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DEVELOPMENT AND FORMULATION OF POLYHERBAL OINTMENT AND EVALUATION OF ANTIOXIDANTS AND ANTIMICROBIAL POTENTIALS

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ABSTRACT

The objective of the present investigation was to formulate polyherbal ointment formulation for antioxidant and antibacterial action. Three plants *Delonix regia* leaves, *Annona squamosa* leaves and *Curcuma longa* rhizomes were used in the study and the methanolic extracts of the plant parts were used as the active ingredient of the ointments.The findings suggest the presence of alkaloids, glycosides, phenolics, terpenoids, proteins and flavonoids in the extracts. The total phenolic content in the extracts was determined using Folin-Ciocalteu method and was found to be 27.12, 31.57 and 22.36 GAE mg/g respectively.The polyherbal ointment formulations were prepared using simple ointment base and varying concentration of the DRE, ASE and CLE. The pH of all the formulations was almost equal to the pH of skin and ranged between 5.2 to 5.5. The spreadability value of the formulations revealed good spreading capacity.The antioxidant activity was determined using hydroxy radical scavenging assay method and reducing power assay method. It was found that formulation F2 was having highest antioxidant capacity of the three formulations in both the methods.The *in vitro* antibacterial action was determined using *Staphylococcus aureus* as the common bacterial strain utilizing disc diffusion method and the formulation F2 exhibited the highest zone of inhibition.

INTRODUCTION

Herbal medicine, sometimes referred to as Herbalism or Botanical Medicine, is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history for their therapeutic or medicinal value. Herbal medicines are plant based medicines made from differing combinations of plant parts. E.g. leaves, flowers and roots. Each part can have different medicinal uses and the many types of chemical constituents require different extraction methods. Both fresh and dried plant matter are used depending upon the herb. Herbal medicines which formed the basis of health care throughout the world since the earliest days of mankind are still widely used, and have considerable importance in international trade. An herb is a plant

or plant part valued for its medicinal, aromatic qualities. Herbal plants contain therapeutically active chemical substances that act upon the body.[1] Herbal medicines widely used in health-care in both developed and developing countries are complex chemical mixtures prepared from plants and are limited in their effectiveness because they are poorly absorbed when taken orally.

The use of medicinal plants has attained a commanding role in health system all over the world. Many countries in the world, that is, two-third of the world's population depends on herbal medicine for primary health care. The reasons for this is because of their better cultural acceptability, better compatibility and adaptability with the human body and pose lesser side effects.[2] These herbal supplements do not have any harmful side effects that might disturb physical health unlike synthetics. For every synthetic drug present there is an alternative herbal drug. Man in his everlasting search for cure of serious illnesses, at last finds his way to our indigenous medicine. Indigenous medicines include Herbal, Ayurveda, Siddha medicine, Unani, ancient Iranian medicine, Islamic Medicine, Traditional Chinese medicine and Traditional African medicine other medicinal practices all over the world.

Advantages of Herbal Medicine

- Herbal medicine have long history of use and better patient tolerance as well as acceptance.
- Medicinal plants have a renewable source, which is our only hope for sustainable supplies of cheaper medicines for the world growing population.
- Availability of medicinal plants is not a problem especially in developing countries like India having rich agro-climatic, cultural and ethnic biodiversity.
- The cultivation and processing of medicinal herbs and herbal products is environmental friendly.
- Cost-effectiveness-prescription drugs cost much more money than herbal medicines.
- Lower side effects herbal medicines are generally a far heal their solution than prescription drugs due to potential harmful side effects caused by unpredicted body chemistry interactions.

Disadvantage of Herbal Medicines

- Procedures for pure and genuine herbs are not available, so sub-standard and spurious herbs are there in the market.
- Identification of exact mechanism and pharmacology of all herbal medicine is not available.
- Adulteration ratio is very high.
- Clinical and toxicological data was not available for all herbal medicine.
- There is no much information available on the safety measures.
- All herbal medicine are not tested with important parameters like microbial content, heavy metals content and pyrogens etc.

Herbal formulation[7]

An herbal formula consists of a selective combination of individual herbal ingredients that are formulated for a specific ailment or group of disease-conditions. When herbs are combined together, they become more

potent and effective within the body than single herb due to their activating or catalyzing influence upon one another. These combinations act as powerful catalysts (with synergistic effects) in order to activate over own individual healing energies (or vital force) which permeate the entire organism and reside in each and every cell in our bodies.

WHO Guidelines for Standardization of Herbal Formulation[4,8]

Standardization is an important aspect for maintaining and assessing the quality and safety of the polyherbal formulation as these are combinations of more than one herb to attain the desire therapeutic effect. Standardization minimizes batch to batch variation, assures safety, efficacy, quality and acceptability of the polyherbal formulations. Standardization involves:

- Quality control of crude drugs material, plant preparations and finished products.
- Stability assessment and shelf life.
- Safety assessment, documentation of safety based on experience or toxicological studies.
- Assessment of efficacy by ethnomedical informations and biological activity evaluations.

Herbal Ointments

Skin care creams are defined as a semi-solid emulsion containing mixtures of oil and water which can be used to moisturizes the skin of the face and any other parts of the body(1).[9] Skin is the organ with the largest surface area in human body, having two main layers, the epidermis which protects our body and prevents water loss, and the dermis containing various glands, blood vessels, and receptors. The integrity of skin damage by various factors, including advanced age, stressful lifestyle, exposure to ultraviolet lights, usage of inappropriate skin cleansing agents, hormonal changes, and others. As a result, many people exhibit mild signs and symptoms of deteriorated skin such as wrinkles and dry skin. A regular skin care is required to prevent inflammation and infections such as acne vulgaris and ulcerations.[10]

Herbal ointments are the medicinal preparations intended to be placed in contact with the various external parts of the human body that manifest beneficial topical actions and provide protection against degenerative skin conditions.[11] The preparations of natural ingredients have been traditionally used for skin care purposes in past centuries. Anti-inflammatory, anti-allergy, moisturizing, pro-collagen, anti-aging, anti-hyper pigmentation, wound healing and free radical scavenge action of herbal ingredient have been discovered by clinical studies.[12]

2. MATERIAL AND METHODS

Collection, identification and preparation of the plant material

The leaves of *Delonix regia* were collected from the local surrounding of Bhopal, Madhya Pradesh in the month of November and authenticated at MFP-PARC, Bhopal. The leaves of *Annonasquamosa* were collected from the local surrounding of Bhopal, Madhya Pradesh in the month of November and authenticated at MFP-PARC, Bhopal. Immediately after collection, the plant material were cleaned with distilled water and dried in shade, powdered using low speed in blender and stored in air tight containers.

Preparation of plant extracts[15]

The powdered material was weighed and individually extracted using hot continuous extraction method utilizing soxhlet apparatus. Briefly, 100 g of the powdered material was filled in the thimble and thimble was placed in the extractor of the soxhlet apparatus. The assembly was fixed properly and 150 mL of solvent (methanol) was flown down the thimble to saturate the material. The heating of the solvent was started when the solvent filled the flask attached to the extractor. Extraction was carried out for 8 h for *Delonixregia*, 11 h for *Curcumalonga* and 6 h for *Annonasquamosa*. The end of extraction was considered when the siphon tube displayed clear solvent.

Phytochemical screening of the extracts[16]

All the extracts (*Delonix regia* extract, DLE; *Annonasquamosa* extract, ASE; *Curcumalonga* extract, CLE) were evaluated by phytochemical qualitative reactions for identifying the presence or absence of usual plant secondary metabolites. The screening was performed for triterpenes/steroids, alkaloids, glycosides, flavonoids, saponins, tannins, and phenolic acids. The color intensity or the precipitate formation was used as analytical responses to these tests.

Total Phenolic Content[17]

For total phenolic content (TPC) determination, 200 µL of extract was mixed in methanol and was mixed with 1.4 mL purified water and 100 µL of Folin-Ciocalteu reagent. After 5 min, 300 µL of 20%Na_2CO_3 aqueous solution was added to it and the mixture was allowed to stand for 2 h. The absorbance wasmeasured at 765 nm with a UV-Vis spectrophotometer. Standard solutions of gallic acid (10-100 ppm)were similarly treated to plot the analytical curve. The control solution contained 200 µL of methanoland suitable reagents, and it was prepared and incubated under the same conditions as the rest of thesamples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the drysample.

Formulation of polyherbal ointment

Ingredients of Simple Ointment Base

Wool fat - 5.0g

Hard paraffin - 5.0g

Cetostearyl alcohol- 5.0g

White soft paraffin- 85.0g

Hard paraffin and cetostearyl alcohol were taken in a porcelain dish and kept on water-bath at 70°C. Wool fat and white soft paraffin were added to this mixture and stirred until all the ingredients werein molten state.

Formula for the ointment

The extracts were incorporated into the ointment base to obtain the polyherbal formulation as per the formula.

Table 6.1 Formula for polyherbal ointment

S. No.	Ingredient	F1	F2	F3
1	DRE (mg)	0.5	0.5	1.0
2	ASE (mg)	0.5	1.0	0.5
3	CLE (mg)	0.5	1.0	0.5
4	Simple Ointment Base (mg)	98.5	97.5	97.0

The extracts were incorporated into the molten ointment base and it was allowed to solidify for preparing the polyherbal ointment. The prepared ointments were characterized for physicochemical properties, antioxidant and antimicrobial actions.

Physiochemical Characterization of Ointment

pH

The pH meter was calibrated using standard buffer solution. About 0.5g of the ointment was weighed and dissolved in 50.0 ml of distilled water and set aside for 2 h and then the pH was measured.

Spreadability

The time required to separate the two slides was measured as spreadability. Spreadability was calculated by following formula

$$S=M\times L/T$$

Where, S= Spreadability

M = Weight tide to the upper slide

L= Length of glass slide

T= Time taken to separate the slides

Pharmacological characterization of the ointments

The polyherbal ointments were evaluated for their antioxidant and antimicrobial actions using *in vitro* models.

Determination of Antioxidant Action[18]

The antioxidant potential of the polyherbal ointment was estimated using reducing power method and hydroxyl radical scavenging assay. The % hydroxyl radical scavenging activity is calculated by the following formula

$$\% HRSA = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Where, HRSA is the Hydroxyl Radical Scavenging Activity, Abs control is the absorbance of control and Abs sample is the absorbance of the extract.

Antimicrobial action of polyherbal ointment[19]

The antibacterial tests were conducted on *Staphylococcus aureus* which is the most common type of bacteria of skin infections. The antibacterial activity of the compounds was assessed by disc diffusion.

Preparation of Nutrient broth:

- Nutrient broth powder – 37.2 g
- Distilled water - 1000 ml

Nutrient broth was prepared by dissolving all the ingredients and adjusting the pH adjusted to 7.2 and autoclaved at 15 lbs pressure for 20 min in an autoclave. One day before the testing, the microorganisms were sub-cultured into sterile nutrient broth and incubated at 37°C for 24 h. The culture growth thus obtained was used as inoculum for the antibacterial testing.

Preparation of nutrient agar media

The nutrient agar media was prepared by using the following ingredients.

- Nutrient agar – 28.6 g
- Distilled water - 1000 ml

The specified amount of nutrient agar powder was dissolved by heating on a water bath. and the volume of final solution is made up to 1000 ml with distilled water. The above prepared nutrient agar media was sterilized by autoclave at 121°C for 20 minutes at 15 lbs pressure.

Preparation of test solution

The test solution was prepared by making solution of ointment in DMSO (600 µg/mL).

Preparation of standard solution

Ciprofloxacin was used as the standard drug at concentration of 100 µg/ml prepared in distilled water.

Procedure of antibacterial testing

The sterilized media (nutrient agar) was cooled to 45°C with gentle shaking for uniform cooling and then inoculated with 18-24 h old bacterial subculture under aseptic conditions in a laminar air flow bench and mixed well by gentle shaking. This was poured in to sterile Petri dishes and allowed to set. After solidification, wells were dug in the plate and the standard drug or the test solution of ointment filled in the

well. These Petri dishes were kept in the laminar air flow unit undisturbed for one-hour diffusion at room temperature and then for incubation at 37°C for 24 h in an incubator. The extent diameter of inhibition after 24 h was measured as the zone of inhibition in millimeters (mm).

3. RESULTS AND DISCUSSION

3.1 Result of Extraction Yields

The extraction yield of various plants used in the study using methanol as the extracting solvent is presented in Table 3.1.

Table 3.1 Extraction yields

Extract	Yield	Appearance	Texture
DRE	12.2	Brown	sticky
ASE	13.6	Green	powder
CLE	17.1	Dark brown	sticky

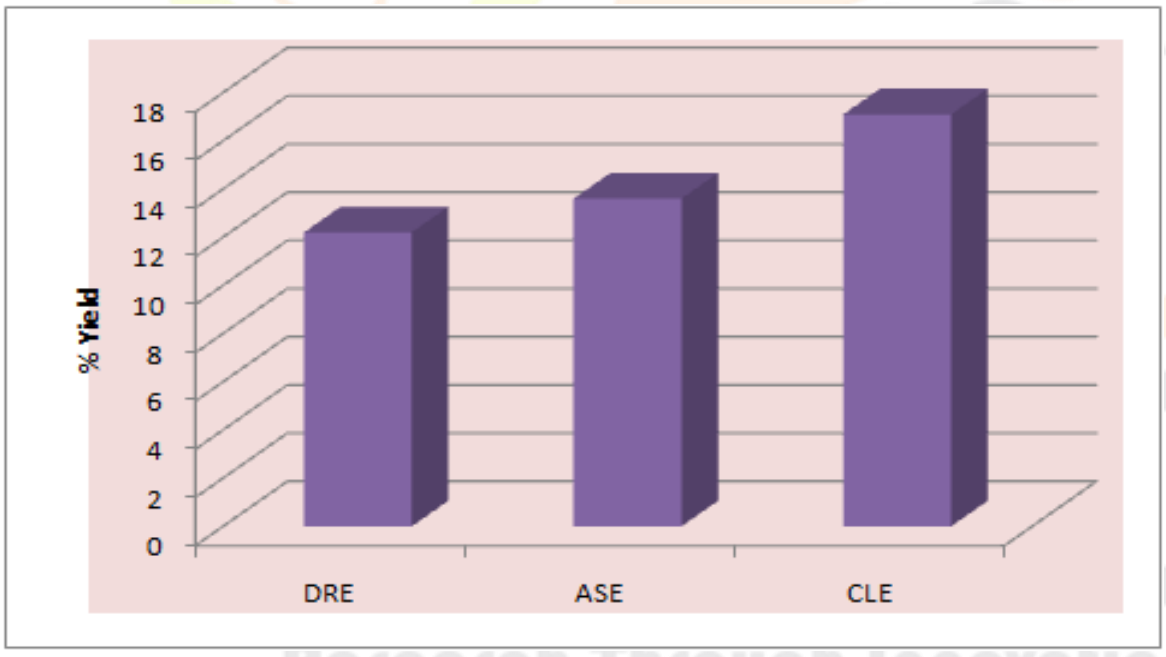

Figure 3.1 Extraction yield of crude extracts in methanol

3.2 Result of Phytochemical Screening

Result of Phytochemical Screening Show in Table no. 3.2

Table 3.2 Phytochemical screening of extracts

Phytochemical Test	Observation	CLE	ASE	DRE
Mayer's reagent	cream colour precipitate	+	+	+
Hager's reagent	yellow colour precipitate	-	+	+
Wagner's reagent	reddish brown precipitate	+	+	+
Dragendorff's reagent	reddish brown precipitate	+	+	+
Froth test	Frothing is seen	+	+	-
Kedde's Test	No color	-	-	-
Bontrager's Test	Rose pink or red color in the ammonical layer	+	+	-
Keller-Kiliani	Color in acetic acid layer	-	-	-
Ferric chloride	Blue green color	+	+	+
Gelatin Solution	White precipitate	+	+	+
Alkaline reagent test	Yellow to red precipitate	+	+	+
Vanillin HCl test	Purplish red color	+	+	+
Shinoda test	red color	+	+	+
Alkaline reagent test	Yellow color that turns red on acidification	+	+	+
Zinc HCl reductino test	red color	+	+	+
Millon's Test	white precipitate, turns red on heating	-	+	-
Ninhydrin Test	Voilet color	-	+	-
Salkowski Test	Yellow color in lower layer	+	+	-

3.3 Total Phenolic content determination

The crude extracts (CLE, DRE and ASE) were processed for determining the total phenolics in them using FolinCiocalteu method. Gallic acid standard curve was prepared at 765 nm and the total phenolic content has been reported as gallic acid equivalent per mg.

Table 3.3 TPC of extracts

Extract	Total phenolic content (GAE mg/g)
DRE	27.12
CLE	31.57
ASE	22.36

3.4 Physicochemical characterization of polyherbal ointments

The polyherbal ointment formulations were prepared using simple ointment base and varying amount of the three extracts. The formulations were characterized for pH and spreadability in order to ascertain its applicability on skin. The physicochemical properties of the ointments are presented in Table 7.5. All the ointments exhibited optimum viscosity and spreadability. The pH of all the formulations was in the range of the pH of the skin (5.0-5.5).

Table 3.4 Physical characterization of polyherbal ointments

Formulation Code	pH	Viscosity (cp)	Spreadability (mm)
F1	5.2	1698	61
F2	5.5	1675	64
F3	5.3	1691	62

3.5 Antioxidant activity of polyherbal ointments

Table 3.5 Reducing Power of F1

Conc (µg/mL)	Absorbance at 700 nm		
	10 min	20 min	30 min
50	0.036	0.031	0.028
100	0.068	0.057	0.049
150	0.105	0.101	0.089
200	0.153	0.142	0.103
250	0.189	0.174	0.135

Values are mean ± SEM of six determinations

Table 3.6 Reducing Power of F2

Conc (µg/mL)	Absorbance at 700 nm		
	10 min	**20 min**	**30 min**
50	0.056	0.051	0.042
100	0.097	0.089	0.078

150	0.139	0.127	0.11
200	0.176	0.168	0.153
250	0.221	0.201	0.191

Values are mean ± SEM of six determinations

Table 3.7 Reducing Power of F3

Conc (µg/mL)	**Absorbance at 700 nm**		
	10 min	20 min	30 min
50	0.042	0.037	0.029
100	0.089	0.07	0.056
150	0.128	0.121	0.105
200	0.165	0.153	0.141
250	0.205	0.191	0.176

Values are mean ± SEM of six determinations

The reducing potential was found to be dose dependent and the followed the order F2>F3>F1. The results indicate a dose dependent hydroxy radical scavenging activity (HRSA) in the ointments and also reveal that the F2 was able to inhibit the hydroxy radical more than the F3 and F1. At concentration of 250 µg/mL,F-2 was able to scavenge the hydroxy radical at par with ascorbic acid (the standard solution).

Table 3.8 % HRSA of F1

Conc (µg/mL)	**HRSA Scavenging %**	
	Extract	**Ascorbic acid**
50	11.1±0.52	89.3±0.23
100	19.6±0.24	-
150	31.3±0.49	-
200	43.4±0.31	-
250	53.1±0.33	-

Values are mean ± SEM of six determinations

Table 3.9 % HRSA of F2

Conc (µg/mL)	**HRSA Scavenging %**	
	Extract	Ascorbic acid
50	18.3±0.46	89.3±0.23
100	33.2±0.21	-
150	56.8±0.61	-
200	70.4±0.49	-
250	87.6±0.67	-

Values are mean ± SEM of six determinations

Table 3.10 % HRSA of F3

Conc (µg/mL)	HRSA Scavenging %	
	Extract	Ascorbic acid
50	16.2±0.46	89.3±0.23
100	33.3±0.16	-
150	46.8±0.53	-
200	67.1±0.31	-
250	81.4±0.29	-

Values are mean ± SEM of six determinations

A comparative analysis of the results of hydroxyl radical scavenging for the different formulations is presented in Figure 7.3.

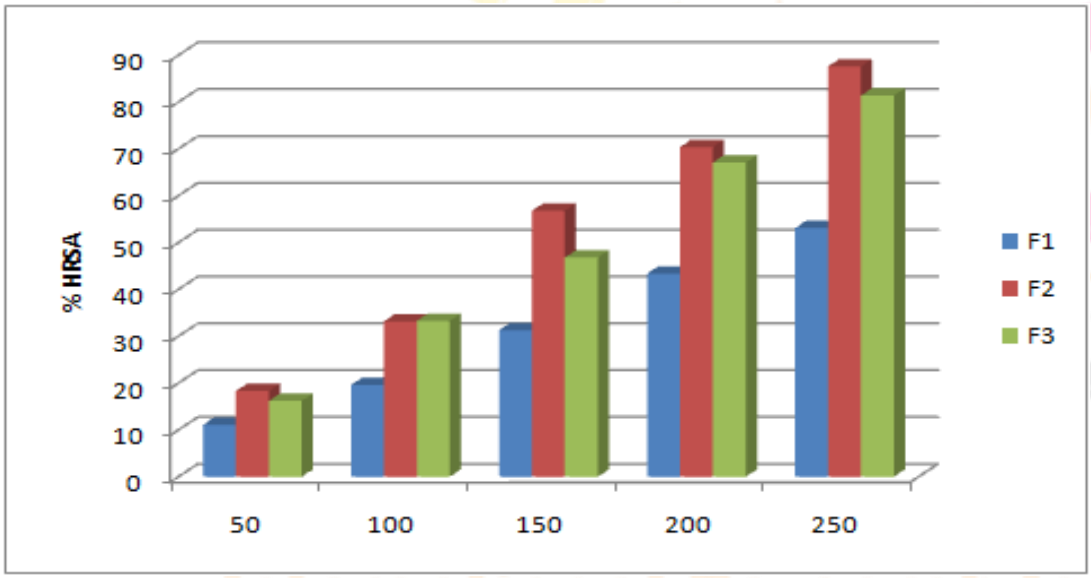

Figure 3.2 Comparative chart of hydroxy radical scavenging by polyherbal formulations

3.6 Antibacterial activity of PHGs

All the polyherbal ointments were subjected to evaluation of *in vitro* antibacterial efficacy in order to establish the best combination for antibacterial activity. The cup and plate method was used for evaluation of the antibacterial action of the ointments. The zone of inhibition (zone in which the growth of microorganism did not occur) was observed for each ointment. Gram positive bacteria like *Staphylococcus aureus* are linked to skin conditions. Hence *S. aureus* was used for testing the antibacterial activity of the ointments. The zone of inhibition was measured for each ointment(figure 7.4) and it was found that the F2exhibited the highest zone of inhibition (table 7.12).

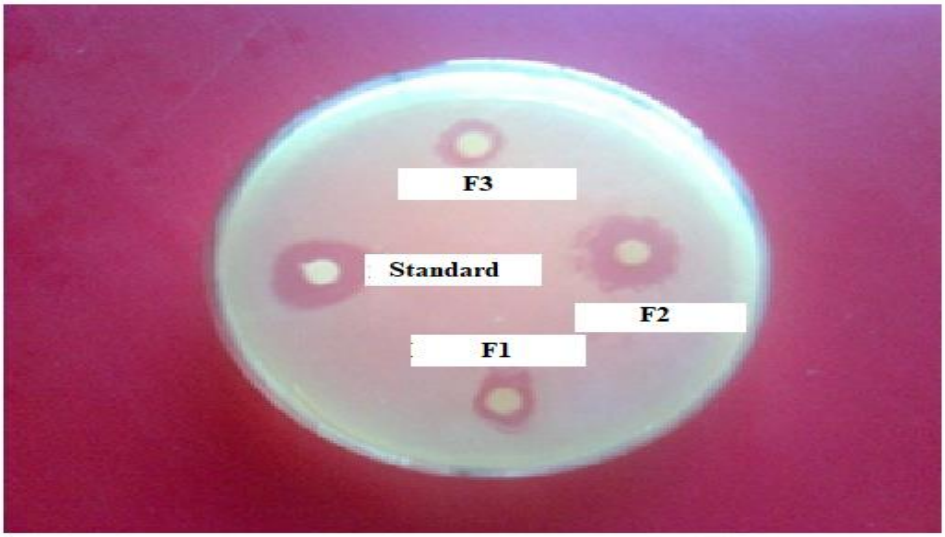

Figure 3.3 : Zone of inhibition of ointments in *S. aureus*inoculated nutrient agar plate

Table 3.11: Antibacterial activity of polyherbal ointments

Formulation Code	Zone of Inhibition (mm)
F1	14
F2	27
F3	22

SUMMARY

The objective of the present investigation was to formulate polyherbal ointment formulation for antioxidant and antibacterial action. Three plants *Delonix regia* leaves, *Annona squamosa* leaves and *Curcuma longa* rhizomes were used in the study and the methanolic extracts of the plant parts were used as the active ingredient of the ointments. The extraction yield was found to be 12.2, 13.6 and 17.1% for DRE, ASE and CLE respectively. The findings suggest the presence of alkaloids, glycosides, phenolics, terpenoids, proteins and flavonoids in the extracts. The total phenolic content in the extracts was determined using Folin-Ciocalteu method and was found to be 27.12, 31.57 and 22.36 GAE mg/g respectively.

The polyherbal ointment formulations were prepared using simple ointment base and varying concentration of the DRE, ASE and CLE. The ointments were assessed for physical characters, antioxidant action and antibacterial actions using in vitro methods. The pH of all the formulations was almost equal to the pH of skin and ranged between 5.2 to 5.5. The spreadability value of the formulations revealed good spreading capacity. The antioxidant activity was determined using hydroxy radical scavenging assay method and reducing power assay method. It was found that formulation F2 was having highest antioxidant capacity of the three formulations in both the methods. The *in vitro* antibacterial action was determined using *Staphylococcus aureus* as the common bacterial strain utilizing disc diffusion method and the formulation F2 exhibited the highest zone of inhibition.

CONCLUSION

Polyherbal topical formulation containing *Delonix regia*, *Annona squamosa* and *Curcuma longa* was successfully prepared with pH within the limits in consonant with stratum corneum. The formulations were found to possess good antioxidant and antibacterial actions. Further designing and *in vivo* studies are needed to optimize the proper ratios of the extracts for preparation of the ointment.

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