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EVALUATION OF WOUND HEALING ACTIVITY OF *TABERNAEMONTANA DIVARICATA* LEAVES IN EXPERIMENTAL RATS

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ABSTRACT

Healthy wistar rats of either sex were selected and were divided according to standard protocol. They were administered single dose of alcoholic extract of *Tabernaemontana divaricata* orally. The wound healing effect of alcoholic extract of *Tabernaemontana divaricata* and its effect in Excision and Incision wound healing was studies in wistar rats. There wound models viz.incision and excision wounds were used in this study. The parameters studied were breaking strength in case of incision wounds. Epithelization and wound contraction in case of excision wound. The ethanolic treated group showed a significant ($p<0.001$) reduction in the wound breaking strength when compared to control group in incision type of wound model co administration of *Tabernaemontana divaricata* had significantly ($P<0.001$) increased the breaking strength of treated group. In excision wound model, the percentage of the wound contraction was significantly ($p<0.05$) increased by *Tabernaemontana divaricata* only on 16th day and also it reversed the Excision suppressed wound contraction on the 16th day. *Tabernaemontana divaricata* significantly ($P<0.001$) reduced for epithelization and reserved the epithelazation delaying effect of significantly ($P<0.001$).

Key Words:- *Tabernaemontana divaricata*, Excision and Incision Wound, Wound contraction, Wound breaking strength.

INTRODUCTION

Wound is made by physical, chemical, thermal, microbic or medical specialty offense to the tissues. The method of healing of wound consists of incorporated cellular & amp; organic chemistry procedures foremost to restoration of structural & amp; purposeful responsibility with regain of strength in scraped tissues. Wounds are typically termed as physical cuts that end in gap/breaking of the skin. The process of wound healing may be a series of freelance and overlapping stages.

Alkaloids and tannins are already reported to promote the wound healing through several cellular mechanisms, chelating of the free radicals and reactive species of oxygen, promoting contraction of the wound and increasing the formation of capillary vessels and fibroblasts. Flavonoids are strong scavengers of reactive oxygen species provide enabling support to the healing process initially by the moderation of superoxide anions and later by enhancing the expression of vascular endothelial growth factor (VEGF), thereby enhancing

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angiogenesis and flow of blood as the wound repair process advances

Tabernae montanan divaricata is a multi-branch evergreen shrub reaching 3-4 meters high, perhaps higher if let be in good conditions. It contains at most 66 indole alkaloids, constituents like enzymes, non-alkaloidal, flavonoids, Phenolic acids, hydrocarbons, phenyl propanoids, terpenoids and steroids

Alkaloids, flavonoids & terpenoids are the main secondary metabolites that exhibit many physiological and pharmacological properties on living cells. T. divaricata flowers contain [3, 4, 14, 19 – tetrahydro-Olivacine, 11-methoxy-[N-methyldihydro - Pericyclivine], 19 - epivoa cangine, Apparicine, Isovoacangine, Isovoacristine, Tabernae montanine. It is traditionally used by people in many parts of the world to treat various disorders like abdominal tumours, arthralgia, asthma, diarrhoea, epilepsy, fractures, eye infections, fever, inflammation, headache, mania, leprosy, oedema, skin disorder, piles, rabies, rheumatic pain, paralysis, ulceration & vomiting. It is also used as anthelmintic, anti-hypertensive, aphrodisiac, diuretic, emmenagogue, hair growth purgative, promoter, and remedy against poisons and tonic for brain, liver & spleen.

MATERIALS AND METHODS

Collection and authentication of plant

The leaves of *Tabernae montana divaricate* were collected locally in the month of February from Govindpura, Bhopal, Madhya Pradesh, India. Herbarium file of plant part was prepared and authenticated by Dr. Zia Ul Hasan (Professor, Department of Botany), Safia College Bhopal, and Madhya Pradesh, India.

Drying and size reduction of plant material

The leaves of Tabernae Montana divaricata were dried under the shade in the laboratory. It was pulverized to moderately coarse powder. The coarse powder of leaves was passed through sieve No. 16 to maintain uniformity and stored in cool and dry place for further study.

Extraction of plant material

Extraction of *Tabernae montana divaricate* leaves of was done by Soxhlet extraction method. Soxhlet apparatus was used for the solvent extraction and ethanol was selected as a solvent for extraction while petroleum ether was used for defatting of the waxy materials. 200 g of coarsely dried powder of leaves was first defatted separately with petroleum ether (50-60 ºC) for 48 hours to remove fatty materials and then extracted with ethanol using soxhlet apparatus for 72 hr at 60-80 ºC. After complete extraction, extract was collected and dried at room temperature for 5 days, the percentage yield of extract was calculated and the dried crude extract was stored in air tight container at 2-8ºC for further study.

Screening of powder (Physiochemical Analysis)

Physiochemical screening of powdered leaves was performed like loss on drying, total ash value, acid insoluble ash value, water soluble ash value and foaming index by the standard reported methods.

Phytochemical analysis

Tests for Alkaloids

Dragendorff's test

In the pipette 1 ml of extract and 1 ml of Dragendorff's reagent (potassium bismuth iodide solution) was added. An orange-red precipitate was appeared which indicated the presence of alkaloids.

Mayer's test

To 5ml of Benedict's reagent, 1ml of extract solution was added and boiled for 2 minutes and cooled. Formation of red precipitate indicated the presence of sugars.

Wagner's test

In the pipette 1 ml of extract and 1 ml of Wagner's reagent (Iodine potassium iodide solution) were added. Reddish brown precipitate indicated the presence of alkaloids.

Tests for Glycosides

Killer-Killani test

2ml of extract was dissolved in Glacial acetic acid to which one drop of 5% FeCl3 and conc. H2SO4 was then added. Reddish brown color was seen at the junction of the two liquid layers and upper appeared bluish green which indicated the presence of glycosides.

Baljet's test

To 1ml of the test extract, 1ml of sodium picrate solution was added and the yellow to orange color revealed the presence of glycoside.

Foam test

0.5gm extract was vigorously shaken with water, formation of a layer of foam indicated the presence of glycosides.

Tests for Carbohydrate

Molisch's test

A small fraction from the respective extract was taken in ethanol and a few drops of 20% w/v solution of α-

napthol in ethanol (90%) were added to it. After shaking well, about 1 ml of concentrated sulphuric acid was allowed to flow carefully by the side of the test tube. A reddish violet ring at the junction of the two layers indicated the presence of carbohydrates.

Fehling's test

Extract was heated with dil. HCL, neutralized with NaOH and Fehling's solution A & B was added to it. Brick red precipitate was formed which indicated the presence of carbohydrates.

Tests for Steroids

Salkowski test

The extract was dissolved in chloroform and equal volume of conc. H_2SO4 was added. Formation of bluish red to cherry color in chloroform layer and green fluorescence in the acid layer represented the steroidal components in the tested extract.

Liebermann-Burchard test

A small portion from each extract was taken with about 1 ml of acetic anhydride and dissolved by warming. The contents were cooled and a few drops of concentrated sulphuric acid were added in each case by the sides of the test tube. Appearance of blue colour indicated the presence of sterols.

Test for Proteins

Biuret test

1ml of 40% sodium hydroxide solution and 2 drops of 1% CuSO4 solution were added till a blue color was produced, and then it was added to 1ml of the extract. Formation of pinkish or purple violet color indicated the presence of proteins.

Tests for Saponins

Foam test

A little fraction from extract was taken with about 2 ml of distilled water. A small quantity of sodium carbonate was then added and shaken. The characteristic foam formation indicated the presence of Saponins. Aqueous and alcoholic extract were tested directly.

Test for Phenolic Compounds and Tannins

i) Little quantity of test extract was mixed with basic lead acetate solution. Formation of white precipitates indicated the presence of tannins.

ii) To 1 ml of the extract, ferric chloride solution was added, formation of a dark blue or greenish black colour product showed the presence of tannins.

iii) Little quantity of test extract was treated with potassium ferric cyanide and ammonia solution. The presence of tannins was indicated by a deep red colour.

iv) To the test extract, strong Potassium dichromate solution was added; a yellow colour precipitate indicated the presence of tannins and phenolics.

Tests for Flavonoids

Shinoda test

To the test solution, few magnesium turnings and concentrated hydrochloric acid was added drop wise, pink scarlet, crimson red or occasionally green to blue colour was seen after few minutes.

Alkaline reagent test

To the test solution, few drops of sodium hydroxide solution was added, intense yellow color was formed which turned colorless on addition of few drops of dilute acid which indicated the presence of Flavonoids.

Lead acetate Test

Extract was treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicated the presence of flavonoids.

Formulation of ointment of ethanolic extract of *Tabernae montana divaricate*

An ointment formulation was prepared for the proposed study i.e. wound healing activity of ethanolic extract *Tabernae montana divaricate* leaves. An ointment of 5% w/w was prepared of plant extract. A defined quantity of carbapol was soaked in distilled water over night before the preparation of ointment to make the ointment base. On next day given quantity of extract & carbapol base was triturated in mortor pestle to make the ointment until extract congeal completely in ointment base at last methyl paraben was added as preservative and triturated for complete mixing. Formulated ointment was observed and evaluated for physiochemical characteristics & stored in wide mouth glass bottle for further In-Vivo study.

Component	***Quantity for 5% w/w ointment***
Test compound	5000 mg
Carbapol gel base	98 gm
Methyl paraben	0.004%
Net content	100 gm

***IN-VIVO* WOUND HEALING ACTIVITY**

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.

Animal care and handling

The animals were carried for experiment from the authorized animal house of Sapience Bioanalytical Research Lab, Bhopal (M. P.). All Wistar albino rats were healthy and 120 gm to 150 gm of body weight. The animals were acclimatized to the standard laboratory conditions in cross ventilated animal house at temperature 25±2°C , relative humidity 44 –56% and light and dark cycles of 12:12 hours, fed with standard pallet diet and water *ad libitum* during experiment. The experiment was approved by the institutional ethics committee of Sapience Bioanalytical Research Lab, Bhopal (M.P.) (Protocol approval no.1413/PO/E/S/11/CPCSEA).

EXPERIMENTAL DESIGN

Excision wound model

In the experiment, a total of 24 rats were used. The rats were divided into 4 groupscomprising of 6 animals in each group as follows:

Group I: Left untreated and considered as control.

Group II: Served as standard and treated with 5% w/w Bitadine ointment daily, OD.

Group III: Treated with 5% w/w ointment of extract once daily.

Group IV: Treated with 5% w/w ointment of extract twice daily.

Procedure

- Hair was removed from the posterior sides of rats using hair remover cream. An area about of 10 mm diameter was measured with sterile scale and this area was marked with a marker pen.
- The rats were anesthetized with ketamine (50mg/kg i.p.). After 15 minutes of anesthesia, the marked area of skin was excised with the help of surgical blade No. 18 and forceps. The skin was removed after creating the wound.
- Formulated ointment and marketed ointment (Betadine) were applied, starting from the day of the operation, till complete epithelialization time. The parameters studied were percent wound closure and epithelialization time.
- The wound was measured using transparency paper, a marker, scale and area was calculated.
- The period of epithelialization was calculated as the number of days required for falling of the dead tissue remnants of the wound without any residual raw wound (with slight modification).
- The percentage wound contraction was determined using the following formula:

$$\textit{Percentage of wound contraction} = \frac{\textit{Initial wound size} - \textit{Specific day wound size}}{} \times 100$$

Incision wound model

- The animals were grouped and treated as mentioned in excision model. The rats were anesthetized with ketamine (50mg/kg i.p.).
- Paravertebral incision of 6 cm length was made through the entire thickness of the skin, on either side of the vertebral column with the help of a sharp scalpel. After complete haemostasis, the wound were closed by means of interrupted sutures placed at approximately 1 cm apart.
- For stitching, stitched with black silk surgical thread (No. 000) and a curved needle (No. 11) were used. Animal were treated daily, as mentioned above under excision wound model from 0th day to 9th post-wounding day.
- One day before performing the experiment (measurement of breaking strength) the sutures were removed from the stitched wounds of rats after recovery. The wound breaking strength was measured on 10th post wounding day.

STATISTICAL ANALYSIS

All the values are expressed as mean standard error of mean (S.E.M.) and analyzed by one way ANOVA and posthoc Tukey multiple comparison test by employing statistical software, Graph Pad InStat 3. Differences between groups were considered significant at $P < 0.05$ levels.

RESULTS

Histopathology of Incision wound model

- Showing hyperkeratosis, dermal fibrosis, focal mononuclear cell infiltration (MNC) in the dermis and peri-follicular areas of Control group.
- Showing no abnormality detection (NAD) in treated standard drug Betadine.
- Showing hyperkeratosis dermal fibrosis and increase in dermal collagen, granulomas around the hair follicles.
- Showing hyper keratosis otherwise no abnormality detection (NAD)
- Showing hyperplasia and hyperkeratosis in epidermis, fibrosis, mild mononuclear cell infiltration, (MNC) and vascular proliferation in dermis in Control rats.

- Showing hyperplasia with crust formation, dermal fibrosis, with mild mononuclear infiltration (MNC) in treated with standard Beta dine drug.

- Showing focal dermal fibrosis, otherwise no abnormality detection (NAD).
- Showing dermal fibrosis and scantly mononuclear cell infiltration (MNC) otherwise no abnormality detection (NAD) treated with *Tabernae montana*.

Table 1. Physiochemical analysis of powder of *Tabernae Montana divaricata* leaves

S.No.	Parameters	Observation (%)
1	Loss on drying	0.6
2	Total ash value	7
3	Acid insoluble ash value	2.6
4	Water soluble ash value	1.25
5	Foaming index	22 (ml)

Table 2. Phytochemical screening of ethanolic extract of *Tabernae Montana divaricata leaves.*

S.No.	Chemical test	Ethanolic extract
1	Carbohydrates	+
2	Tannins	+
3	Alkaloids	+
4	Flavonoids	+
5	Glycosides	+
6	Steroids and sterol	-
7	Proteins and amino acids	-

Table 3. Effect of extract of Leave of *Tabernae montana divaricate* on excision wound model

Groups	% Wound contraction				Epithelisation period (Days)
	4^{th} day	8^{th} day	12^{th} day	16^{th} day	
Control	9.75±1.44	24.42±1.49	44.42±1.56	62.55±1.12	22.66±0.44
Standard	19.33±0.23 a***	41.66±0.45 a***	71.0±1.43 a***	83.0±2.76 a***	18.66±0.54 a***
Extract 5% OD	16.33±1.03 a***	32.29±0.38 a**,b**	55.33±2.54 a***,b***	70.33±1.66a**,b**	20.0±0.68a*
Extract 5% BID	18.36±1.22 a***	39.05±0.58a***	64.66±1.32 a***,b*	78.0±2.27 a***	19.0±0.62 a***

Values are mean ± SEM from a group of four animals. *p<0.05, **p<0.01 and***p<0.001

a- Significance difference in compare to untreated group

b- Significance difference in compare to standard treated group.

Table 4. Effect of extract of Leave of *Tabernae montana divaricata* on Incision wound model.

Groups	Treatment	Wound breaking strength (g)
I	Control	233.66±4.66
II	Standard	277.0±4.6a***
III	Extract 5%, OD	255.0±5.9a***, b***
IV	Extract 5%, BID	265.3±4.43a***,b*

Values are mean ± SEM from a group of four animals*p<0.05, **p<0.01, ***p<0.001

a- Significance difference in compare to untreated group

b- Significance difference in compare to standard treated group

Initial wound size

Fig 1. Marked area of skin for excision wound

Fig 2. Creation of wound on rat skin

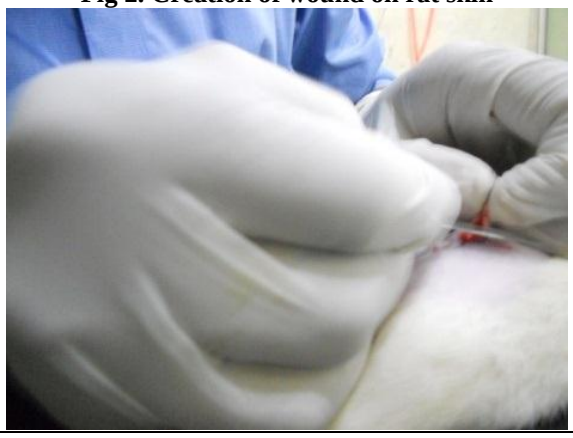


Fig 3. Measurement of wound breaking strength

Fig 4. Showing hyperkeratosis, dermal fibrosis, focal Mono nuclear cell infiltration (MNC) in the dermis and perifollicular areas of incision wound model of control rats.

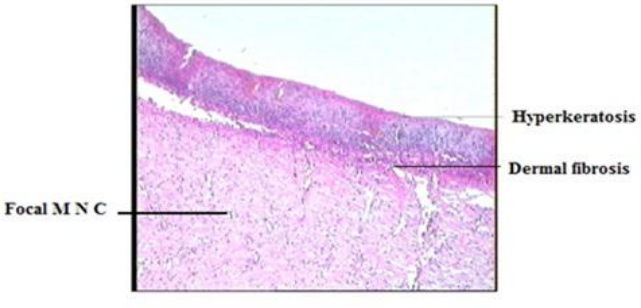


Fig 5. Showing no abnormality detection (NAD) in incision wound model of rats treated with standard drug (Betadine).

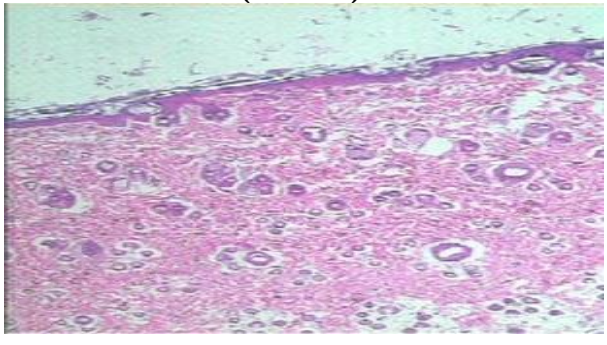


Fig 6. Showing hyperkeratosis, dermal fibrosis and increase in dermal collagen, granulomas around the hair follicles in incision wound model of rats treated with *Tabernae montana divericata.*

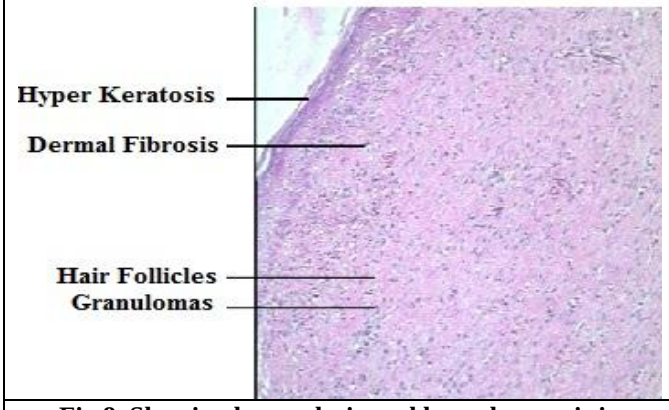


Fig 7. Showing hyperkeratosis, otherwise no abnormality detection (NAD) in incision wound model of rats treated with *Tabernae montana divericata.*

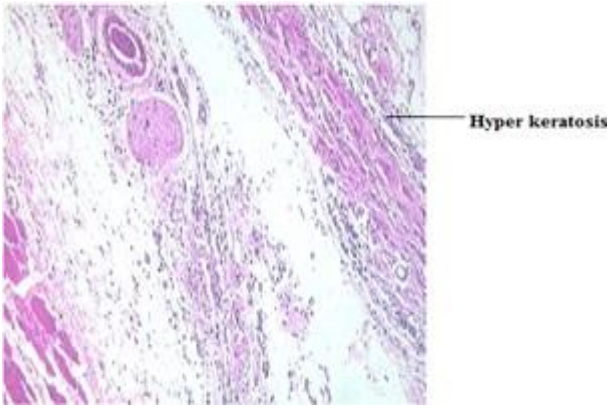


Fig 8. Showing hyperplasia and hyperkeratosis in epidermis, fibrosis, mild mononuclear cell infiltration (MNC) and vascular proliferation in dermis in excision wound model of control rats

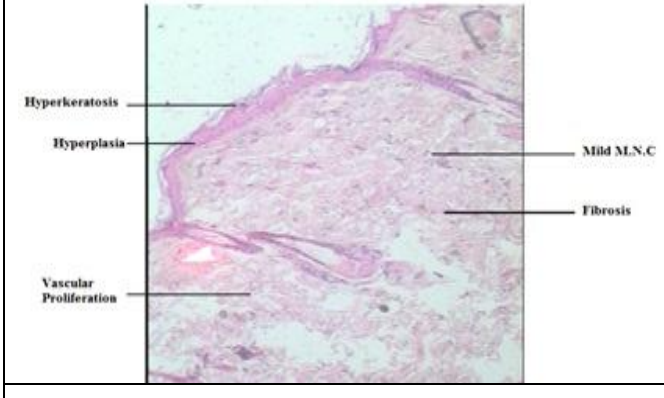


Fig 9. Showing epidermal hyperplasia with crust formation Dermal fibrosis with mild mononuclear cell infiltration (MNC) in excision wound model of rats treated with standard Betadine drug.

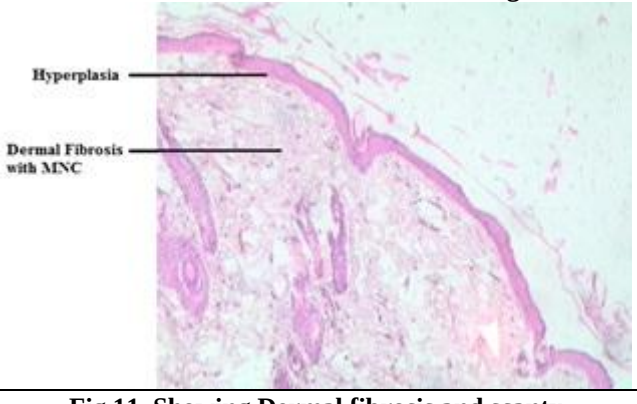


Fig 10. Showing focal dermal fibrosis. Otherwise no abnormality detection (NAD) in excision wound model of rats treated with *Tabernae montana divericata.*

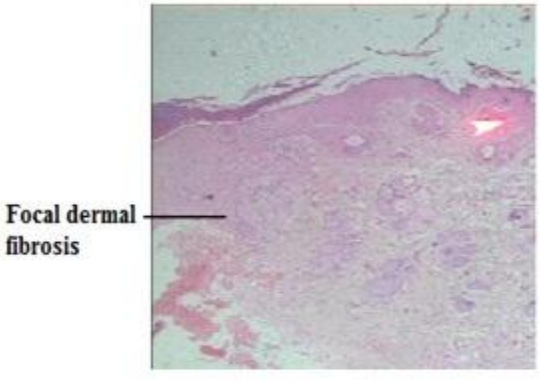
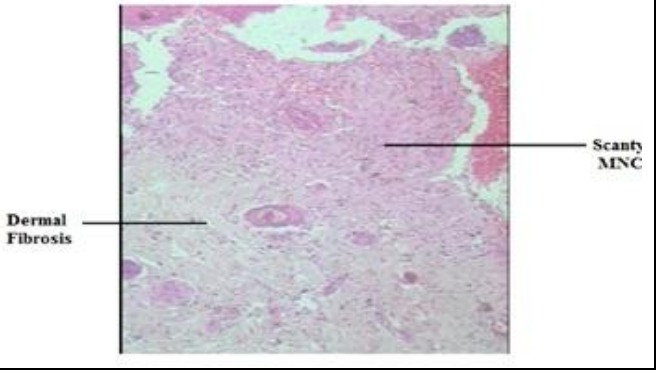


Fig 11. Showing Dermal fibrosis and scanty mononuclear cell infilteration (MNC).otherwise no abnormality detection (NAD) in excision wound modal of rats treated with *Tabernae montana divericata.*



DISCUSSION

The coarse powder of the shed dried parts of the plant was subjected to extraction by using soxhlet apparatus using ethyl acetate as solvent. In the extract yield was obtained in alcoholic extract that was 8.14%.

After the extraction, phrmacognostical evaluation was done including determination of Ash value and moisture content was determined. Extract was subjected to various chemical tests for preliminary identification of various phytoconstituents. The extract contains carbohydrates, tannins, flavonoids, glycosides, alkaloids etc.

Wound healing involves a cascade of events characterized by completion of biological processes in a certain order and a certain time frame. These events represent the restructuring of the damaged tissue in an attempt to restore as normal a condition as is possible. The natural response of a living organism is to repair the wounds in the shortest time possible and to re-establish the normal continuum of the structures.

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 epithelialization.

Tensile strength of wound represents the promotion of wound healing. Usually wound healing agents promote the gaining of Tensile strength. Tensile strength (the force required to open the healing skin) was used to measure the amount of healing.

The studies on excision wound healing model reveal that all the groups showed day to day decrease in wound area. However, on 16th post wounding day, control animals group-I showed 62.55% of wound contraction whereas group-II standard group animals showed that of 83.0% and extract treated group-III & IV exhibited that of 70.33& 78.0 % wound contraction respectively. When compared with the standard, the activity of formulation of extract was found to be lesser. It was also observed that reducing the epithelization period of 5% extract OD (20.0) and 5% extract BID (19.0) group in comparison to control group (Table-7.5). The time required for complete epithelization of the excision wound is an important parameter to assess the wound healing process.

The promotion of wound healing activity is also well gazed by its tensile strength of the incision wound. Generally wound-healing agents have the properties to enhance the deposition of collagen content, which provides strength to the tissues and forms cross-linkages between collagen fibers. The tensile strength of the extract treated (5%w/w OD) groups was found to be (255.0gm) which was higher than that of extract treated (5%w/w BID) group (265.3gm). Standard group showed maximum tensile strength (277.0gm) of on 10th post wounding day, which indicate wound healing strength of the extract.

CONCLUSION

The results of the present study revealed that, animals treated with 5% extract of *Tabernae montana divaricata* leaves showed faster rate of epithelialization in excision wound model and tensile strength in incision wound model. The wound healing effect of extract may be attributed to the presence of phytoconstituents like alkaloids, triterpenoids, tannins and flavonoids in the extract which are known to promote the wound healing process mainly due to their antimicrobial property.

Thus, wound-healing property of the extract 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. At this stage, it is difficult to say which component(s) of the extract are responsible for the wound healing activity. However, further photochemical studies are needed to isolate the active compound(s) responsible for these pharmacological activities.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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