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PHARMACOLOGICAL EVALUATION OF WOUND HEALING ACTIVITY OF POLYHERBAL FORMULATION OF *CALENDULA OFFICINALIS* AND *IXORA CHINENSIS* FLOWERS EXTRACTS

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ABSTRACT

The use of herbal plant extracts in wound healing is known through decades, but it is necessary to provide scientific data through reverse pharmacology. The present research aims to formulate and evaluate an herbal gel containing *Calendula officinalis* and *Ixora chinensis* flowers methanolic extract. The extracts were subjected to various physicochemical evaluations. The present study was to determine qualitative phytochemical analysis, *in vitro* antioxidant, anti-bacterial and wound healing activities. Qualitative phytochemical screening of *Calendula officinalis is* showed the presence of active metabolites such as alkaloids, flavonoids, proteins, tannin, saponin, protein, glycosides and phenols is presented and *Ixora chinensis* showed the presence of active metabolites such as flavonoids, steroids, carbohydrates, phenols and alkaloids. DPPH radical scavenging activity of *Calendula officinalis* extract exhibited percent inhibition 60.32% and its IC50 value was found to be 49.38µg/ml and *Ixora chinensis* extract exhibited percent inhibition 60.15% and its IC50 value was found to be 49.56µg/ml. Ascorbic was used as a reference compound which exhibited percent inhibition 85.16% and showed IC50 value of 22.67µg/ml. The pH of all prepared formulation ranged from 6.3-6.7. The measurement of viscosity of the prepared gel were found to be formulation 1-5455, formulation 2-5509 and formulation 3-5691 cps. Spreadability of different gel formulation was studied. The formulations produced good spreadability. Skin irritation test indicate that prepared gels were not produce irritation, redness, or edema on application and free from dermatological reaction. Antibacterial activity was performed against *E. coli* by well diffusion assay. Formulation 3 and standard gel showed best zones of inhibition. Further we were performed the wound healing studies like contraction of wound model for 21 days. The wound contraction studies revealed that the wound contraction increases on increasing the concentration of herbal extract. The study also reveals that the better activity of polyherbal formulation may be due to the synergistic action of the plant's constituents present in the formulation. Thus, the prepared topical gels possess a versatile approach in healing the wound contraction.

KEYWORDS: *Calendula officinalis, Ixora chinensis,* Qualitative phytochemical analysis, *In vitro* antioxidant, Anti-bacterial, Wound healing activities.

INTRODUCTION

Wound healing is a process of reconstruction of injured skin, coordinated by interaction of various epithelial and mesenchymal cells with cytokines, chemokines and growth factors.[1] Keratinocyte growth factor (KGF) is a paracrine growth factor synthesized by fibroblasts, endothelial cells, smooth muscle cells and dendritic epidermal T-cells.[2] KGF also known to induce mitogen activated protein activation and directly acts as angiogenic factor *in vitro*.[3] Natural plant products play major role in proliferation of fibroblasts and keratinocytes.[4] Plant products were reported to contain growth factors, cell signaling molecules and cell adhesion molecules.[5] India is the largest producer of medicinal herbs and appropriately called the Botanical Garden of the world.[6] Since ancient times plants have been traditionally used in therapeutic practices for the treatment of different types of ailments.[7-10] There are a number of crude drugs where the plant source has not yet been scientifically identified. A phytochemical is a natural bioactive compound found in plants foods that works with nutrients and dietary fibre to protect against diseases. Many researchers suggest that, phytochemical working together with nutrients found in fruits, vegetables and nuts. They can have complementary and overlapping mechanism of action in the body including antioxidant effect.

Calendula officinalis L. (marigold) belongs to the Asteraceae/Compositae family, which is native to Central Europe and Mediterranean. Its flower oil is the main preparation used in cosmetic products, and contains several bioactive compounds, including terpenoids and terpenes (mainly bisabolol, faradiol, chamazulene, arnidiol and esters), carotenoids (mainly with rubixanthin and lycopene structures), flavonoids, (mainly quercetin, isorhamnetin and kaempferol aglycones) and polyunsaturated fatty acids, (mainly calendic acid).[11-14] *Calendula officinalis* L. has been used for medical purposes since the XII century. The plant is reported to present several biological activities namely angiogenic, vascular regeneration, analgesic, antimicrobial, antioxidant and immunomodulatory.[15-18] In cosmetic products, calendula is used in formulations for sensitive skin and soothing products (e.g., after-sun products) among a variety of presentations, including skin, eye, hair and bath products, with recognized safety for use in cosmetics.[19] *Ixora chinensis*, belonging to the Rubiaceae family, is widespread in China and India. As one of the most important traditional medicines, it is used as a remedy for a wide range of diseases such as hypertension, abnormal menstruation, skin and external diseases, rheumatism and gastralgia.[20] The aim of this work was to determine the quality (types) of bioactive compounds, *in vitro* antioxidant, anti-bacterial and wound healing activities of herbal gel.

MATERIAL AND METHOD

Plant material

The medicinal plant *Calendula officinalis* and *Ixora chinensis* (300 gm) was collected. After cleaning, plant parts (flowers) were dried under shade at room temperature for 3 days and then in oven dried at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration. Medicinal plant *Calendula officinalis* and *Ixora chinensis* was authenticated by a plant taxonomist in order to confirm its identity and purity.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals and solvent used in this study were of analytical grade.

Extraction

In present study, plant material was extracted by continuous hot percolation method using Soxhlet apparatus. Powdered material of *Calendula officinalis* and *Ixora chinensis* was place in thimble of soxhlet apparatus. Soxhlation was performing at 60°C using petroleum ether as nonpolar solvent. Exhausted plant material (marc) was dry and afterward re-extracted with methanol solvent. For each solvent, soxhlation was continued till no visual colour change was observed in siphon tube and completion of extraction was confirmed by absence of any residual solvent, when evaporated. Obtained extracts was evaporate using rotary vacuum evaporator (Buchi type) at 40°C. Dried extract was weighed and percentage yield for each extract was determined. Prepared extracts were observed for organoleptic characters (percentage yield, colour and odour) and was packed in air tight container and labelled till further use.[21]

Phytochemical investigation

Experiment was performed to identify presence or absence of different phytoconstituents by detailed qualitative phytochemical analysis. The colour intensity or the precipitate formation was used as medical responses to tests.[22,23]

DPPH free radical scavenging assay

The antioxidant activity of *Calendula officinalis* and *Ixora chinensis* extracts was determined by using the DPPH free radical scavenging assay. 1mg/ml methanol solution of extracts/standard was prepared. Different concentration of *Calendula officinalis* and *Ixora chinensis* extracts/standard (20-100µg/ml) were prepared from 1mg/mL stock solution and 2mL of 0.1mM solution of DPPH was added. The obtained mixture was vortexed, incubated for 30min in room temperature in a relatively dark place and then was read using UV spectrophotometer (Shimadzu 1700) at 517nm. For control, take 3 ml of 0.1mM DPPH solution and incubated for 30 min at room temperature in dark condition. Absorbance of the control was taken against methanol (as blank) at 517nm.[24] Percentage antioxidant activity of sample/standard was calculated by using formula:

% Inhibition = [(Ab of control- Ab of sample)/ Ab of control x 100]

Formulation of topical gel

Initially carbopol-934 was immersed in 50 mL of warm water (A) for 2hr and was homogeneously dispersed using magnetic stirrer at 600 rpm. In separate container carboxymethyl cellulose and methyl paraben was added into 50 ml warm water (B) and stirred continuously to make stiff gel. Both the mixtures A and B were mixed with the continuous stirring. Then triethanolamine (Drop wise) was added to neutralize the pH and formulations I, II, were 1% of each concentration of extract and formulation III was 2% concentration (i.e. 1% of each extract) were incorporated into the dispersion to obtained gel. At this stage, permeation enhancer (Propylene glycol) was added. The final dispersion was agitated until smooth gel was formed without lumps.[25]

Table 1: Composition of prepared herbal gel.

Name of Ingredient	Formulation I	Formulation II	Formulation III
Carbopol 940	1 gm	1 gm	1 gm
Carboxymethyl cellulose	1 gm	1 gm	1 gm
Propylene glycol	0.5 ml	0.5 ml	0.5 ml
Methyl paraben	0.2 ml	0.2 ml	0.2 ml
Calendula officinalis	1 gm	----	1 gm
Ixora chinensis	----	1 gm	1 gm
Triethanolamine	q.s	q.s	q.s
Water	100 ml	100 ml	100 ml

Characterization of extracts loaded gel formulation[26-28]

Physical appearance

The prepared gel formulations were evaluated for appearance, Colour, Odour, and homogeneity by visual observation.

pH determination

pH of the formulation was determined by using Digital pH meter (EI).

Viscosity determination

The viscosity of the gel formulations was determined using Brookfield viscometer with spindle no. 61at 100 rpm at the temperature of 25^0C.

Spreadability

An ideal topical gel should possess a sufficient spreading coefficient when applied or rubbed on the skin surface. This was evaluated by placing about 1 g of formulation on a glass slide. Another glass slide of the same length was placed above that, and a mass of 50 mg was put on the glass slide so that the gel gets sandwiched between the two glass slides and spreads at a certain distance. The time taken for the gel to travel the distance from the place of its position was noted down. Spreadability was determined by the following formula

$$S= M*L/T$$

Where, S-Spreadability, g.cm/s M-Weight put on the upper glass L-Length of glass slide T-Time for spreading gel in sec.

Skin irritation test

The intact skin of Wistar rats of either sex with average weight 150-200 g was used. The hairs were removed from the rat 2-3 days before the experiment. The gel was applied on the properly shaven skin of rat. The animals were treated daily for 2-3 days, and undesirable skin changes, i.e., change in colour, change in skin morphology was checked for a period of 24h and erythema and edema on the treated skin were examined.

***In-vivo* wound healing activity**

Animals protocol

IAEC Approval All animal experiments were approved by Institutional Animal Ethics Committee (IAEC).

Animal used

Weight: 200±50 gm
Strain Wistar rat
Sex: Either
Animals were housed in a group of six in separate cages under controlled conditions of temperature (22 ± 2°C). All animals were given standard diet (golden feed) and water regularly.

Excision model

The back of the animals was shaved and sterilized with 70% ethanol before 7 X 7 mm excision wound going to be create by a surgical blade from a predetermined shaved area on the back of each animal. The wound left undressed to the open environment and no local or systemic antimicrobial agents used. This model is used to monitor the rate of wound contraction. A progressive decrease in the wound area was monitored periodically at every 4th day interval. The wound contractions are measured by a tracing paper on the wounded margin and calculated as percentage reduction in wound area. The actual value is converted into percentage value taking the size of the wound at time of wounding as 100 %. The animals were randomly divided into 5 groups and each group containing 6 animals.[29] The treatments of each gel (500 mg/rats) were applied topically once a day.
Group I: Control group.
Group II: Test group treated with *Calendula officinalis* gel (Formulation I)
Group III: Test group treated with *Ixora chinensis* gel (Formulation II)
Group IV: Test group treated with (*Calendula officinalis* and *Ixora chinensis gel*) (Formulation III)
Group V: Gentamicin gel (Reference standard marketed preparation).

Wound contraction rate

The wound contraction rate was measured. It is the percentage reduction of wound size. It can also be treated as a percentage of wound protection. By using a transparency paper and a suitable marker, at a specified interval, the decrease in size of wounds was monitored and accordingly, the percentage of wound closure is accessed, which indicates the formation of fresh epithelial tissue to heal the wound. Wound contraction is expressed as a reduction in the percentage of the original wound size.

Anti-bacterial activity

Well diffusion assay

Culture of bacterial strains (*E. coli*) was spread on the Nutrient agar media (NAM). The wells were then formed for the inoculation of the samples (extract gel) given in the different concentrations, volume make-up was done till 1 ml. 100µl of the sample was loaded. The plates were allowed to incubate at 37^0 C for 48-72 hours for the best results. The bacterial suspension was standardized to 10^8CFU/ml of bacteria and kept into the shaker. Then, 100µl of the inoculum from the broth (containing 10^8 CFU/ml) was taken with a micropipette and then transferred to fresh and sterile solidified agar media plate. The agar plate was inoculated by spreading the inoculum with a sterile spreader, over the entire sterile agar surface. Four wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer. Each well was filled with different concentration of formulations (1, 2 and 3). It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24 hours at 37^oC. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of tested compounds. The zone of inhibition (ZOI) was observed and measured in mm. Zones were measured to a nearest millimetres using a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, non-reflecting background. The diameters of the zone of complete inhibition (as judge by unaided eye) were measured, including the diameter of the well.[30]

RESULTS

In phytochemical extraction the percentage yield is very crucial in order to determine the standard efficiency of extraction for a specific plant, various sections of the same plant or different solvents used. The yield of extracts received from the *Calendula officinalis* and *Ixora chinensis* is shown in Table 2. Qualitative phytochemical screening of *Calendula officinalis is* showed the presence of active metabolites such as alkaloids, flavonoids, proteins, tannin, protein, glycosides and phenols is presented Table 3 and *Ixora chinensis* showed the presence of active metabolites such as flavonoids, steroids, carbohydrates, phenols and alkaloids Table 4. In the present investigation, the *in vitro* anti-oxidant activity of extracts of *Calendula officinalis* and *Ixora chinensis* was evaluated by DPPH radical scavenging activity. The results are summarized in Table 5. The gel was evaluated based on color, appearance, and homogeneity. When tested, gel turned out to be a somewhat yellowish color. The findings were shown in Table 6. The pH of all prepared formulation ranged from 6.3- 6.7. The pH of the prepared gel formulation was considered to be acceptable to avoid the risk of irritation upon application to the skin. The results were shown in Table 7. Viscosity is an important property of fluids which describes a liquids resistance to flow and is related to the internal friction within the fluid. This rheological property helps in determining consistency and also the diffusion rate of drug from gel. The measurement of viscosity of the prepared gel was done with Brookfield viscometer with spindle no: 61. Spreadability denotes the extent of area to which the gel readily spreads on application to skin or the affected part. Spreadability of different gel formulation was studied. The formulations produced good spreadability. Results of skin irritation test indicate that prepared gels were not produce irritation, redness, or edema on application and free from dermatological reaction. Wound contraction is another parameter used to assess wound healing. Significant wound contraction was shown in Table 8 & Figure 1. The *in vitro* antibacterial activities of the extracts of Formulation 1, Formulation 2, Formulation 3 and standard gel have been investigated. Antibacterial activity was performed against *E. coli* by well diffusion assay. Formulation 3 and standard gel showed best zones of inhibition Table 9 & Figure 2.

Table 2: Percentage yield of crude extracts of *Calendula officinalis* and *Ixora chinensis* extracts.

S.no	Plant name	Solvent	Theoretical weight	Yield(gm)	% yield
1	*Calendula officinalis*	Pet. ether	299	1.47	0.49%
2		Methanol	289.12	6.55	2.26%
3	*Ixora chinensis*	Pet. ether	298	1.35	0.45%
4		Methanol	299.3	5.09	1.70%

Table 3: Phytochemical testing of *Calendula officinalis.*

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	Methanolic extract
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Present
1.2	Mayer's reagent test	Absent	Present
1.3	Wagner's reagent test	Absent	Present
1.3	Hager's reagent test	Absent	Present
2.	**Glycoside**		
2.1	Borntrager test	Present	Present
2.2	Legal's test	Present	Present
2.3	Killer-Killiani test	Present	Present

3.	**Carbohydrates**		
3.1	Molish's test	Absent	Absent
3.2	Fehling's test	Absent	Absent
3.3	Benedict's test	Absent	Absent
3.4	Barfoed's test	Absent	Absent
4.	**Proteins and Amino Acids**		
4.1	Biuret test	Absent	Present
5.	**Flavonoids**		
5.1	Alkaline reagent test	Present	Present
5.2	Lead Acetate test	Present	Present
6.	**Tannin and Phenolic compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponin**		
7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Present	Absent
8.2	Libbermann-Burchard's test	Present	Absent

Table 4: Phytochemical testing of and *Ixora chinensis.*

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	Methanolic extract
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Absent
1.2	Mayer's reagent test	Absent	Absent
1.3	Wagner's reagent test	Absent	Absent
1.3	Hager's reagent test	Absent	Absent
2.	**Glycoside**		
2.1	Borntrager test	Absent	Absent
2.2	Legal's test	Absent	Absent
2.3	Killer-Killiani test	Absent	Absent
3.	**Carbohydrates**		
3.1	Molish's test	Present	Present
3.2	Fehling's test	Present	Present
3.3	Benedict's test	Present	Present
3.4	Barfoed's test	Present	Present
4.	**Proteins and Amino Acids**		
4.1	Biuret test	Absent	Absent
5.	**Flavonoids**		
5.1	Alkaline reagent test	Absent	Present
5.2	Lead Acetate test	Absent	Present
6.	**Tannin and Phenolic Compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponin**		
7.1	Foam test	Absent	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Absent	Present
8.2	Libbermann-Burchard's test	Absent	Present

Table 5: % Inhibition of ascorbic acid, methanol extract of *Calendula officinalis* and *Ixora chinensis* using DPPH method.

S. No.	Concentration (µg/ml)	% Inhibition		
		Ascorbic acid	Methanol extract of *Calendula officinalis*	Methanol extract of *Ixora chinensis*
1	20	50.752	43.891	44.168
2	40	55.591	49.621	49.460
3	60	66.129	50.918	51.403
4	80	72.150	55.567	54.535
5	100	85.161	60.324	60.151

6	Control	0.930	0.925	0.926
IC 50		22.67	49.38	49.56

Table 6: Organoleptic properties.

S. No	Parameters	Results
1.	Appearance	Semisolid gel
2.	Color	Slightly yellowish
3.	Homogeneity	Absence of aggregates

Table 7: pH, viscosity and spreadability test.

S. No	Formulation	pH	Viscosity determination (cps)	Spreadability Test (gm.cm/sec)	Skin Irritation Study
1.	Formulation1	6.3	5455±0.71	11.99	Not irritant observed
2.	Formulation 2	6.5	5509±0.56	11.19	Not irritant observed
3.	Formulation 3	6.7	5691±0.82	13.20	Not irritant observed

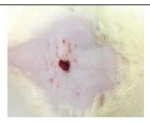
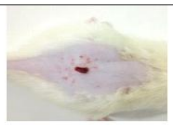
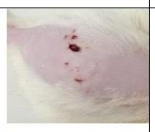
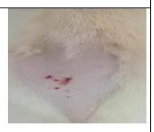
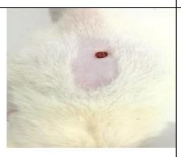
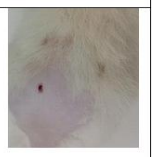
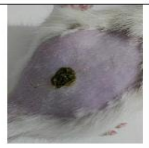
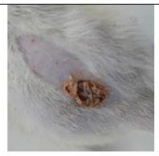
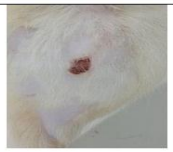
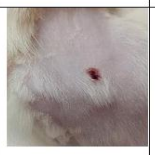
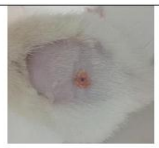
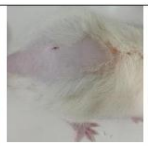
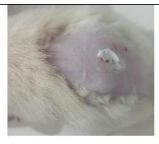
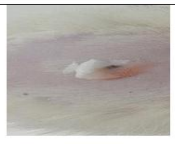
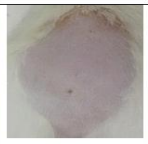
Table 8: Percentage wound closure in various treatment groups.

Sr. No.	Formulation	Area of wound during different days of observation (%)				
		4 days	8 days	12 days	16 days	21days
1	Control	8.31±0.712	8.45±0.814	8.49±0.782	8.39±0.884	8.42±0.981
2	Formulation I	8.99±0.404	25.11±0.435	38.99±0.707	58.99±0.542	68.94±0.553
3	Formulation II	8.37±0.782	21.11±0.523	50.44±0.551	67.40±0.553	74.86±0.582
4	Formulation III	11.07±0.859	29.12±0.952	56.43±0.667	74.10±0.335	80.99±0.382
5	Reference Standard (Gentamicin gel)	12.41±0.743	33.08±0.727	62.02±0.642	91.98±0.643	94.74±0.252

Table 9: Antimicrobial activity.

S. No	Sample name	Zone of Inhibition (mm)
1	Formulation 1	10 mm
2	Formulation 2	13 mm
3	Formulation 3	15 mm

Table 10: Images of wound closure in various treatment groups.

Group	4 Day	8 Day	12 Day	16 Day	21 Day
Control					
Formulation I *Calendula officinalis gel*					
Formulation II *Ixora chinensis gel*					
Formulation III Polyherbal gel					
Reference Standard(Gentamicin gel)					

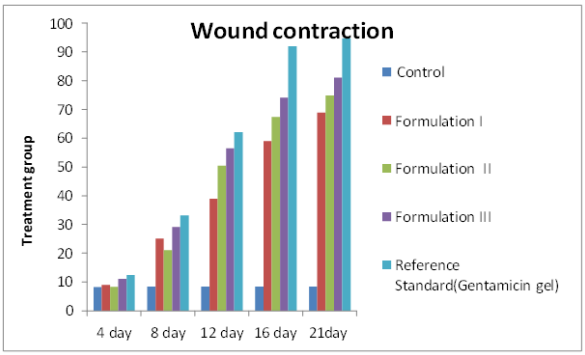


Figure 1: Evaluation of wound healing activity.

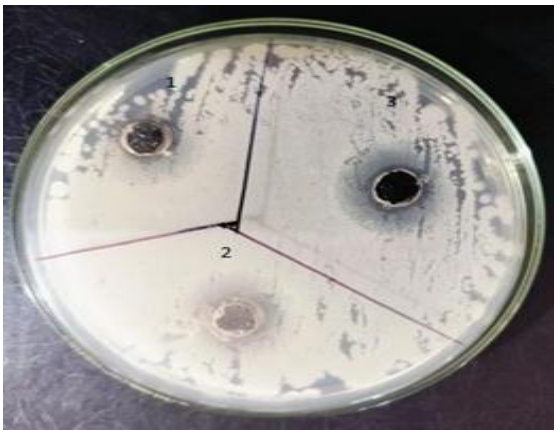


Figure 2: Anti-bacterial activity.

DISCUSSION

Qualitative phytochemical screening of *Calendula Officinalis is* showed the presence of active metabolites such as Alkaloids, flavonoids, proteins, tannin, saponin, protein, glycosides and phenols is presented and *Ixora chinensis* showed the presence of active metabolites such as flavonoids, steroids, carbohydrates, phenols and alkaloids. DPPH radical scavenging activity of *Calendula Officinalis* extract exhibited percent inhibition 60.32% and its IC 50value was found to be 49.38µg/ml and *Ixora chinensis* extract exhibited percent inhibition 60.15% and its IC 50value was found to be 49.56µg/ml. Ascorbic was used as a reference compound which exhibited percent inhibition 85.16% and showed IC 50 value of 22.67µg/ml. The pH of all prepared formulation ranged from 6.3- 6.7. The pH of the prepared gel formulation was considered to be acceptable to avoid the risk of irritation upon application to the skin. The measurement of viscosity of the prepared gel was done with Brookfield viscometer with spindle no: 7. the results were found to be Formulation 1- 5455, Formulation 2 - 5509 and Formulation - 5691 cps. Spreadability denotes the extent of area to which the gel readily spreads on application to skin or the affected part. Spreadability of different gel formulation was studied. The formulations produced good spreadability. Skin irritation test indicate that prepared gels were not produce irritation, redness, or edema on application and free from dermatological reaction. The *in vitro* antibacterial activities of the extracts of Formulation 1, Formulation 2, Formulation 3 and standard gel have been investigated. Antibacterial activity was performed against *E. coli* by well diffusion assay. Formulation 3 and standard gel showed best zones of inhibition. Further we were performed the wound healing studies like contraction of wound model for 21 days. For wound healing activity, the extracts were loaded in the gel. The development wound curing activity by excision model was evaluated by wound shrinkage of the excision wound of different groups Group I: Control group, Group II: Test group treated with *Calendula Officinalis* gel (Formulation I), Group III: Test group treated with *Ixora chinensis* gel (Formulation II), Group IV: Test group treated with (Polyherbal gel) (Formulation III), Group V: Gentamicin gel (Reference Standard Marketed Preparation). The wound contraction studies revealed that the wound contraction increases on increasing the concentration of herbal extract. The study also reveals that the better activity of polyherbal formulation may be due to the synergistic action of the plant's constituents present in the formulation. Thus, the prepared topical gels possess a versatile approach in healing the wound contraction.

CONCLUSION

Herbal extracts gel containing the extracts of *Calendula officinalis* and *Ixora chinensis* promotes wound healing in Wistar rats with a comparison of the synthetic gel formulation. The wound contraction rate was higher in the treatment of gel containing the combination of *Calendula officinalis* and *Ixora chinensis.*

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