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A phytochemicals based formulation induced faster wound healing of excision wounds in *wistar* rats

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ABSTRACT

The present study involved development and scientific validation of wound healing potential of three phytochemical-based formulations at different concentration in experimental rat model. Formulation-I consisted of ethanolic extracts of *T. erecta*, *T. procumbens*, *A. vera* and *C. longa*. Formulation-II consisted of ethanolic extracts of *A. indica, F. benghalensis*, *A. vera* and *C. longa*. Formulation-III contained ingredients of both Formulations-I and II. The extracts were analyzed quantitatively, revealing that *T. erecta* had the highest content of total phenols (41.61±0.10 mg GAE/g) and flavonoids (52.50±0.53 mg QE/g) and tannins were found in *T. procumbens* (59.61±0.15 mg TC/g). Wound healing functions of formulations were assessed by topical application on excision wounds made in *Wistar* rats. The healing process was monitored by measuring the degree of wound contraction on alternate days. The polyherbal Formulation-III (5% concentration) was found to achieve complete wound contraction (Significant pd"0.0001) on 18th day in comparison to other Formulations. The study demonstrated that the Formulation-III was found to have the most potential in achieving rapid wound healing rate.

Figures : 03 References : 33 Tables : 05
KEY WORDS : Excision wound, Phytochemicals, Polyherbal formulation, Wound healing.

Introduction

Wounds are physical skin injuries that result in an opening or break of the skin that disrupts structural and functional integrities of the skin tissues. These alterations may or may not be associated with the loss or damage of underlying connective tissues (bone, cartilage, fat, blood and lymphatic tissue). Wound healing is the process involving four overlapping phases, *viz.,* hemostasis, inflammation, proliferation and remodeling[4,6,14]. Speedy and rapid wound healing is necessary for the survival and protection against the edema and skin ulcers which may prove lethal to the organism on no treatment[18].

The *Tagetes erecta*, *Tridax procumbens*, *Azadirachta indica, Ficus benghalensis*, *Curcuma longa* and *Aloe vera* exhibit a wide spectrum of biological activities and are used as traditional medicines for household remedies against various human ailments in Ayurveda[20]. *T. erecta* also known as Marigold (Family-Asteraceae)[32] and has anti-microbial, anti-inflammatory, hepatoprotective, anti-parasitic, anti-septic, anti-oxidant, analgesic and wound healing properties[2,9]. Studies have

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TABLE-1: Herbal components of F-I, F-II and F-III

F-I	F- II	F-III
T. erecta	-	*T. erecta*
T. procumbens	-	*T. procumbens*
-	*A. indica*	*A. indica*
-	*F. benghalensis*	*F. benghalensis*
A. vera	*A. vera*	*A. vera*
C. longa	*C. longa*	*C. longa*

shown that it can increase platelet and white blood cell count and reduce bleeding and clotting times[16]. Topical application of *T. erecta* flower paste has resulted in faster wound healing in animals and extracts have also exhibited oral anti-ulcer activity[7]. The *T. procumbens* (Ghamara) belonging to the Asteraceae family has been extensively used in the Ayurvedic system of medicine for treatment of cuts, wounds and burns[33] due to the presence of pharmacological activities like anti-inflammatory, anti-bacterial and anti-oxidant potentials[8]. The herb contains bioactive components belonging to alkaloids, phenols, flavonoids, carotenoids, B sitosterol, fumaric acid, luteolin, quercetin, tannin, *etc.*[30]. Neem, scientifically known as *A. indica* (Family- Meliaceae), contains bioactive compounds in its every part like seeds, leaves, roots, bark and trunk. *A. indica* possesses anti-inflammatory, anti-fungal, and anti-bacterial properties that aid in wound healing. It also contains amino acids, vitamins, and main active ingredients such as nimbidin, nimbin and nimbidol that play important role in wound healing processes particularly in the proliferation phase, formation of collagen and angiogenesis[14,29]. The *F. benghalensis* (Family-Moraceae), also known as the Indian Banyan tree, has been used in traditional medicine to treat conditions such as dysentery, diabetes and nervous disorders. Its parts have antimicrobial, antioxidant, anti-inflammatory, anti-ulcer, and wound healing properties[15]. A study reported that ethanolic and aqueous extracts of *F. benghalensis* leaves demonstrated a decrease in epithelization and an increase in the rate of wound contraction in an excision wound model[10]. The *A. vera* plant from the Liliaceae family has been traditionally used to treat burns, allergic reactions, arthritis, indigestion, ulcers, diabetes, skin diseases, and digestive system inflammation[1]. *A. vera* extract has anti-inflammatory properties, promote collagen synthesis, skin regeneration and blood supply essential for wound healing[12]. It contains bioactive compounds such as flavonoids, alkaloids, tannins, terpenoids, polyphenols, amino acids and vitamins and reduces wound-related bacterial infections[11]. The *C. longa* (family- Zingiberaceae) is well known for its anti-microbial functions[22]. Curcumin has a wide range of biological effects including anti-inflammatory, anti-oxidant, anti-tumor, anti-bacterial and anti-viral activities[21]. Curcuminoids, the active ingredients of *C. longa* are known to be beneficial in treatment of skin diseases and enhance the wound healing and skin regeneration[28].

Several other herbs/plants have been reported to play a crucial role in wound healing process[26]. Polyherbal formulations not only accelerate wound healing process with minimum or no side effects *i.e.,* they are safe, non-toxic and can be administered over longer periods[25]. Hence the polyherbal formulations would have longer acceptability in treatment of wounds with high efficacy. More than 70% of wound healing pharma products are of plant origin[24]. The present study demonstrated superior wound healing potentials of different polyherbal formulations on excision wounds made on Wistar rats.

Materials and Methods

Collection and identification of plant materials

Mature *A. indica*-leaves, *T. procumbens*-whole plant*, F. benghalensis*-bark, and *A. vera*-leaves were collected from 'Charak Udhyan' (Medicinal plants garden) of Jiwaji University, Gwalior. The used

TABLE-2 : Quantitative analysis of phytochemical extracts

Extracts	Total Phenols (mg GAE /g)	Total Flavonoids (mg QE / g)	Total Tannins (mg TC / g)
T. erecta	41.61±0.10	52.50±0.53	9.920±0.24
T. procumbens	17.14±0.23	34.00±0.52	59.61±0.15
A. indica	15.15±0.53	9.420±0.60	14.17±0.60
F. benghalensis	12.23±0.90	8.970±1.12	44.01±0.41
A. vera	34.28±0.36	40.27±1.00	19.51±0.32

Results are expressed in Mean ± SEM (n=3)

T. erecta-flowers were collected from the temple of the Jiwaji University campus. The plant specimens were authenticated by the Institute of Ethnobiology, Jiwaji University. The dried plants/plant parts were mounted as herbarium specimens in the Institute and have been assigned code names *viz.*, *T. erecta* (IOE-438), *A. indica* (IOE-439), *T. procumbens* (IOE-440), *F. benghalensis*, (IOE-435) and *A. vera* (IOE-437). Curcumin purchased from Pukhraj herbals, Mandsaur India and it was used directly.

Preparation of extracts

The selected parts of plants/herbs (*T. erecta*-flowers, *T. procumbens*-whole plant, *A. indica*-leaves and *F. benghalensis*-bark) were washed. Materials were dried at room temperature in shade for 7 to 10 days then grounded to powder separately using a mechanical grinder. Crude powder was Soxhlet extracted separately using 95% ethanol. The extract was dried up at *45ºC in a hot air oven for 2-3 days. F*resh *A. vera* leaves gel was ground to homogenous gel and left for 24 hours on a magnetic stirrer adding 95% ethanol for uniform mixing. The solution was centrifuged at 10,000 rpm for 30 min., supernatant was dried under the hot air oven at *45ºC* for 2-3 days, and the powder was collected and stored at 4°C in an airtight bottle.

Characterization

Quantitative analyses

The total phenolic constituents of various extracts were determined by *Folin-Ciocalteu* method[27] using Gallic acid as standard and results are expressed as mg of gallic acid equivalents per g (mg GAE/g) of extract. The flavonoid constituents were determined by colorimetric assay[27] using Quercetin as standard and the results are expressed as mg QE/g of extract. The tannins were determined by the *Folin-Ciocalteu* method[3]. Tannic acid (100 to 1000µg/ml) was used as a standard. The tannin content was expressed as mg of TA/g of extract.

Preparation of polyherbal ointment/formulation

The base was prepared by melting cetyl alcohol and soft wax paraffin, followed by the addition of liquid paraffin. Each polyherbal formulation (I, II, and III) was created by mixing specific amounts of ingredients/ extracts (refer to Table 1) and then homogenized to form a smooth ointment suitable for topical application on wounds. Four different concentrations (2%, 5%, 10%, and 25% w/w) of each formulation (F-I, F-II and F-III) were made and stored at room temperature in plastic containers.

Experimental rodents

Wistar rats weighting about 200-250g obtained from Animal facility of the Defence Research Development Establishment (DRDE), Gwalior were acclimatized at 25°C±2°C and with a humidity of 50% - 60% for two weeks' time before making excision wounds. The animals were fed on standard pellet diet and provided water *ad libitum*.

Creation of excision wounds on *Wistar* rats

Rats were anesthetized with diethyl ether, hair of the dorsal thoracic region was shaved off with an electrical shaver and disinfected with 70% alcohol and a skin area of 2 X 2 cm^2 (~400 mm^2 diameter circular

area) on the dorsal thoracic region was excised with surgical scissor[33]. The day of wound creation was considered a zero-day. The formulation was topically applied evenly on the wound area, once a day till complete healing of the wound achieved.

Experiment- I: A total of six groups (6 rats in each group) were made as detailed below-

Group 1- Normal Control (Base)

Group 2- Reference (Betadine)

Group 3- Formulation-I (2%)

Group 4- Formulation-I (5%)

Group 5- Formulation-I (10%)

Group 6- Formulation-I (25%)

Experiment- II: A total of six groups (6 Rats in each group) were made as detailed below-

Group 1- Normal Control (Base)

Group 2- Reference (Betadine)

Group 3- Formulation-II (2%)

Group 4- Formulation-II (5%)

Group 5- Formulation-II (10%)

Group 6- Formulation-II (25%)

Experiment- III: A total of six groups (6 Rats in each group) were made as given detailed below-

Group 1- Normal Control (Base)

Group 2- Reference (Betadine)

Group 3- Formulation-III (2%)

Group 4- Formulation-III (5%)

Group 5- Formulation-III (10%)

Group 6- Formulation-III (25%)

Assessment of wound area contraction

Wound area was marked by tracing the raw wound area on transparent paper with a permanent marker and the area was measured on graph paper. The wound area was measured at 4 day intervals till complete healing was achieved following topical application of a given formulation. Photographic image of wound area from a fixed distance of 15 cm was taken and documented. Percentage of wound contraction was calculated by using the formula given below[33].

$$\text{Wound contraction \%} = \frac{[\text{Initial wound area} - \text{Wound area following treatment}]}{\text{Initial wound area}} \times 100$$

The study protocol was approved by the Institutional Animal Ethics Committee of Jiwaji University (Number- IAEC/JU/21).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Prism software Inc., La Jolla, Ca). The results were analyzed using one-way analysis of variance (ANOVA). P values *pd"0.05, **p<0.01, ****p<0.001, ****p<0.0001. All values are presented as mean ± the standard error of the mean (SEM).

Results

Quantitative analyses

Quantitative estimation of total phenols, flavonoids and tannin were done on ethanolic extracts of all phytochemical ingredients and results are shown in the Table 2. The highest content of total phenols (41.61±0.10 mg GAE/g) and flavonoids (52.50±0.53 mg QE/g) were found in *T. erecta*. Maximum amount of tannins were recorded from *T. procumbens* (59.61±0.15 mg TC/g), followed by *F. benghalensis* (44.01±0.41 mg TC/g), *A. vera* (19.51±0.32 mg TC/g), *A. indica* (14.17±0.60 mg TC/g) and *T. erecta* (9.920±0.24 mg TC/g).

Wound healing potentials of different phytochemical formulations

Wound contractions recorded on topical application with different concentrations *viz.,* 2%, 5%, 10% and 25% of F-I as measured on day 20th were 85.35%, 100%, 94.04% and 88.50% respectively. F-I 5% on topical application showed the maximum wound contraction (100%) which is significant (pd"0.001) as compared to the reference (Table-3 and Fig. 1).

Wound contractions recorded on topical application with different concentrations of F-II *viz.*, 2%, 5%, 10% and 25% measured on day 22nd were 88.75%, 100%, 98.02% and 82.12% respectively. F-II 5% on topical application showed the maximum wound contraction (100%) which is significant (pd"0.01) as compared to reference (Table-4 and Fig. 2).

Wound contractions recorded on topical application with different concentrations of F-III *viz.*, 2%, 5%, 10% and 25% on day 18th were 91.03%, 100%, 97.37%, and 87.50% respectively. F-III at 5% on topical application showed the maximum wound contraction (100%) which is highly significant (pd"0.0001). 5% of F-III showed superior wound contraction from 4th day onwards and angiogenesis was significantly higher than

TABLE-3: Wound healing potential of F-I on excision wounds in *Wistar* rat model

Experimental Groups	Wound area (mm^2)					
	0^{th} day	4^{th} day	8^{th} day	12^{th} day	16^{th} day	20^{th} day
Group 1 (Control)	400.0±2.2 (0.0%)	386.7±6.6 (3.33%)	336.5±22.0 (12.98%)	256.2±6.6 (35.95%)	190.0±5.7 (52.50%)	121.5±6.6 (69.63%)
Group 2 (Reference)	398.0±4.2 (0.0%)	373.3±6.6 (6.21%)	336.7±12.0 (15.40%)	246.7±20.2 (38.02%)	127.1±13.2 (68.09%)	62.3±5.6*a (84.35%)
Group 3 F- I (2%)	400.0±5.6 (0.0%)	370.0±11.5 (7.50%)	333.2±14.5 (16.70%)	273.5±8.5 (31.63%)	130.3±102 (67.43%)	59.2±8.2*a (85.20%)
Group 4 F- I (5%)	400.0±4.5 (0.0%)	321.2±8.1 (19.70%)	246.7±4.6 (38.33%)	113.2±10.4 (71.70%)	23.5±15.3 (94.13%)	0.0±0.0***a*b (100%)
Group 5 F- I (10%)	396.0±8.9 (0.0%)	350.0±5.1 (11.62%)	296.7±9.3 (25.08%)	198.4±10.5 (49.90%)	89.2±11.5 (77.47%)	23.6±8.3**a (94.04%)
Group 6 F- I (25%)	398.0±4.6 (0.0%)	370.2±5.3 (6.98%)	315.0±4.2 (20.68%)	234.0±10.5 (41.2%)	121.3±4.8 (69.52%)	45.5±7.2**a (88.50%)

Data are expressed as mean ± SEM. Wound area is expressed in $mm^{2.}$ Figures in parentheses indicate percentage of wound area and is taken as measure of wound healing * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data were analyzed by One way ANOVA, **a** = compared with control , **b** = compared with the reference.

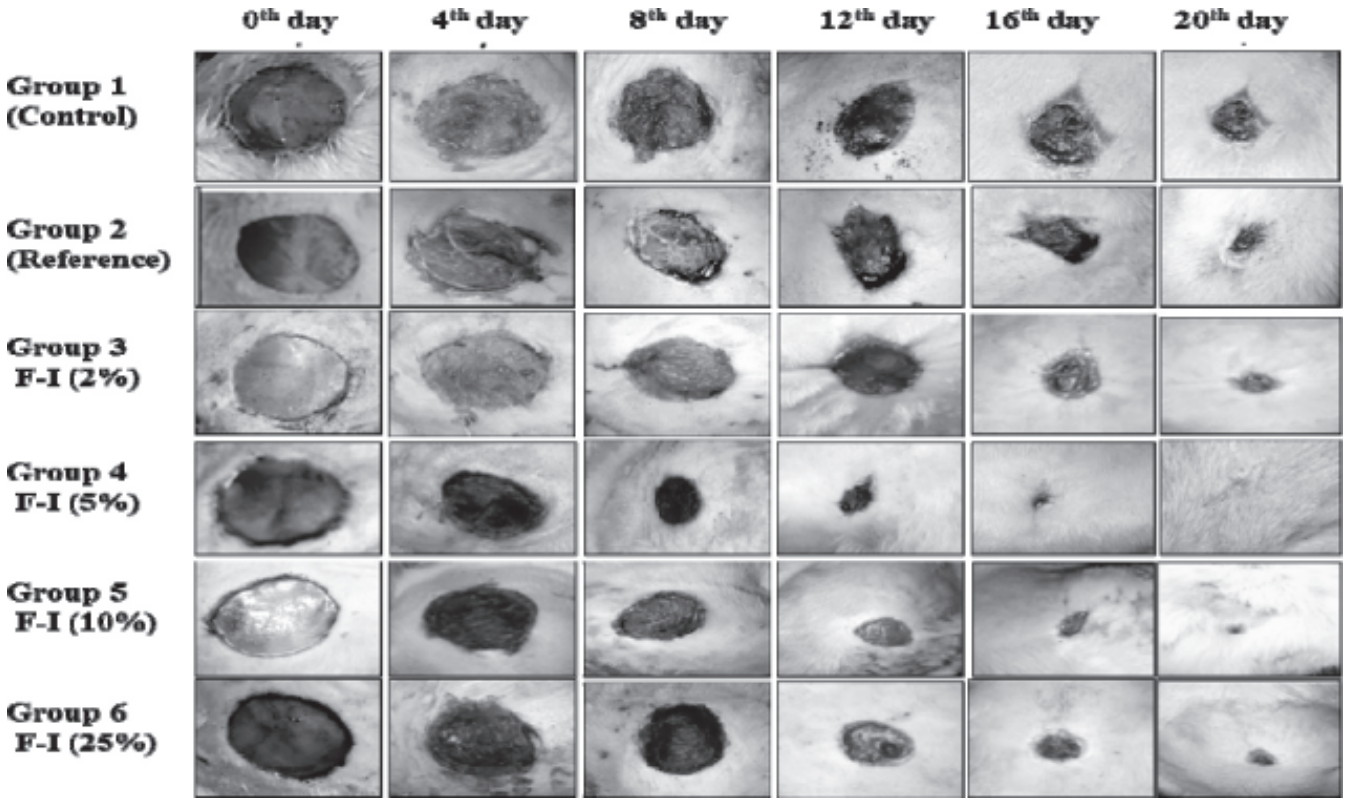

Fig. 1: Images of wound areas in different groups of experiment I at different time intervals

TABLE-4: Wound healing potential of F-II on excision wounds in *Wistar* rats

Experimental Groups	Wound area (mm^2)						
	0th day	4th day	8th day	12th day	16th day	20th day	22nd day
Group 1 (Control)	400.0±2.2 (0.0%)	386.7±6.6 (3.33%)	336.5±22.0 (12.98%)	256.2±6.6 (35.95%)	190.0±5.7 (52.50%)	121.5±6.6 (69.63%)	76.5±4.2 (80.55%)
Group 2 (Reference)	398.0±4.2 (0.0%)	373.3±6.6 (6.21%)	336.7±12.0 (15.40%)	246.7±20.2 (38.02%)	127.1±13.2 (68.09%)	62.3±5.6 (84.35%)	10.8±4.8 (97.11%)
Group 3 F- II (2%)	401.0±2.2 (0.0%)	360.0±1.2 (10.22%)	315.3±10.3 (21.37%)	247.2±12.4 (38.35%)	163.3±6.4 (59.28%)	89.3±9.7 (77.73%)	40.5±10.2*a (88.75%)
Group 4 F- II (5%)	400.8±6.3 (0.0%)	343.3±8.4 (14.35%)	276.4±9.3 (31.04%)	196.0±10.5 (51.10%)	62.7±11.8 (84.36%)	13.1±4.3 (96.73%)	0.0±0.0**ab (100%)
Group 5 F- II (10%)	399.0±5.3 (0.0%)	366.7±5.8 (8.10%)	318.0±4.5 (20.30%)	266.3±11.5 (33.26%)	196.3±11.0 (50.73%)	66.5±14.2 (83.33%)	7.9±5.6*a (98.02%)
Group 6 F- II (25%)	400.0±8.5 (0.0%)	386.7±6.5 (3.33%)	336.5±4.8 (15.88%)	293.2±6.5 (26.7%)	240.0±4.3 (40.0%)	153.3±6.2 (61.68%)	73.0±7.1*a (82.12%)

Data are expressed as mean ± SEM. Wound area is expressed in mm^2. Figures in parentheses indicate % of wound area and is taken as measure of wound healing * $p<0.05$, ** $p<0.01$. Data were analyzed by One way ANOVA, **a** = compared with control, **b** = compared with the reference.

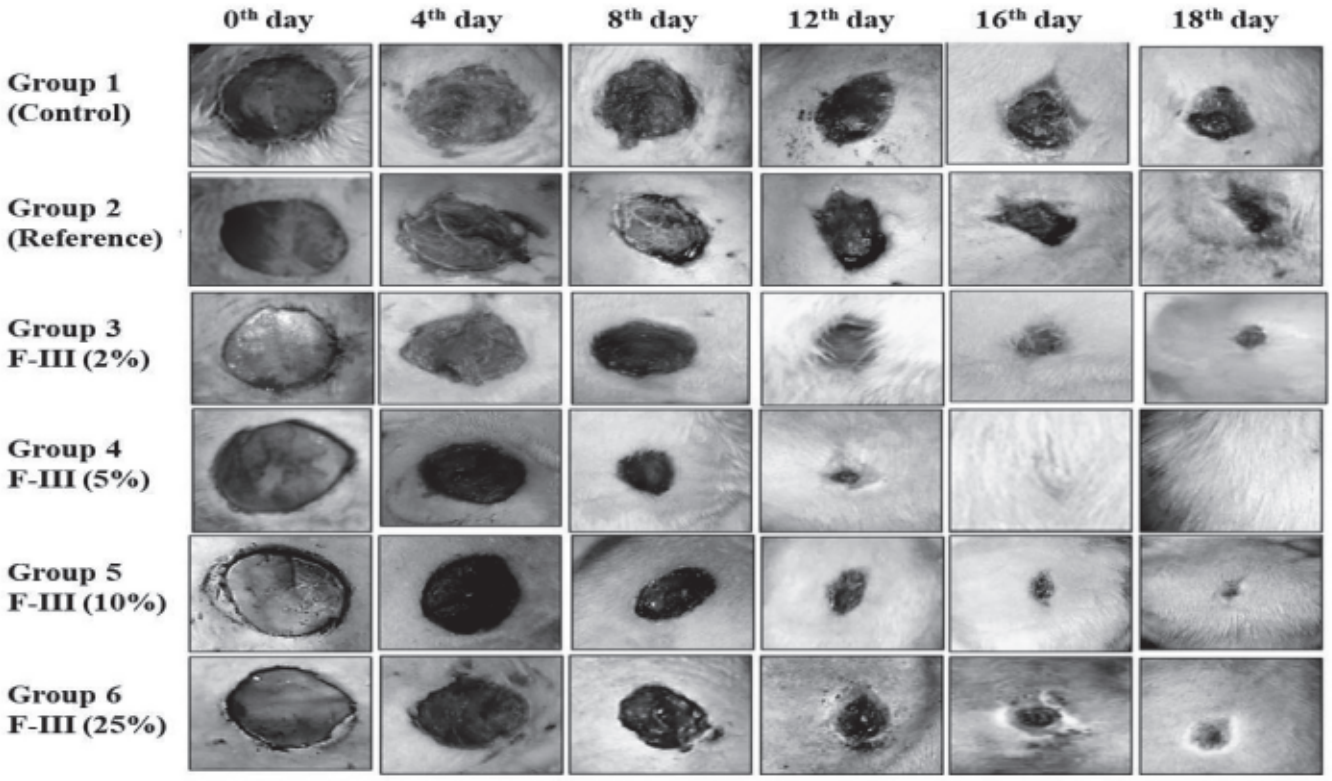

Fig. 2: Images of wound areas in different groups of experiment II at different time intervals

TABLE-5: Wound healing potential of Formulation III on excision wound in *Wistar* rats

Experimental Groups	Wound area (mm^2)					
	0^{th} day	**4^{th} day**	**8^{th} day**	**12^{th} day**	**16^{th} day**	**18^{th} day**
group 1 (Control)	400.0±2.2 (0.0%)	386.7±6.6 (3.33%)	336.5±22.0 (12.98%)	256.2±6.6 (35.95%)	190.0±5.7 (52.50%)	157.5±6.6 (60.63%)
Group 2 (reference)	398.0±4.2 (0.0%)	373.3±6.6 (6.21%)	336.7±12.0 (15.40%)	246.7±20.2 (38.02%)	127.1±13.2 (68.09%)	82.3±6.6*a (79.32%)
Group 3 F-III (2%)	395.6±3.5 (0.0%)	355.3±6.6 (10.26%)	280.4±11.5 (29.17%)	190.0±10.0 (52.01%)	73.3±13.2 (81.49%)	35.5±6.6**ab (91.03%)
Group 4 F- III (5%)	402.0±6.0 (0.0%)	310.0±11.5 (22.90%)	206.7±8.8 (48.59%)	70.0±11.5 (82.59%)	2.5±11.8 (99.38%)	0.0±0.0****ab (100%)
Group 5 F- III (10%)	398.9±8.5 (0.0%)	340.0±5.7 (14.77%)	280.0±8.8 (30.81%)	151.3±12.0 (62.07%)	37.8±15.2 (90.52%)	10.5±6.6***a**b (97.37%)
Group 6 F- III (25%)	396.0±7.2 (0.0%)	346.7±6.6 (12.47%)	273.0±5.7 (31.08%)	198.0±5.7 (52.0%)	98.8±4 (75.18%)	49.5±6.6**a*b (87.50%)

Data are expressed as mean ± SEM. Wound area is expressed in mm^2. Figures in parentheses indicate % of wound area and is taken as measure of wound healing *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001. Data analyzed by One way ANOVA, **a** = compared with control (Base), **b** = compared with the reference.

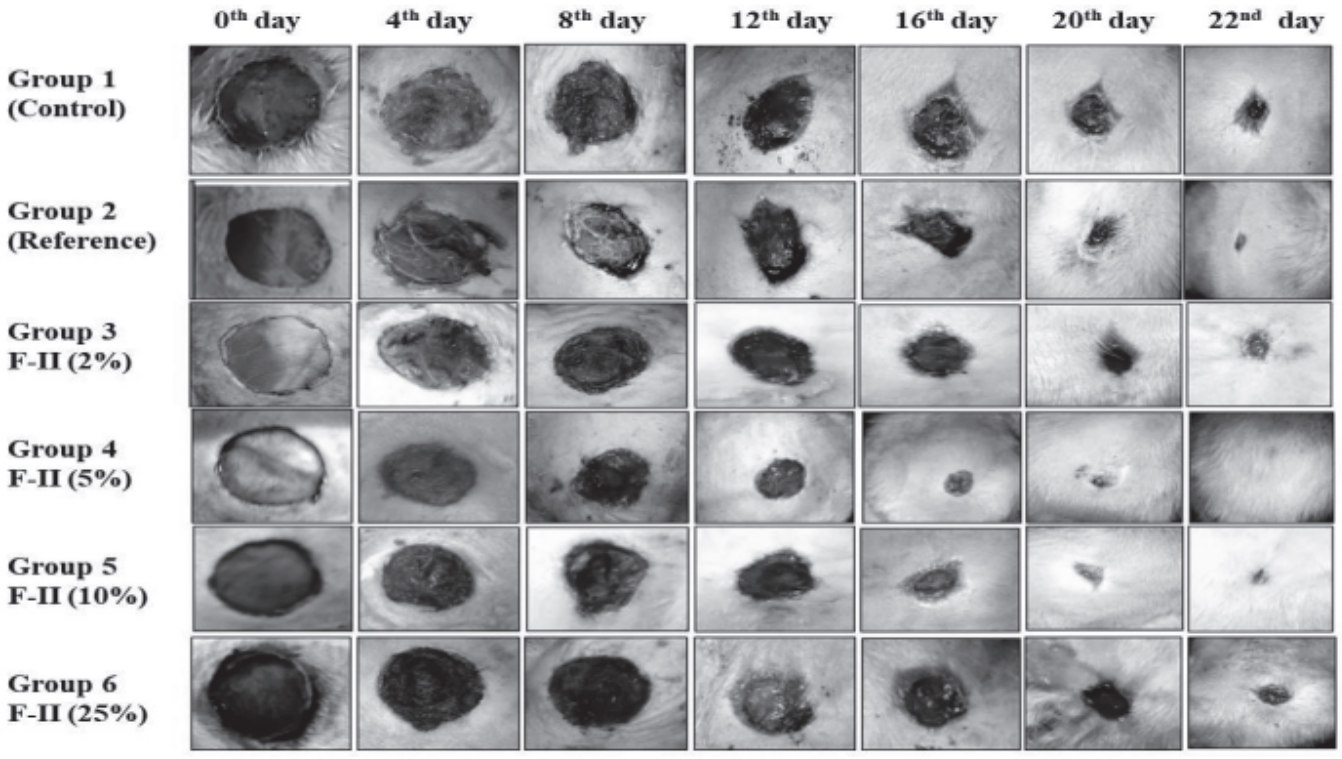

Fig. 3: Images of wound areas in different groups of experiment III at different time intervals

in control (Table 5 and Fig. 3). The 5% F-III showed significantly faster completely wound contraction at 18th day with absence of scar compared to F-I (20th day) and F-II (22nd day) and reference group.

Discussion

Good wound care is essential for effective management. Wound healing involves replacing damaged skin tissue through biological processes like clot formation and tissue generation. Antiseptics may have a toxic effect on tissues and are not suitable for open wounds. It's advised to use them with caution as their toxicity might outweigh any benefits[13]. Some reports suggest that they may exhibit cytotoxicity and are advised against their application on open wounds. Wound healing agents primarily function in the Inflammatory and Proliferative phases[17]. Common antiseptics may have a toxic effect on tissues and caution is advised when using them. It's recommended to combine traditional and modern wound healing agents for optimal care.

Various studies have reported an improved wound-healing process following topical application of herbal products[31]. The results of the present study demonstrated wound healing abilities of ethanolic extracts of a specified set of plants/herbs as Formulations in excision wounds, as evident by the generation of granulated tissue and remarkable increase in the rate of wound contraction compared to the reference formulation *viz.,* Betadine. Phytochemicals-based formulations appear to influence one or more stages resulting in faster wound closure when compared to the control and reference groups.

Medicinal plants have different phytochemicals as secondary metabolites. Phytochemicals such as flavonoids, phenols and tannins present in phytochemical formulations are shown to possess anti-microbial, anti-inflammatory, anti-oxidant activities and are thus responsible for wound healing activity[5], because Tannins have strong astringent property and promote capillary vasoconstriction, which decrease vascular permeability and cause a local anti-inflammatory effect[23]. Flavonoids are well known for their antioxidant potential and antibacterial properties which promote the wound healing process by wound contraction, increased rate of epithelialization and raise the level of hydroxyproline supporting homeostasis[19].

The results of this study seem to confirm the use of F-III consisting of *T. erecta, T. procumbens, A. indica, F. benghalensis, C. longa* and *A. vera* for faster and effective treatment of excision wounds followed by F-I and F-II. The extracts of these plants can be developed into phytomedicines for wider application in the management of wounds.

Conclusion

After conducting experiments on excision wounds, it can be concluded that the topical application of all phytochemical formulations -I, II, and III separately shows significant wound healing activity. This was evidenced by faster generation of fibrocollagenous tissue, neovascularization, epithelialization, and anti-bacterial functions. F-III (5%) proved to be superior in achieving faster wound healing than the other formulations (F-I and F-II) studied. Since *Tagetes erecta, Tridax procumbens, Azadirachta indica, Ficus benghalensis, Aloe vera* and *Curcuma longa* are widely available and abundant, they could provide a fairly economical wound healing agent for wider application in wound care management. Further studies are required to identify the active compounds participating in processes of wound healing.

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