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Formulation and Evaluation of Polyherbal Ointment for Wound Healing and Antimicrobial Activity

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ABSTRACT

Herbal therapy and herbal drugs predominates in traditional medicine as well as in alternative medicine practiced in the developed world. Among the various indications where traditional herbal medicines are used, skin related disorders is ranked top. Thus, the main objective of the present study is to formulate and evaluate a polyherbal ointment for wound healing and antimicrobial activity. Ointments were formulated using hydroalcoholic extracts (soxhlet extraction) of *Piper nigrum* and *Curcuma longa* were evaluated for its physicochemical property, wound healing and antimicrobial activity. Ointments were prepared using different concentrations of the extracts such as 2%, 4% w/w by fusion method using emulsifying ointment as base. Formulations were tested for its physicochemical properties like pH, spreadability, extrudability and viscosity and gave satisfactory results. The prepared formulations were also stable at various temperature. Further, extract were evaluated for its antimicrobial activity against Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis and Proteus vulgaris by paper disc diffusion method. All the extract showed predominant activity against selected species. Formulations were also evaluated for wound healing activity. Hence an attempt was made to formulate a polyherbal ointment, and to evaluate for its physical parameter, the formulated ointment was compared with the standard ointment (povidone iodine). Overall result of this study reveals that this is an effective polyherbal ointment.

Keywords: *Piper nigrum, Curcuma longa,* Wound healing activity, Antimicrobial activity.

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1. INTRODUCTION:

There are many causes for the increased use of the herbal medicines. These may scale from the appeal of products from 'nature' and the understanding that such product are 'steady' (or at least 'safer' than conventional medicines, which are often contemptuously referred to as 'drugs'), to more complex reasons related to the theoretical views and religious beliefs of individuals. (1)

The emphasis on use of medicinal plants had either to be placed on the treatment rather than prevention of diseases. Over 90% of traditional medicine remedies contain medicinal plants, the medicinal plants that have been implicated with preventive measures in diseases control strategies. (2)

The 16th and 17th centuries were golden era of herbal medicines. The more and more plants incorporated during 18th and 19th centuries in America. After that, scientists started making synthesizing plant compounds by their own.(3)

The wound may be defined as a loss or breaking of cellular and anatomical or functional continuity of living tissues. Wound healing is a biological process that is initiated by trauma and often terminated by scar formation. Thus healing is essentially a survival mechanism and represents an attempt to maintain normal anatomical structure and function. (4)

The word antimicrobial was derived from the Greek words anti (against), mickros (little) and bios (life) and refers to all agents that act against microbial organisms. The introduction of antimicrobial agents into general clinical use represents one of the land- Mark medical advances of modern medicine.(5)

Pharmaceutical semisolid preparations include ointments, pastes, cream, emulsions, gels, and rigid foams. Their common property is the ability to cling to the surface of application for reasonable duration before they are washed

or worn off. This adhesion is due to their plastic, rheological behavior, which allows the semisolids to retain their shape and cling as a film until acted upon by an outside force, in which case they deform and flow. (6)

Many medicaments meant for topical application to intact or broken skin or to mucous membranes, have been presented in the form of semisolid consistency variously designated as ointment, creams, salves, pastes etc. and used mainly as protective or emollient for the skin. (7)

The skin has three layers. Beneath the surface of the skin are nerves, nerve ending, glands, hair follicles and blood vessels. A typical human skin surface is known to include, on the normal 40-70 hair follicles and 200-300 sweat ducts on every square centimeter of the skin. (8)

2. MATERIAL AND METHODS:

2.1 Collection of plant material

The fruits of *Piper nigrum* and rhizome of *Curcuma longa* were purchased from the local market of Ahmednagar district of Maharashtra.

2.2 Extraction process

The fruits of *piper nigrum* and the rhizome of *Curcuma longa* were procured and pulverized in electrical grinder. About 150 gm of powdered Fruits and rhizomes were used for extraction, powder were pass through 120 mesh sieve to remove fine powder and coarse powder and coarse powder was used for extraction. (9) The solvent used for extraction was alcohol & water (7:3).

Technique: Soxhlet apparatus

The powdered fruits of *Piper nigrum* and rhizomes of *Curcuma longa* were extracted with solvent for removal of coloring matter by defatting process using continuous soxhlet extraction method. After complete defatting the defatted powder were condensed with solvent for 30 hrs. Extraction temperature was maintained at 50^0 c. The extract was filtered and concentrated to get thick paste and after it freeze dried to get powder. The extract was stored in air tight container. (10)

2.3 Formulation of ointment:

Table.1 Formula for Ointment base

Sr. No.	Ingredients	Quantity
01	Stearic acid	15 g
02	White wax	2 g
03	Yellow Vaseline	8 g
04	Triethanolamine	1g
05	Propylene glycol	8 g
06	Purified Water	quantity sufficient to 100 g

Table.2 Formula for Herbal Ointment

Sr. No.	Extract Use	Quantity of extract in gm for given formula	
		F1(2%)	**F2(4%)**
1.	Hydroalcoholic extract of *Piper nigrum*	0.5 gm	1 gm
2.	Hydroalcoholic extract of *Curcuma longa*	0.5 gm	1 gm
3.	Ointment Base	Q.S.100 gm	Q.S.100 gm

2.3 Evaluation of Ointment Formulation:

a. Color and Odour: -

Color and Odour of all ointments was examined by visual examination. (11)

b. pH:-

The pH of ointment formulation was determined by using digital pH meter. 1gm of ointment was dissolved in 100 ml of distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate and average values were depicted. (12)

c. **Spreadability Test: -**

Spreadability is expressed in terms of time in seconds taken by two slides. To flip out or from cream when placed in between the the slides under the direction of certain load. Laser the time taken for separation of two slides, better the spread ability. It is calculated by using the formula. S = M x L/T, where, M = Weight tied to upper slide, S = Spread ability of formulation, L = Length of Glass Slides, T = Time taken to separate the slides. (12)

d. Extrudability:

A closed collapsible tubes containing ointments was pressed firmly at the cramped end. When the cap was removed, ointment extruded until pressure dissipated. Weight in grams required to extrude 0.5 cm ribbon of ointment in 10 sec was determined. (13)

e. Viscosity:-

Viscosity of ointment was measured by using Brookfield viscometer with spindle # 7.

f. Accelerated stability study:

A physical stability test of herbal ointment was carried out at 8^0C for 45^0C and stability was carried out for one month. The different parameter such as color, odor, texture, traces of gritty particles, skin irritation test were studied for all formulation at first month. [13]

2.4 Pharmacological activity:

2.4.1 Experimental animals:

Wistar albino rats weighing between 150-180 gm were obtained from Laxmi bio farms private limited, Pune. The rats were housed in cleaned metallic cages and kept in well ventilated room and allowed to acclimatized to the laboratory condition for one week before being used. They were fed with standard animal pellet and had free access to water and libitum. The animal were randomly divided into four groups. The protocol of the experiment (1942/PO/Re/S/17/CPCSEA/2018/02/02) was approved by Institutional Animal Ethics Committee (IAEC) of Pravara Rural College of Pharmacy, Loni and were conducted in accordance with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.4.2 Excision wound model

Excision wounds were used for the study of rate of contraction of wound and epithelization. Animal were anesthetized with Thiopental sodium and the hairs on the skin of the back, shaved with sterilized razor blade. A circular wound of about 500mm^2 area and 2mm depth was excised rats, 5cm away from the ear. The entire wound was left open. The was done topically in all the cases. The wounds were traced on transparent tracing paper by permanent marker on the day of wounding and subsequently on alternate days until healing were complete. Wound area was measured on days0, 3, 6, 9, 12, 15, 18 for all the groups. [14, 15]

2.4.3 Treatment protocol

The excision wound models rats were randomly divided into four groups (n=6/ groups). Total 24 Rats.

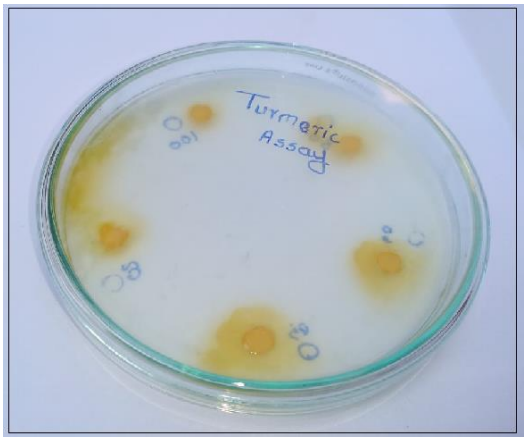
Treatment was given in following manner;

Table.3 Treatment protocol

Sr.No.	Name of groups	Treatment
1.	Vehicle control	ointment base
2.	Standard	Povidone iodine ointment
3.	Test I (2%)	Hydroalcoholic extract of *Piper nigrum* and *Curcuma longa* (0.5 gm)
4.	Test II (4%)	Hydroalcoholic extract of *Piper nigrum* and *Curcuma longa* (1 gm)

2.4.4 Antimicrobial activity

In-vitro antimicrobial screening was performed by paper disc diffusion method. The antimicrobial activity was performed on the hydroalcoholic extract of the rhizome of *Curcuma longa and* Fruits of *Piper nigrum.* Activity was performed against four pathogenic bacteria like *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and Proteus Vulgaris* (Two-Gram positive and two-Gram negative bacteria). Sterile discs (6 mm diameter) made of Whatman filter paper were impregnated with 20µl of different concentration of both the test extract (20mg/ml, 40mg/ml, 60mg/ml, 80mg/ml, 100mg/ml) and air dried to eliminate residual solvent and then was placed on the agar media In addition to these disc loaded with hydroalcoholic and disc loaded with Amoxycillin were used as negative and positive control. Plates were then incubated in incubator at 37°C for 24 h. After incubation both the plates were observed for zones of inhibition, and their diameter were measured including the diameter of the disc. [16]



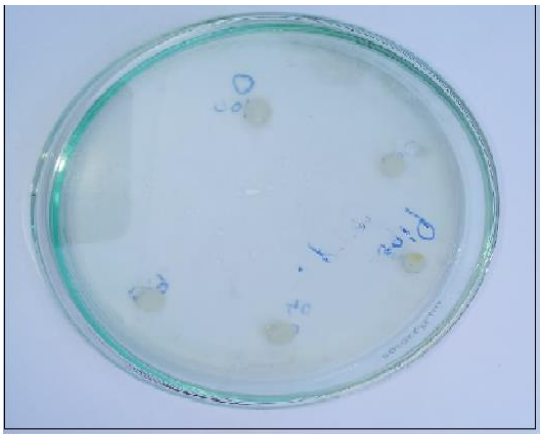


Fig.1 Antimicrobial activity of *Curcuma longa* Fig.2 Antimicrobial activity of *Piper nigrum*

2.4.5 Determination of Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) of the hydroalcoholic extract of *Curcuma longa* and *Piper nigrum* were determined by Serial dilution method. Nutrient broth media were prepared and 5ml of media is taken in each of the test tube. Hydroalcoholic extract of fruits and rhizome is prepared of different concentration (100mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ ml, 6.25 mg/ml and 3.125 mg/ml 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml). Freshly grown bacterial strains of Pseudomonas aeruginosa is inoculated in broth media and grown at 37ºC for 18 hrs. Each tube was inoculated with different concentration of both the extracts prepared at different concentration. All the tubes were incubated at 37ºC on a shaker with 140 rpm for 24 h. Presence of turbidity denoted presence of microorganism in the test tube after the period of incubation, whereas the complete absence of any turbidity indicates complete inhibition of microbial growth. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC. After incubation, the bacterial growth was measured at 660 nm. The % of inhibition was calculated by using the formula below: [16]

% Inhibition = OD of culture with sample/ OD of culture without sample x 100

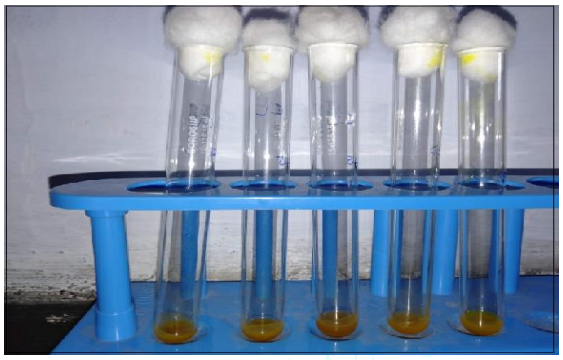


Fig.3 MIC of *Curcuma longa*

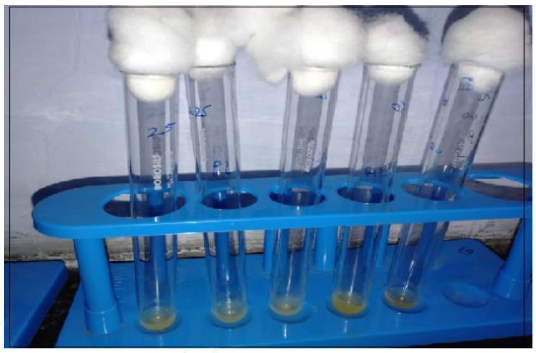


Fig.4 MIC of *Piper nigrum*

3. Statistical analysis

Data obtained were analyzed using One Way Analysis (ANOVA) followed by dunnet test and expressed as mean ± SEM. Differences between means were regarded significant at P<0.001.

4. RESULTS

4.1 Evaluation of Ointment

Accelerated stability study- The ointment was found to be physically stable at different temperatures. There were no changes in the spreadability, irritant effect even after exposure to different temperatures. For one month stability study at different temperature conditions viz. room temperature, 8^0C and 45^0 C, different parameters were studied and result observed as below.

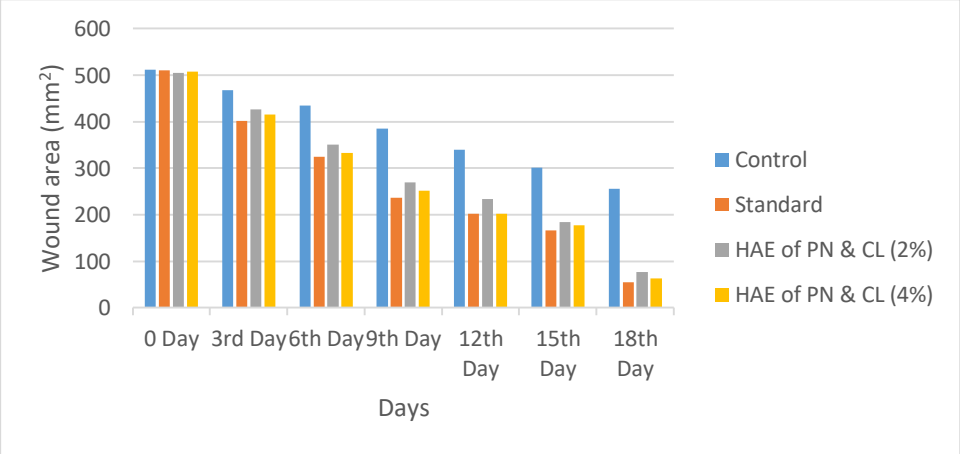
Table.4 Physicochemical Evaluation Parameters for Ointments:

Sr.no	Parameter	Ointment formulation	
		F1	F2
1.	Nature	Semisoild	Semisoild
2.	Color	Yellowish brown	Yellowish brown
3.	Odour	Characteristic	Characteristic
4.	Texture	Gummy	Gummy
5.	Trace of gritty particles	No	No
6.	Skin irritation	No	No
7.	PH	6.90	6.90
8.	Spreadability	6.31	6.31
9.	Extrudability	0.5 gm	0.5 gm
10.	Viscosity	5.48 cp	5.48 cp

4.2 Wound healing activity

Table.5 Effect of polyherbal formulation on healing of excision wound model

Treatment	Wound area (mm^2)			
Groups	**Control**	**Standard**	**HAE of PN & CL (2%)**	**HAE of PN & CL (4%)**
0 Day	511±0.91	510±0.62	505±0.76	508±0.82
3rd Day	468±0.63**	402±0.51**	426±0.43**	416±0.42**
6th Day	435±0.52***	325±1.05***	351±0.48***	333±0.52***
9th Day	385±0.59***	237±0.79***	270±0.52***	251±0.63***
12th Day	340±0.83***	203±0.39***	233±0.46***	202±0.55***
15th Day	301±0.86***	166±1.20***	184±0.47***	177±0.45***
18th Day	256±1.69***	54.3±0.48***	76.3±0.41***	62.9±0.36***
Period of epithelization in days	13	6	11	8



Graph.1 Effect of Standard, Test 1 and Test 2 on wound area

4.3 Antimicrobial activity

Table.6 Zones of Inhibition Shown by *Piper nigrum* on bacterial strains

Pathogens	Zones of Inhibiton in mm at different concentration						Negative Control
	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml	Positive Control	
Staphylococcus aureus	4.9	4.9	4.9	5.0	5.0	5.0	Nil
Bacillus substilis	Nil	Nil	Nil	Nil	Nil	7.0	Nil
Pseudomonas aeruginosa	4.0	4.1	5.0	5.0	6.0	8.0	Nil
Proteus vulgaris	Nil	Nil	Nil	Nil	Nil	6.0	Nil

Table.7 Zones of Inhibition Shown by *Curcuma Longa* on bacterial strains

Pathogens	Zones of Inhibition in mm at different concentration						Negative Control
	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml	Positive Control	
Staphylococcus aureus	4.0	4.1	4.8	5.8	6.0	6.0	Nil
Bacillus substilis	3.2	3.2	4.8	5.0	5.0	7.0	Nil
Pseudomonas aeruginosa	4.0	4.1	5.0	5.0	6.0	6.0	Nil
Proteus vulgaris	Nil	Nil	Nil	Nil	Nil	7.0	Nil

Table.8 Minimum Inhibitory Concentration for hydroalcoholic extract of *Piper nigrum*

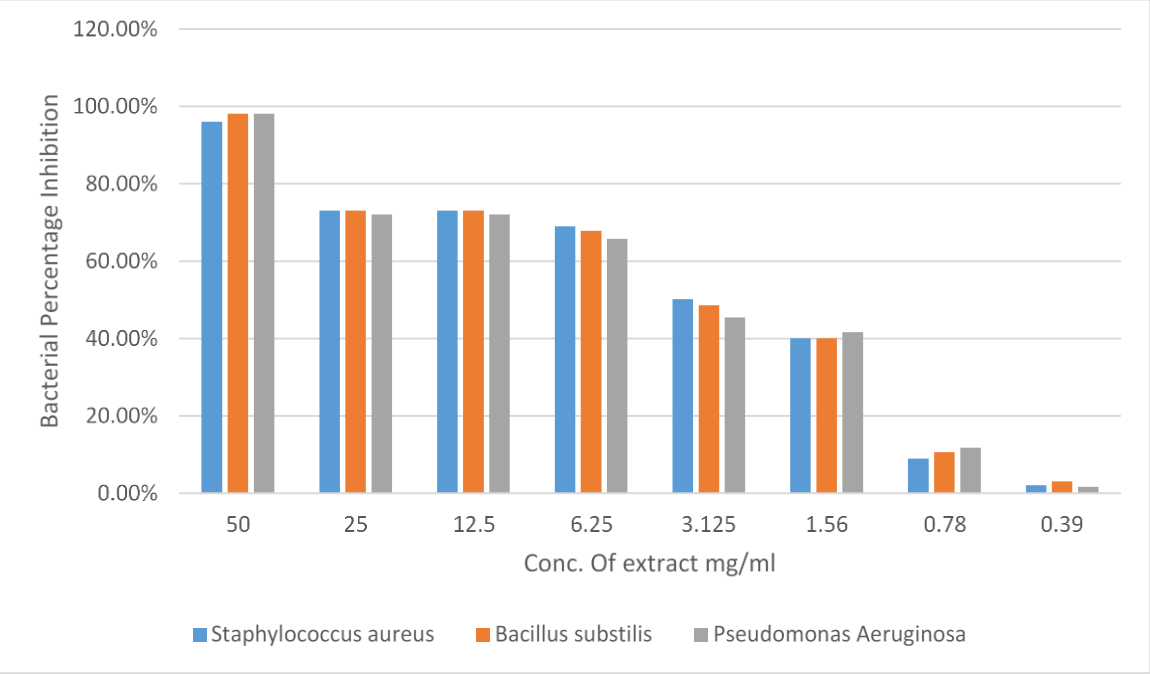
Sr no.	Conc. Of extract mg/ml	Bacterial Percentage Inhibition		
		Staphylococcus aureus	***Bacillus substilis***	***Pseudomonas Aeruginosa***
1	Control	0	0	0
2	100	96.013%	98.058%	98.089%
3	50	73.023%	73.049%	72.046%
4	25	73.023%	73.049%	72.046%
5	12.5	68.964%	67.874%	65.765%
6	6.25	50.167%	48.632%	45.432%
7	3.125	40.068%	40.067%	41.670%
8	1.56	9.008%	10.680%	11.809%
9	0.78	2.101%	3.112%	1.673%
10	0.39	0	0	0

Minimum inhibitory concentration for hydroalcoholic extract of *Piper nigrum* can be considered as 25mg/ml for all 3 bacterial strain.

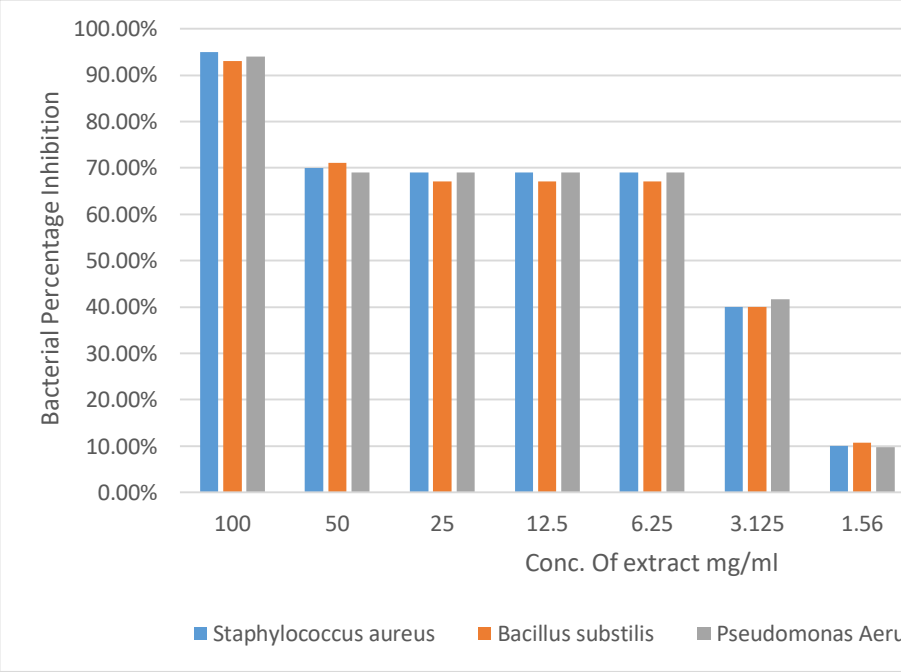
Table.9 Minimum Inhibitory Concentration for hydroalcoholic extract of *Curcuma longa*

Sr no.	Conc. Of extract mg/ml	Bacterial Percentage Inhibition		
		Staphylococcus aureus	***Bacillus substilis***	***Pseudomonas Aeruginosa***
1	Control	0	0	0
2	100	95.023%	93.058%	94.089%
3	50	70.023%	71.049%	69.046%
4	25	69.067%	67.084%	69.087%
5	12.5	69.067%	67.084%	69.087%
6	6.25	69.067%	67.084%	69.087%
7	3.125	40.068%	40.067%	41.670%
8	1.56	10.008%	10.670%	9.809%
9	0.78	3.111%	2.112%	3.773%
10	0.39	0	0	0

Minimum inhibitory concentration for hydroalcoholic extract of *Curcuma longa* can be considered as 12.5mg/ml for all 3 bacterial strains.



Graph.2 Graphical representation of *Piper nigrum* extract at various concentration of the extract on pathogens



Graph.3 Graphical representation of *Curcuma longa* extract at various concentration of the extract on pathogens

Effect of the hydroalcoholic extracts of *Piper nigrum* and *Curcuma longa* on wound area (mm^2) Table- 5. The initial wound areas selected for the study were in the range of 500 mm^2. In standard group the wound area decreased 54.3 mm^2on the 18th day. In hydroalcoholic extract (4% ointment) treated steadily decreased and it was 62.9 mm^2on the 18th day. Thus the hydroalcoholic extract (4% ointment) compared to standard restored the wound area. In present investigation the hydroalcoholic plant extracts of *Piper nigrum* and *Curcuma longa* was tested against three pathogenic bacterial strains viz. *Staphylococcus aureus, Bacillus substilis* and *Pseudomonas Aeruginosa.* The antibacterial efficacy of the extracts of *Piper nigrum* and *Curcuma longa* was quantitatively assessed on basis of inhibition zone and MIC (Minimum inhibitory concentration). The zone of inhibition by diffusion method shown by both extracts on various concentration given in table 6 & 7. The MIC value for *Piper nigrum* and *Curcuma longa* was found to be 25mg/ml and 12.5 mg/ml respectively.

5. DISCUSSION

The phytochemical study of the plants *piper nigrum* and *Curcuma longa* revealed the presence of various active constituents such as alkaloids, flavonoids, steroids and tannins. In excision wound model standard was effective than test. As in the antimicrobial activity plants extract showed significant activity. So further they were investigated for the formulation of ointment and then evaluated for wound healing activity. The present study involved excision wound model. In excision wound model animals were treated with polyherbal ointment showed better healing.

6. CONCLUSION

Herbs are plants that content healing properties and can treat a number of health problems. The study on selected plants for the formulation proved the potential for therapeutic use of wound healing and antimicrobial purpose. Plants used in work were *Piper nigrum* and *Curcuma longa* extracted by using hydroalcoholic solvent (7:3) and extract was used to formulate ointment. Ointments were evaluated for physical parameters which revealed that all the values were within acceptable limits. The herbal formulation shows significant Wound healing and antimicrobial activity.

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