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EVALUATION OF ETHOSOMAL GEL FORMULATION OF *MANGIFERA INDICA* ON EXCISION WOUNDS IN WISTAR RATS

Pharmaceutical

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ABSTRACT

The objective of the study was to evaluate the potential effect of ethosomal formulation of leaf extract of *Mangifera indica* on excision wounds in Wistar rats. The methanolic extract *of Mangifera indica* leaves was prepared by maceration. Three different formulations of ethosomes (100 mg, 200 mg, 300 mg) were prepared and the entrapment efficiency was 65.1%- 96.54% with average vesicle size of 926 nm. Wistar rats weighing between 180 -200 gms were taken and were divided into 5 groups (n=6). Silver nitrate ointment was used as a standard. The circular excision wound model was created on the backside of the rat neck and the percent wound area, and period of epithelisation were determined and study was conducted till the wound is healed. The % wound contraction (200 mg) was significantly reduced to 14.5 %. The present study revealed that ethosomal gel with the test drug has significantly influenced the wound healing property.

KEYWORDS

Ethanol, Lecithin, Ethosomes, *Mangifera indica,* wound healing

INTRODUCTION:

Wounds are physical injuries, which lead to open or broken skin. So, it is essential for proper healing of wounds for the restoration of disrupted anatomical stability and disturbed functional status of the skin. Repair of injured tissues occurs as a sequence of events, which includes inflammation, proliferation, and migration of several types of cells. The inflammation stage begins immediately after injury, first with vasoconstriction that favours homeostasis and releases inflammatory mediators.[1] In countries like India and china, there are less known plants for treating wounds. However, in Africa and other Asian countries, traditional forms of medicine were practised for centuries for their potential in the treatment of disorders associated with wounds. Among various herbs, *Mangifera indica* was found to be an important herb in ayurvedic and indigenous medical systems for over 4000 yrs.[2]

Mangifera indica plant which belongs to the family Anacardiaceae, grows in tropical and sub-tropical regions. Various parts of the plant were commonly used in folk medicine for a wide variety of remedies like treating diarrhoea, asthma, hypertension, insomnia, improves strength and immunity, paralysis, neuropathy, skin self-repair, skin disorders, bleeding disorders, constipation, bloating and used as an astringent.[3] Leaves and bark contain mangiferin (flavonoid, natural xanthone C-Glycoside), mangiferolic acid, homonangiferin and indicenol. Leaves have tannins, flavonoids, steroids, cardiac glycosides, alkaloids and carbohydrates. For external application like excessive bleeding, injury and wounds, dry powders of bark, flower, leaves and seeds can be applied.[4]

Tropical preparations such as creams, ointments, and gels may be prepared where they can be spread to local inflammation sites. The main limiting factor of transdermal drug delivery is epidermal barrier, which can be overcome by ethosomes when compared to transdermal drug delivery.[5]

Ethosomes are non-invasive delivery carriers that enable drugs to reach the deep skin layers and /or the systemic circulation. Even after exhibiting the promising therapeutic effects, most of the phytoconstituents fail to achieve bioavailability because of poor absorption. Incorporation of these plant actives or extracts into vesicular carriers vastly improves their absorption and consequently bioavailability. Indirectly ethosomes has become an area of research in herbal formulations because of their enhanced skin permeation and improved entrapment efficiency.[6]

As wounds result in various forms of traumatic insults to the skin, these insults may be burns, accidental mechanical injuries and diabetic wounds. When wounds are formed different skin layers get injured and wound healing process starts with systematic formation of skin layers as a response to severe local inflammation. Any treatment, which can produce wound healing response without any issues of infection and scar formation is considered as a best wound healing formulation. In the current study, an attempt was made to prepare novel wound healing formulations to produce best therapeutic effect. These formulations were studied in skin excision wound healing model in Wistar rats. Hence, an in-depth study was done on the wound healing activity of ethosomal gel prepared from leaf extract of *Mangifera indica*.[7]

MATERIALS AND METHODS:

Collection of Plant material and Preparation of extract:

Mangifera indica leaves were collected from local market, Hyderabad, India and were further authenticated by Dr. Madhava Chetty, Botanist, Tirupati, Andhra Pradesh. All the other solvents and reagents were of analytical grade. Fresh leaves were chopped into small pieces, air dried grounded in to fine powder. Powder weighing 650 grams was macerated with 5 litres of pure methanol for 7 days at room temperature. Later it was filtered and the extract was concentrated under reduced pressure below 500^0 C in rotary vacuum evaporator. It was kept in petri dish for air drying to remove the traces of methanol and finally a concentrated extract is formed.[8]

Preliminary Phytochemical analysis:

The leaf extract of *M.indica* was screened for the presence of various phytoconstituents like alkaloids, carbohydrates, phenolics, flavonoids, glycosides and tannins.

Preparation of ethosomes:

Lipid and cholesterol were measured accurately and dispersed in water by stirring it on a magnetic stirrer for 30 min heating at 40°C. Organic phase containing 100 mg of extract was added to ethanol and to this propylene glycol was added, kept for stirring separately. Lipid solution was added drop by drop to the organic phase and kept for stirring on a magnetic stirrer for 1 hr, 12 different ethosomal formulations were prepared using different concentrations of lipid (100-400mg) and ethanol (10-40%). The optimized formulation was chosen and further ethosomal preparations of other doses (100 mg, 200 mg, 300 mg) were formulated. The formulations with high entrapment efficiency and drug release were selected to incorporate in to gel formulations.[5]

Evaluation of prepared ethosomes:

The morphology of samples were visualised by scanning electron microscopy (SEM, Hitachi S-3700N), gives a three-dimensional

image of the globules. Zeta potential was determined using Zeta sizer (HORIBA SZ-100). Measurements were performed on the same samples prepared for size analysis. Entrapment efficiency of *Mangifera indica* ethosomal vesicles was determined by centrifugation and estimated using UV visible spectrophotometer at 214 nm. From this, the entrapment efficiency was determined by the following equation -

$$EE\% = \frac{\text{(Total drug) - (free drug)}}{\text{Total drug}} \times 100$$

Formulation of gels

Gels were prepared by adding dispersing gelling agent carbopol 940 to distilled water. Then the mixture was allowed to swell overnight. The mixture was neutralized by drop wise addition of triethanolamine. Then, glycerol was added to gel to balance its viscosity. To this gel solution optimized ethosomal dispersion was added and mixed properly. Mixing was continued until a transparent gel appeared. Paraben was added as a preservative. The prepared gels were filled in glass vials and stored at 4-8^0 C.[9]

Evaluation of prepared gels [11]

Physicochemical properties

The appearance was checked visually. They are light greenish in colour. The pH was checked using pH meter (Systronics digital pH meter). Viscosity of prepared formulations was prepared by Brookfield Synchro Electric Viscometer (LVDV Pro II), spindle S64 (small sample adaptor) and the angular velocity was increased from 5, 10, 50, 100 rpm and values were noted. Drug content was estimated spectrophotometrically, 100 mg of the formulation was taken and dissolved in methanol and filtered. The volume was made up to 100ml with methanol. The resultant solution was suitably diluted with methanol and absorbance was measured at 212 nm.

In-vitro drug release

The Franz diffusion cell consisted of two compartments (cells). Upper one is donor cell, consisting of two open ends and lower one is receptor cell, with one open end capacity of 15 ml. One end of the donor compartment was covered with Himedia dialysis membrane (cut off molecular weight 12000-14000), which was previously soaked in warm water and placed on the receptor compartment. The receptor cell contained a small magnetic bead and was rotated at a constant speed. The temperature in the donor and receptor cells was maintained at 37^{0}C, with the help of a thermostat. Phosphate buffer 7.4 was placed in the receptor cell. A 5ml sample of each formulation was transferred to the diffusion cell. 3ml samples were withdrawn from the receptor cell at specified time intervals. Each time immediately after the removal of the sample, the medium was compensated with the fresh media. The samples were analysed for drug content using a UV-Visible spectrophotometer at 212 nm.[10]

Acute skin irritation study for topical formulations:

Skin irritation test was performed following OECD guidelines 404. In skin irritation test, total 9 rats were taken of either sex weighing between 150-180 gms. Animals were divided into three groups of 3 each. Hairs were depleted from the back of the rats with the help of depilatories and area 4 cm^2 was marked on both the sides. One side served as control while the other as test. Test substance was applied and the substance should be attached to the skin. The animals were observed for 14 days for signs of oedema and erythema.

***WOUND HEALING ACTIVAITY*:**

***Animals*:**

Wistar rats of either sex weighing about 125-150 g were obtained from National Institute of Nutrition, Hyderabad. They were kept in quarantine for acclimatization in the animal cages of animal house of Sri Venkateshwara College of Pharmacy at ambient temperature of 22^0 C + 2^0 C and relative humidity with 12 h each of dark and light cycles, were fed pelleted diet and water ad *libitum*. The experimental protocol was duly approved by IAEC (Institutional Animal Ethical Committee) of CPCSEA (Committee for purpose of control and supervision of Experimentation on animals) through its reference no: IAEC/SVCP/2016/005, Dated: 27/2/16.

2.10.2. Experimental design:

The animals were divided into five groups of 6 rats each (n=6)

Group I: Control with wound alone

Group II: Test group with wound and treated with Formulation 1 (Eg1), 100 mg

Group III: Test group with wound and treated with Formulation 1 (Eg2), 200 mg

Group IV: Test group with wound and treated with Formulation 1 (Eg3), 300 mg

Group V: Test group with wound and treated with Standard drug - Silver nitrate

The three test formulations (EG1, EG2 and EG3) of different doses were tested for wound healing activity.

***Excision Wound*:** The wound procedures were carried out using Ketamine (1 ml / kg body weight) as an anesthetic agent to animals in excision wound model. Using commercial depilatory, the hair was removed from the dorsal portion of the rats. A circular piece of full thickness (approximately 500 mm^2) was cut off from a pre-determined area on the back of rat. Wounds were traced on 1 mm^2 graph paper on the day of wounding and subsequently on days specified, until healing was complete. All the animals were undergone with wound induction and were pooled. During the surgical procedures, all the surgical items and materials used were sterilized. The test formulations (EG1, EG2 and EG3) were applied topically; the untreated control was treated with sterile 200 µl normal saline solution.[11]

Treatment with test formulations: After creating the excision wound, topical formulations were applied from day '0'onwards for a period of 21 days. From day '0' to day '21', wounds were regularly observed and the wound areas were measured. At designated time periods (0, 7, 14 and 21 days) images of the wounds were captured at same magnification and resolution from the anesthetized animals and percent wound areas were calculated. The wound area or dimensions were measured by keeping transparent papers on the wound and marking the wounds. Number of days required for falling of scab without any residual raw wound gave the period of epithelization. At the end of the experiment the rats were sacrificed and tissues of wound were collected for H & E (Hematoxylin and Eosin) staining.

% Wound area = [initial wound size – specific day wound size / initial wound size] X 100

***Histo pathological analysis*:** After collecting the wound tissues, they were fixed in buffered formalin and processed. Tissue Sections of 5 microns were prepared and stained with H&E. The stained slides were analyzed by histopathologist. The results were analyzed.

***Statistical analysis*:** The results of the studies were expressed as Mean±SEM. Data analysis was done by one-way analysis of variance (ANOVA). Probability values $P<0.01$ were considered significant when compared to control group.

RESULTS

The microscopic evaluation showed the surface morphology of ethosomes. It was observed that most of the vesicles were spherical in shape with a zeta potential of -8.8 mv. The vesicular size of the ethosomes significantly increased and it was 926 nm with increase in phospholipid concentration and decreased with increased concentration of ethanol.

The entrapment efficiency of ethosomes was observed in the range of 65.31-89.38%. The entrapment efficiency was found to be higher for the F10 formulation. The entrapment efficiency was influenced by amounts of ethanol, lecithin and cholesterol which were used for preparation. Of all the factors examined the concentration of ethanol was found to influence the entrapment efficiency to a significant increased level due to the formation of thinner membrane.

Table 1 Entrapment efficiency and % drug release of different formulations

S.NO	FORMULATION CODE	ENTRAPMENT EFFICIENCY	% DRUG RELEASE
1	F1	65.31±0.22	63.98±0.37
2	F2	68.42±0.5	72.2±0.54
3	F3	70.88±0.31	74.75±0.2

4	F4	71.5±0.66	72.85±0.72
5	F5	68.65±0.26	73.53±0.24
6	F6	72.73±0.9	77.06±0.14
7	F7	70.82±0.67	80.58±0.21
8	F8	75.2±0.36	76.8±0.12
9	F9	82.4±0.44	82.62±0.73
10	F10	89.58±0.26	87.79±0.50
11	F11	84.33±0.45	86.5±0.42
12	F12	86.21±0.33	87.88±0.5

In the *in vitro* drug release, the cumulative percentage drug release from various ethosomal formulations was done. The formulation F10 showed higher drug release of 87.79 % in 8 hrs. Therefore, F10 has been selected for formulating the ethosomal gel and based on this; different doses of 100, 200 and 300 mg f drug extract were also formulated.

In the evaluation of ethosomal topical gel, all the formulations were found to be opaque, light greenish in colour, odourless, semi solid in nature and had smooth appearance.

Table 2 Evaluation of physicochemical properties of gel formulations

Formulation	Colour	Appearance	Spread ability (g.cm/sec)	pH	Viscosity (cps)	Drug content %
EG1	Greenish	Homogenous	35.07+0.86	5.6	2399	74.67
EG2	Greenish	Homogenous	33.72+0.52	5.8	2574	78.92
EG3	Greenish	Homogenous	34.62+0.67	5.5	2250	82.31

The pH for all the formulations exhibited in the range of 5.4-6.2. The formulations were analysed Spectro photometrically at 212 nm. All the formulations were found to possess uniform drug content.

The viscosity of all the gel formulations ranged from 2250- 2574 cps. The viscosity of the formulations decreased on increasing the shear rate i.e. pseudo plastic behaviour was noted. In the *in vitro* drug release, the cumulative percentage drug release after for 8 hrs was highest for all the three doses of extracts using 1% carbopol. The drug content of the gels ranged between 74.67-82.31 %.

The herbal ethosomal gel was subjected to acute skin irritation studies and wound healing activity.

Preliminary Phytochemical Investigation: The preliminary phytochemical investigation of leaf extract of *Mangifera indica*showed the presence of alkaloids, carbohydrates, phenolics, flavonoids, glycosides and tannins.

Acute skin irritation study of topical formulations:
There were no signs of oedema and erythema was observed till 14 days which indicated that absence of skin toxicity after topical application of formulations.

Wound healing activity:
Plants have immense potential for the management and treatment of wounds. Many plants were used by tribal and folklore in many countries for the treatment of wounds and burns. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms.

Table 3 Wound healing activity: Effect of test formulations on individual wound area in mm

Groups	Day 0	Day 3	Day 7	Day 10	Day 14	Day 21
I	4.09±3.17	4.0 ± 5.0	3.5 ± 0.97	3.01 ± 0.75	1.90 ± 0.07	1.6 ± 0.2
II	3.95 ± 3.12	3.65 ± 0.59	2.48± 0.47	2.26 ± 1.21	1.27± 0.51	0.1 ± 1.7
III	4.29 ± 1.83	3.86 ± 1.6	2.47 ± 0.2	1.76 ± 0.42*	0.69±0.08*	0.06 ± 0.2*
IV	4.12 ± 1.17	3.57 ± 1.59	2.42± 0.26	1.19 ± 0.8*	0.97± 0.86*	0.28 ± 1.3*
V	4.27 ± 1.44	4.14 ± 1.17	3.39 ± 0.6	2.88 ± 0.04	1.68 ± 0.35	0.62±0.49*

The data are expressed in Mean ± S.E.M; n=6 in each group; *p<0.01, significant, compared to control and standard drug.

Table 4 Effect of test formulations on percent wound area

Groups	Day 0	Day 3	Day 7	Day 10	Day 14	Day 21
I	100±3.17	97.74± 5.0	85.79± 0.97	73.59± 0.75	46.43±0.07	18.91 ± 0.2
II	100 ± 3.12	92.7± 0.59	62.94± 0.47	57.41± 1.21	32.26±0.51*	2.5±1.7*
III	100 ± 1.83	89.91 ± 1.6	57.62 ± 0.2*	41.10±0.42*	16.18±.08*	1.45 ± 0.2*
IV	100 ± 1.17	86.79±1.59	58.81±0.26*	28.95 ± 0.8*	23.52±0.86*	6.85 ± 1.3*
V	100 ± 1.44	96.89± 1.17	79.25±0.6*	67.49± 0.04*	39.27±0.35*	14.6±0.49*

The data are expressed in Mean ± S.E.M; n=6 in each group; *p<0.01, significant, compared to control and standard drug.

Table 5 Effect of test formulations on Epithelization period (days)

Groups	Period of epithelization (number of days)
I	21.33±0.21
II	19.83 ± 0.30*
III	14.33 ± 0.42*
IV	18.11 ± 0.33*
V	20.12 ± 0.46

The data are expressed in Mean ± S.E.M; n=6 in each group; *p<0.01, significant, compared to control and standard drug

In the wound healing activity, the wound area in groups II, III, IV and V was significantly (*p<0.01) decreased from 10th day onwards, which continued on 14th and 21st days. However, amongst all the test groups, group III was shown a subsequent reduction on 14th and 21st day as compared to the other groups. The percent wound area was also calculated and was observed a profound decrease from day 7th onwards as compared to the other groups. Similarly, the mean period of epithelization was found to be significant (*p<0.01) in all the treated groups while group III showed only 14.33 as compared to the control animals. It showed that group III treated rats had faster healing capacity as compared to the other treated groups.

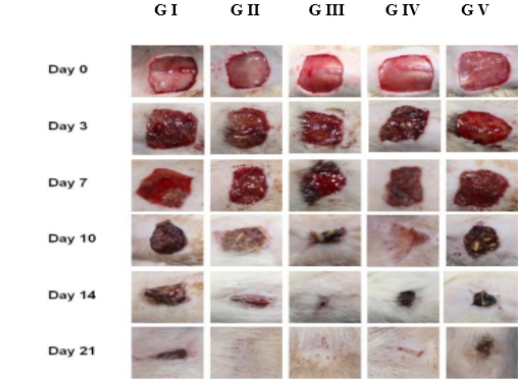

Fig. 1. Representative wound images from untreated control and 3 different test formulation groups with standard at 0, 3,7, 10, 14 and 21st day post wound induction.

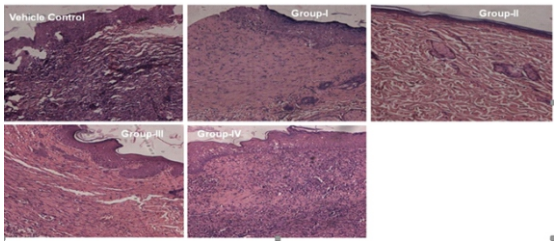

Fig. 2. Representative H& E stained images of wound sections obtained from distinct groups at 21stday post treatment.

Group I (Control): Moderate to severe inflammation along with infiltration of inflammatory cells noticed in the dermal and sub cutaneous layer of skin.

Group II (100): Inflammatory region in the dermal region are completely healed. Higher magnification of wound was healed in epidermal layer and dermal region.

Group III (200): Wound completely healed with matured adult fibrous tissue.

Group IV (300): Massive proliferation of fibrous tissue, with healing of the wounded dermal region of skin.

Group V (Standard): Epidermal layer of skin normal. Higher magnification of dermal and subcutaneous inflammatory region with infiltration of lymphocytes noticed.

DISCUSSION

Novel drug delivery system offers potential advantages over traditional route. These advantages include avoidance of first pass metabolism, sustainable duration of action, minimization of side effects, improvement in pharmacological response and high patient compliance. In the present study, the prepared ethosomes were evaluated for vesicle shape, vesicle size, entrapment efficiency and % drug release. Based on the entrapment efficiency and in vitro drug release F 10 ethosomal formulation was selected and incorporated into gel form. Wound represents a major health problem, both in terms of morbidity and mortality. The results of the excision wound healing activity depicted that the ethosomal gel of *M.indica* showed potential anti-healing effect in experimental rats. The histopathological studies revealed that animals treated with gel formulations at different doses showed marked effect on wounds. Among all the four test groups, except test group V (Standard), remaining test groups (II, III and IV) observed to show significant wound healing response compared to untreated control group. Group III was found to be promising as compared to other treated groups. The tissue section of group III showed that the wound was completely healed with matured adult fibrous tissue. Formation of fibrous tissue is a reparative or reactive process. Similarly, the histological examination of wound tissues suggested that there was promising restoration of wounded tissues in EG1 2, 200 mg treated group compared to rest of the treated groups. Though the group II, IV treated animals showed almost equal wound healing response compared to group III treated group, the histopathological studies demonstrated that group III was best among all the four treated groups.

In recent years, oxidative stress has been implicated in a variety of degenerative processes and diseases which include acute and chronic inflammatory conditions such as wound healing. The preliminary phytochemical analysis of *Mangifera indica* leaf extract revealed the presence of saponins, glycosides, flavonoids and alkaloids. As flavonoids, play an important role in combating oxidative stress, inflammation and may be responsible for wound healing process. As the plant possesses astringent property, they stop bleeding and heal wounds quickly. The rationale for application of topical medications is prophylaxis and treatment of infection in compromised skin. These preparations allow avoiding systemic toxicity and are most effective in the earlier stages of healing. Results of the present investigation clearly elicit the wound healing property of ethosomal herbal formulation of *Mangifera indica.*

CONCLUSION

Mangifera indica ethosomal gel has shown accomplished wound healing property in terms of wound contraction and epithelisation. These findings are confirmed with the histopathological studies. These observations and findings suggest that the formulation is useful as a therapeutic agent in the treatment of wounds and superiority when compared to the standard drug. The formulation has commercial potential.

Competing interests

The authors declare that they have no conflicting interests.

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