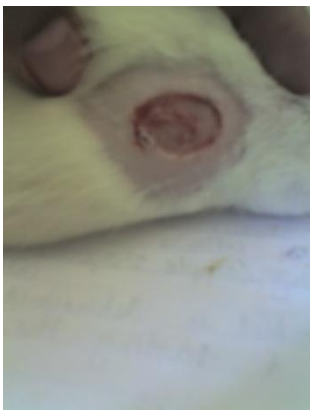
Day 01(Group III)

Group III (16th day)

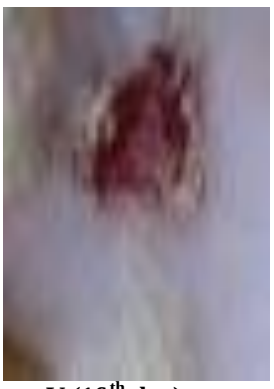
Day 01(Group IV)



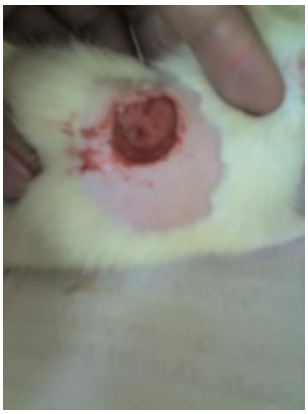
Group IV(16th day)



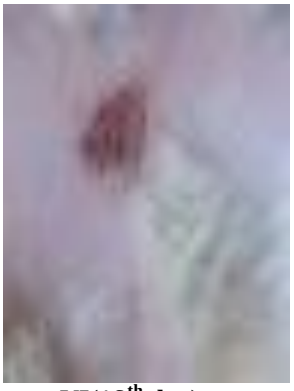
Day 01(Group V)



Group V (16th day)

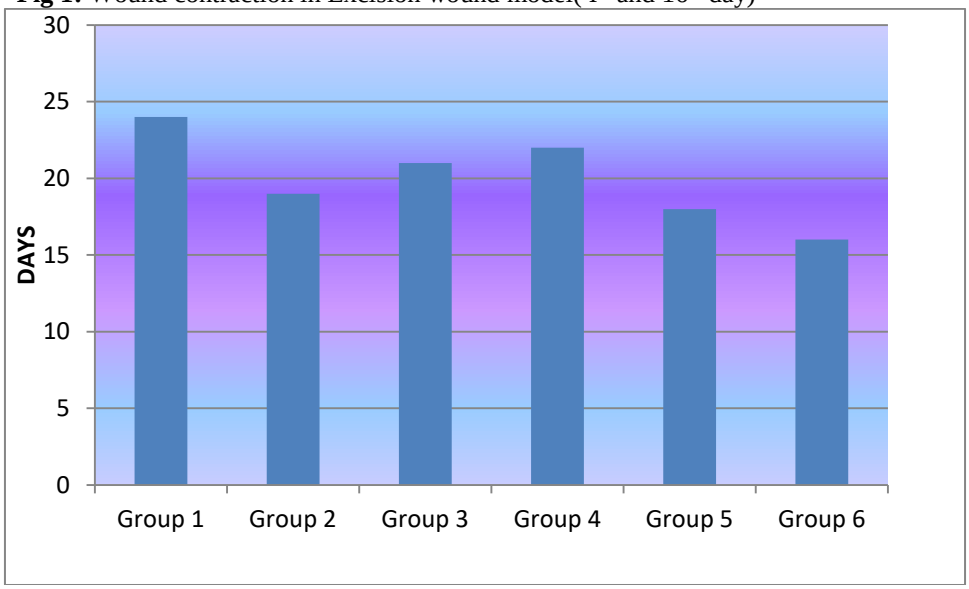


Day 01(Group VI)



Group VI(16th day)

Fig 1: Wound contraction in Excision wound model(Ist and 16th day)

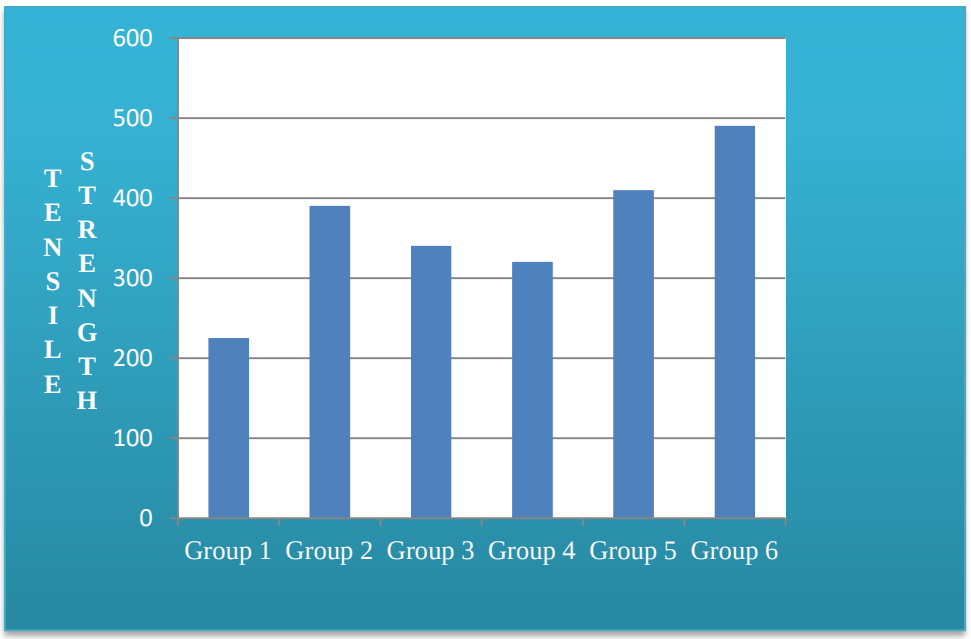


Graph1: Epithelization period (days) in Excision wound healing model

Groups	Tensile strength (in Grams)
Control	225.16±3.51
Test I	390.29±2.56
Test II	340.39±2.40
TEST III	320.45±2.71
POLYHERBAL	410±2.71
STANDARD	490.50±2.71

Table4:Tensile Strength in incision wound model

≠ Result expressed as Mean Area ± S.E.M. (Standard Error Mean)
* P≤ 0.01 indicates significant when compared with control

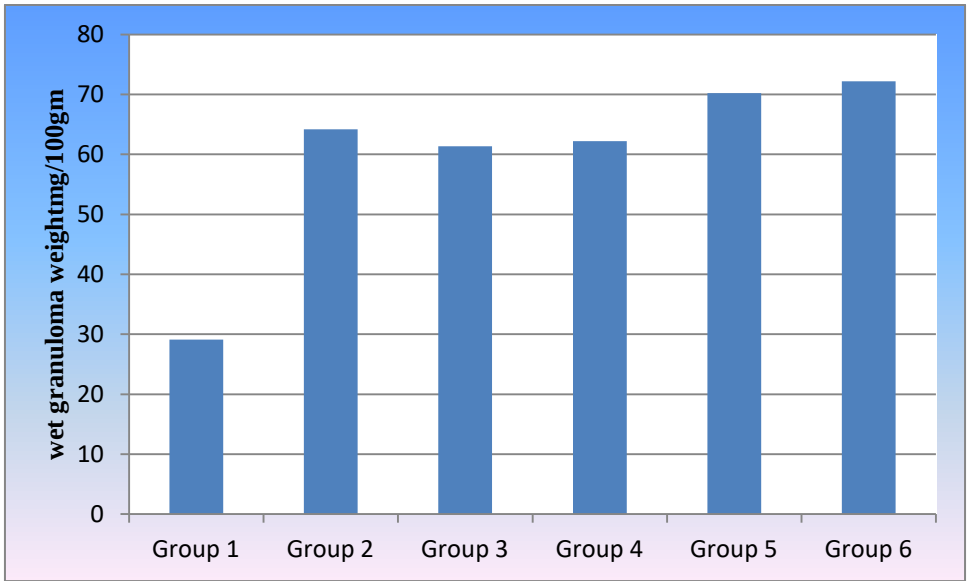


Graph2: Tensile strength (gm) in incision wound healing model

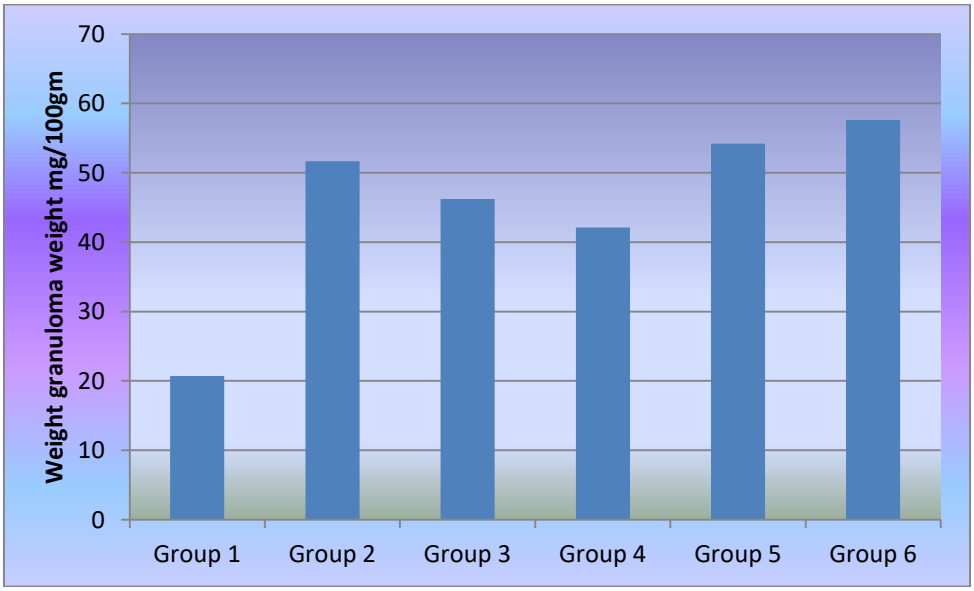
Group(n)	Wet granuloma weight(mg/100gm	Dry granuloma weight(mg/100gm)	Hydroxiproline(mg/gm of tissue)
Control	29.12.±1.20	20.71±3.20	34.70±4.92
Test I	64.19±2.12	51.66±1.23	55.12±3.23
Test II	61.36±0.91	46.22±0.82	40.20±1..62
Test III	62.19±0.82	42.12±1.93	39.21±2.12
Polyherbal	70.23±2.22	54.19±1.22	59.22±1.43
Standard	72.19±1.66	57.62±2.12	69.35±3.22

Table 5: Dry, Wet granuloma weight and Hydroxyproline estimation in Dead space wound healing model

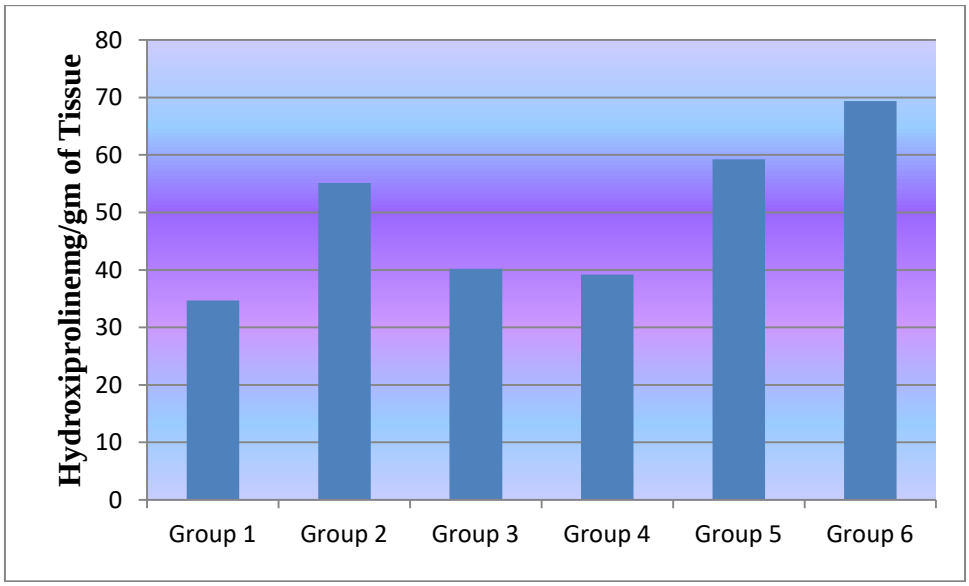
≠ Result expressed as Mean Area ± S.E.M. (Standard Error Mean)
* P≤ 0.01 indicates significant when compared with control



Graph3: Effect of extract on wet granuloma weight



Graph 4: Effect of extract on dry granuloma weight

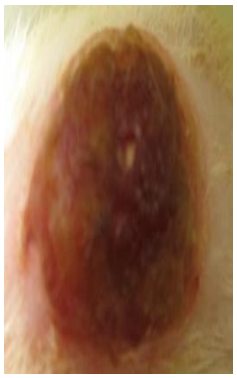


Graph5: Effect of extract on Hydroxiproline estimation

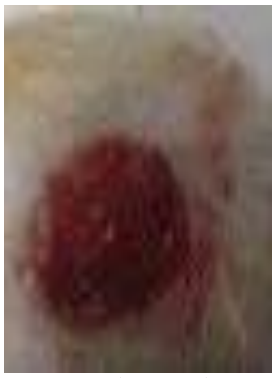
	Area of wound closure (sq mm ± S.E.M)					
Groups	**Initial**	**4th day**	**8th day**	**12th day**	**16th day**	**Epithelization period (Days)**
I (CONTROL)	5.92±0.72	20.19±0.92	40.22±1.80	60.11±1.21	70.19±1.20	24
II (TEST-I)	13.11±0.92	38.92±1.21	55.12±0.92	74.12±0.86	84.99±0.72	19
III (TEST-II)	10.14±1.22	30.11±1.22	42.94±1.11	60.12±0.66	69.12±1.29	21
IV (TEST-III)	12.11±1.92	30.22±2.12	40.12±2.32	55.12±0.98	65.11±0.98	22
V (Polyherbal)	13.12±2.12	39.99±0.98	60.11±0.52	76.12±1.92	87.11±1.13	18
VI (silvesulphadiazain)	12.13±2.12	40.24±1.24	66.12±1.29	91.92±0.92	99.19±0.71	17

Table 6: Percentage wound contraction in Burn wound model

Initial wound area approx. 500 sq mm
≈ n = 6 animals in each groups.
≠ Result expressed as Mean Area ± S.E.M. (Standard Error Mean)
* P≤ 0.01 indicates significant when compared with control.



Day 01(Group I)



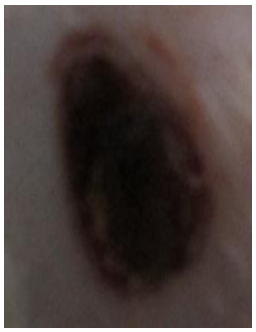
Day16 (Group I)

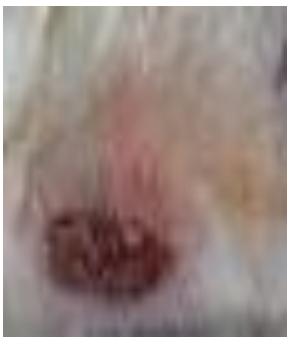

Day01 (Group II)

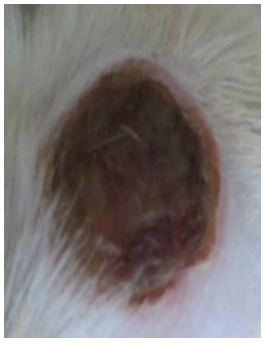

Day16 (Group II)

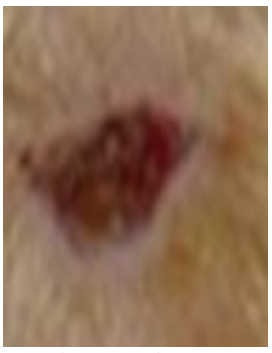

Day01 (Group III)

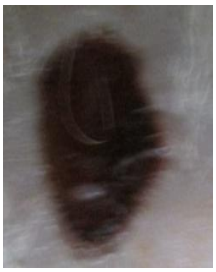

Day16 (Group III)


Day01 (Group IV)

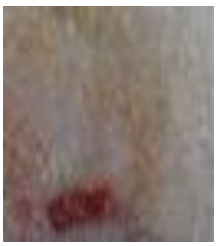

Day16 (Group IV)


Day01 (Group V)


Day16 (Group V)

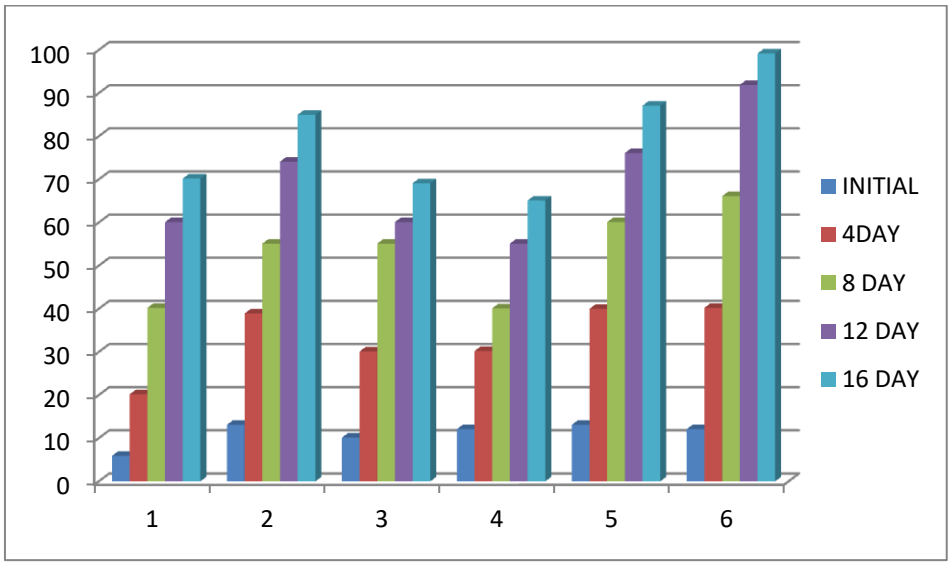


Day01 (Group VI)

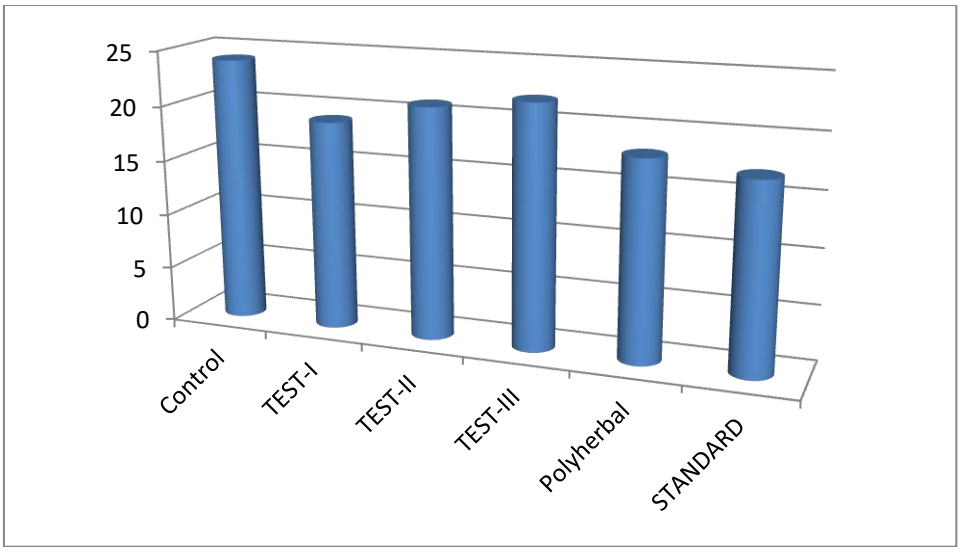


Day16 (Group VI)

Fig 2: Wound contraction in Burn wound model (Ist and 16th day)



Graph 06: percentage wound contraction in burn wound model



Graph 07: Epithelisation period(days) in burn wound model

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