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DEVELOPMENT AND EVALUATION OF ANTIMICROBIAL OINTMENT FORMULATION CONTAINING EXTRACTS OF OCIMUM SANCTUM, ANTHOCEPHALUS CADAMBA, ALLIUM SATIVUM AND ORIGANUM VULGARE

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ABSTRACT

Most of the antibiotics were originally derived from micro-organisms while the chemotherapeutic agents are from plants. Along with other dosage forms, herbal drugs are also formulated in the form of ointment. An ointment is a viscous semisolid preparation used topically on a variety of body surfaces. The objective of the study was to formulate and evaluate the antimicrobial herbal ointment from the local medicinal plants. The hexane, ethyl-acetate and ethanolic extracts of the selected plants were taken in different ratio randomly and the antimicrobial tests of the combinations were carried out. The most effective combination was then determined by comparing the results of the zone of inhibition given by the 12 different extracts ratios on *Staphylococcus aureus* (MTCC-3160), *Escherichia coli* (MTCC-1652) for antibacterial and *Aspergillus niger* (MTCC-282) for antifungal were used. Then the minimum inhibitory concentration of the effective combination was found out. The ointment base was prepared and formulation of ointment was done by incorporating the active ingredients in most effective ratio in the base by trituration. After the completion of the formulation, quality of the ointment was assessed in terms of irritancy, pH, viscosity, spreadability and stability studies. Comparative antimicrobial studies for prepared formulations with marketed antibiotic discs and herbal ointment.

KEY WORDS: Antimicrobial activity, Human pathogens, Herbal ointment, MIC, MBC and MFC, Comparative antimicrobial studies.

INTRODUCTION

In the recent years, there has been a gradual revival of interest in the use of medicinal plants in developing countries because herbal medicines have been reported safe without any adverse side effect especially when compared with synthetic drugs. Thus a search for new drugs with better and cheaper substitutes from the plant origin is a natural choice[1]. Herbal remedies for skin care with antibacterial and antifungal activities are prepared from a variety of plant parts such as leaves, stem, root, bark or fruit. These medicines are administered topically and may be applied in the form of cream, lotion, gel, soap, solvent extract or ointment, and have been establish to possess antimicrobial properties. Gels, creams and soap formulations containing a variety of plant extracts have been used to treat various skin disorders caused by microbial infections. Most of the skin infections are caused by fungi and bacteria, *Staphylococcus aureus* and *Streptococcus species*[2].

Benefits of Herbal Cosmetics

A number of people with sensitive skin don't want to use chemical sunscreens due to concern about skin exposure to unknown chemicals. Topical cosmetic formulations are the most preferred treatments asked by patients and are also often most prescribed by family physicians and dermatologists. Patients feel more comfortable using topical therapies because they have milder side effects, are easier to use, are generally less expensive and are more readily available.

The study of the present review focuses on the potential of herbal extracts for cosmetic purposes and the present disorder under study can be given enhanced therapy by reducing the adverse effects of allopathic medication by the use of natural herbs. Synthetic drugs may bring on well- known and documented harmful, side-effects whereas the use of natural herbs and their derivatives many have some synergistic effect which is not known. A sherbs are natural products they would get ready absorbed and assimilated by the body, these contain several active topical ointment formulation for anti-microbial activity by the use of Ocimum sanctum, Anthocephalus cadamba, Allium sativum and Origanum vulgare[3].

MATERIAL AND METHODS

Chemicals

The following ingredients were used for the preparation of nutrient agar media and Potato dextrose media: Agar, Peptone, Sodium chloride, Beef extract, Potato, dextrose, water. All other chemicals and analytical reagents were purchased from Hi-media, India, unless stated otherwise. Mature plants of Anthocephalus cadamba (FRLH-53753), Allium sativum (FRLH-53754), Origanum vulgare (FRLH-53755) and Ocimum sanctum (FRLH- 53756), were used for this study was collected from Institute of Ayurveda and Integrative Medicine –Bengaluru and authentified by Mrs. S. Noorunnisa Begum.

Culture and Maintenance of microorganisms

Pure cultures of all experimental bacteria; *Staphylocous aureus* (MTCC No.3160), *Escherichia coli* (MTCC No.1652) and fungi *Aspergillus niger* (MTCC No.282) were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The pure bacterial cultures were maintained on nutrient agar medium and fungal culture on potato dextrose agar (PDA) medium. Each bacterial and fungal culture was further maintained by sub culturing regularly on the same medium and stored at 4^{o}c before use in experiments.

Preparation of plant extract

In vivo leaves of *Anthocephalus cadamba*[4], *Allium sativum*[5], *Origanum vulgare*[6], *Ocimum sanctum*[7] were washed for 2-3 times with tap water and finally with distilled water and then allowed to dry at 50^{o} C for overnight and finally milled to a coarse powder (Sieve no 80). 100 gm of powdered material was soxhlet extracted with different solvents like, Hexane, Ethyl acetate, Ethanol 80% and water 20% (12 hours each). All the extracts were evaporated in vacuum under reduced pressure. All extracts were stored in sterile glass bottles at room temperature until screened.

PRELIMINARY PHYTOCHEMICAL SCREENING[8, 9] (QUALITATIVE ANALYSIS)

The preliminary phytochemical studies were performed for testing different chemical groups present in hexane, ethyl acetate, and ethanol extracts. The chemical group tests were performed and are presented in results.

Antimicrobial studies

Culture and maintenance of microorganisms

Pure cultures of all experimental bacteria; *Staphylococcus aureus* (MTCC No.3160), *Escherichia coli* (MTCC No.1652) and fungi *Aspergillus niger* (MTCC No.282) were procured from Institute of microbial technology (IMTECH), Chandigarh. The pure bacterial and fungal cultures were maintained on nutrient agar medium and potato dextrose agar (PDA) medium. Each bacterial and fungal culture was further maintained by sub culturing regularly on the same medium and stored at 4ºC before use in experiments.

Microbiological screening

Antimicrobial activities of different extracts were evaluated by modified Olurinola[10, 11] agar well diffusion method modified and Minimum inhibitory concentration (MIC) [12].

Media Preparation and Its Sterilization

Antimicrobial susceptibility was tested on solid Agar-agar media (gm/l: beef extract, 3g; peptone, 5g; sodium chloride, 5g; agar, 20g) and for fungus PDA(39 gm/l) was used for developing surface colony growth. The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values were determined by serial micro dilution assay. The suspension culture, for bacterial cells growth was done by preparing 2% Lauria Broth (w/v), and for fungus cells growth, 2.4% (w/v) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared was then sterilized by autoclaving the media at (121°C) for 20 min.

Agar well diffusion method[13]

The antimicrobial activity was tested against crude hexane, ethyl acetate and ethanol extracts of *Ocimum sanctum, Anthocephalus cadamba, Allium sativum and Origanum vulgare.* The inoculation of microorganism was prepared from bacterial culture. The inoculums suspension was spread uniformly over the agar plates using spreader, for uniform distribution of bacteria. Subsequently, using a sterile borer, well of 0.7 cm diameter was made in the inoculated media in addition to 100 mg/ml of each plant extract was aseptically filled into the well with the dilution of different stock solution each plant extracts. Later the plates were placed at room temperature for an hour to allow diffusion of extract into the agar. Then the plates were incubated for 24 h at 37ºC for room temperature. The results were recorded by measuring the diameter of inhibition zone at the end of 24 - 48 h. zone of inhibition surrounding the well was measured using a transparent ruler and the diameter was recorded in mm.

Minimum Inhibitory concentration

The minimum inhibitory concentration is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates. This was determined from readings on the culture plates after incubation. The most commonly employed methods are the tube dilution method and agar dilution methods. Serial dilutions are made of the products in bacterial and fungal growth media. The test organisms are then added to the dilutions of the products, incubated, and scored for growth. This procedure is a standard assay for antimicrobials. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. Clinically, the minimum inhibitory concentrations are used not only to determine the amount of antibiotic that the patient will receive but also the type of antibiotic used, which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents. The minimum inhibitory concentrations (MIC), MBC and MFC were performed by well diffusion method. The different plant extracts *viz*. hexane, ethyl acetate and Ethanol extracts were taken at different concentrations of *Tulsi* (8, 12, 16 and 24 µg/ml), *Cadamba* (10, 15, 20 and 25 mg/ml), *Garlic* (5, 10, 15 and 20%), Oregano (3, 6, 12 and 25 µg/ml) and serial dilution of the extract with luria broth for bacterial culture and for fungus, potato dextrose broth medium with respective inoculum were used. The plates were incubated for 24 – 48 h at 28ºC, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

Determination of MIC

The minimum inhibitory concentrations (MIC), MBC and MFCs were performed by a serial dilution technique using Agar well diffusion method. The different plant extracts viz. Hexane, Ethyl acetate, Ethanol (80%) and water (20%), were taken (1 mg/ml) and serial dilution of the extract with luria broth for bacterial culture and for fungus, potato dextrose broth medium with respective inoculum were used. The pertiplates were incubated for 72 hours at 28ºC, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

Determination of MBC

The MBCs were determined by serial sub-cultivation of 2 µl into micro titer plates containing 100 µl of broth per well and further incubation for 72 hours. The lowest concentration with

no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. All experiments were performed in duplicate and repeated three times.

Determination of MFC

The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of a 2 µl into microtiter plates containing 100 µl of broth per well and further incubation 72 hours at 28ºC. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. All experiments were performed in duplicate and repeated three times

PREPARATION OF ANTIMICROBIAL OINTMENT[14]

1. Polyherbal antimicrobial ointment was prepared by trituration method. In this method the ointment base was weighed and triturates the solid medicament with a small amount of the base by using mortar and pestle until a homogenous product is formed.
2. Add remaining quantities of the base until the medicament is uniform mixed with it. Thus the ointment was prepared.

FORMULATION DESIGN (10 g)

F1	F2	F3	F4	F5	F6
A.C (H)	T (H)	A.C (H)	T (H)	A.C (H)	T (H)
A.C (E.A)	T (E.A)	T (E.A)	A.C (E.A)	A.C (E.A)	T (E.A)
A.C (E)	T(E)	A.C (E)	T(E)	A.C (E)	T(E)
O.V (H)	G (H)	G (H)	O.V (H)	G (H)	O.V (H)
O.V (E.A)	G (E.A)	O.V (E.A)	G (E.A)	G (E.A)	O.V (E.A)
O.V (E)	G (E)	G (E)	O.V (E)	G (E)	O.V (E)

Formulation Design

T = *Tulsi*, A.C = *Anthocephalus cadamba*, G = *Garlic*, O.V = *Origanum vulgare*,

H = Hexane, E.A = Ethyl acetate, E = Ethanol.

EVALUATION OF OINTMENT[15]

ACID VALUE

2 g of the ointment was approximately weighed and dissolved in 50 ml of a mixture of equal volumes of ethanol (95%) and ether, previously neutralized with 0.1 M potassium hydroxide using phenolphthalein solution as indicator. 1ml of phenolphthalein solution was then added

and titrated with 0.1 M potassium hydroxide until the solution remains faintly pink after shaking for 30sec. The acid value was calculated from the expression.

Acid value = 5.61 n/w

Where, n = the volume (ml) of 0.1 M potassium hydroxide required;

w = the weight in gram of the substance.

SAPONIFICATION VALUE

1gm of ointment was approximately weighed and introduced into a 200 ml borosilicate glass flask fitted with a reflux condenser. 25 ml of 0.5 M ethanolic potassium hydroxide was added with a little pumice powder and boiled under reflux on a water-bath for 30 min. 1 ml of phenolphthalein solution was then added and titrated immediately with 0.5 M hydrochloric acid. The operation was repeated by omitting the substance being examined. Saponification value was determined by using the formula:

Saponification value = 28.05 (b-a)/w

Where, w = weight of the substance in gram.

VISCOSITY

Viscosity of the different formulations was determined using Brookfield viscometer with spindle TF-96 at 60 rpm at temperature 37±0.5 °C.

SPREADABILITY

Spreadability was done by using slide over slide method. In this method required quantity of ointment formulation was applied to one slide and to the other slide particular weight was tied. The time taken to slip off the slide from one slide was noted. The spreadability coefficient was calculated by using the formula,

S=ML/t

Where, M = weight tied to the slide

L = length of the slide

t = time

pH

Weighed 1 g of ointment mixed with 9 ml of water and pH of the resulting ointment taken with a pH meter.

Comparative antimicrobial studies for prepared formulation with marketed antibiotic discs and herbal ointment.

Disk diffusion method[16, 17]

In vitro antibacterial and antifungal activities were examined for prepared herbal ointment formulation from the *Ocimum sanctum, Anthocephalus cadamba, Allium sativum, Origanum vulgare* plant extracts. Comparative antimicrobial studies were done for prepared ointment formulation with marketed antibiotic discs **(ciprofloxacin and fluconazole)** and other selected formulations comparative antimicrobial studies were done for prepared ointment formulation with herbal ointment **(vetex)** formulation. All the ointment were screened for their antibacterial and antifungal activities against the *Escherichia coli, Staphylococcus aureus* and the fungi *Aspergillus niger*, were investigated by the agar disk diffusion method.

In the disk diffusion method the sterile nutrient agar plates were prepared. Then test organism was taken from the stock (broth) and swabbed on the agar medium in aseptic condition. The filter paper disc of 0.7 cm diameter (Whatman No.1 Filter paper) were prepared and sterilized. Residue from each formulation was dissolved in (DMSO) Dimethyl sulfoxide (10 mg/ml) and various concentrations of 50 µl/ml, 100µl/ml, and 150µl/ml of each was pipetted onto sterile paper discs (0.7 cm diameter) placed on the surface of inoculated agar plates. The sterile impregnated disc with ointment formulation were placed on the agar surface with framed forceps and gently pressed down to ensure complete contact of the disc with the agar surface. Plates were incubated at 37°C for 24 h. For the determination of zone of inhibition, the antimicrobial activity of all the ointment formulations was compared with standard antimicrobial agents like ciprofloxacin, fluconazole and vetex ointment. The sensitivities of the microorganism species to the ointment formulations were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks.

Stability study determination[18]

The formulations were subjected to accelerated stability testing study according to ICH guidelines for finished pharmaceutical products. The formulations were placed at $40^{\circ}C \pm 2^{\circ}C$ for a period of 2 months and sample were evaluated for various parameters like pH, viscosity, Spreadability and organoleptic property at an interval of 15 days.

Observation and Result

In the present investigation, the inhibitory effect of different extracts (viz. Hexane, Ethyl acetate, Ethanol (80%) and water (20%) of in vivo leaves from *Anthocephalus cadamba,*

Allium sativum, Origanum vulgare and Ocimum sanctum were evaluated against both fungicidal and bacterial strains. The antimicrobial activity was determined using agar well diffusion method and micro dilution method summarized in Table 1-2. The activity was quantitatively assessed on the basis of inhibition zone and their activity index was also calculated along with minimum inhibitory concentration (MIC).

MATERIALS & METHOD OF EXTRACTION

BOTANICAL NAME	PART OF PLANT	METHOD OF EXTRACTION	% YIELD
Ocimum sanctum	Leaves	Soxhlet extraction at 70-80ºC	23.75
Anthocephalus cadamba	Leaves	Soxhlet extraction at 70-80ºC	30.50
Allium sativum	Pearls	Soxhlet extraction at 70-80ºC	27.92
Origanum vulgare	Leaves	Soxhlet extraction at 70-80ºC	26.80

Table no: 9. Materials & Method of Extraction

PRELIMINARY PHYTOCHEMICAL SCREENING (QUALITATIVE ANALYSIS)

S no.	Test	*Ocimum sanctum*	*Anthocephalus cadamba*	*Allium sativum*	*Origanum vulgare*
1.	**For carbohydrate**				
a.	Molisch test	+	+	+	+
b.	Benedict test	+	+	-	+
c.	Fehling test	+	+	-	+
2.	**For alkaloid**				
a.	Dragandroff test	+	+	+	+
b.	Hagers test	+	+	+	-
c.	Wagners test	+	-	+	+
3.	**For flavanoid**				
a.	Shinoda test	+	+	+	-
4.	**For saponin**	+	+	-	+

Table no: 10. Preliminary Phytochemical Screening

Antimicrobial studies

In the present investigation, the inhibitory effect of different extracts viz. hexane, ethyl acetate, ethanolic extracts of *in vivo* leaves from *Anthocephalus cadamba, Allium sativum, Origanum vulgare and Ocimum sanctum* were evaluated against both fungicidal and bacterial strains. The antimicrobial activity was determined using agar well diffusion method and

micro dilution method summarized in (Table no: 11-12). The activity was quantitatively assessed on the basis of inhibition zone and their activity index was also calculated along with minimum inhibitory concentration (MIC).

Measurement of antimicrobial activity by using Agar well diffusion method[13]

The antimicrobial potential of both the experimental plants was evaluated according to their zone of inhibition against various pathogens and the results (zone of inhibition). The results revealed that all the extracts are potent antimicrobials against all the microorganisms studied. Among the different solvents extracts studied hexane and ethyl acetate showed high degree of inhibition followed by ethanolic extracts. For all the tested microorganisms hexane and ethyl acetate showed maximum antibacterial activity in from *Anthocephalus cadamba*, *Allium sativum*, *Origanum vulgare and Ocimum sanctum*. For the antibacterial activity, the hexane extract the maximum inhibition zone diameter was obtained in *S. aureus* and in *E. coli* with diameter 2.36 ± 0.585mm, 2.26 ± 0.493mm, respectively. Similarly, ethyl acetate extract showed maximum inhibition zone with diameter of 1.2 ± 0.1mm in *E. coli* and 1.63 ± 0.351 mm in *S. aureus*. The ethanolic extract (0.26 ± 0.057 mm) showed restrained and minimum activity, respectively. (Table no: 11. Fig no: 10. (A-D)).

For the antifungal activity, *A. niger* (0.93 ± 0.251 mm) showed efficient antifungal activity for hexane plant extract and comparing to the ethyl acetate and ethanolic extracts. Ethyl acetate extract showed maximum inhibition zone with diameter of (0.83 ± 0.416 mm) and ethanolic extract showed lowest inhibition zone (0.36 ± 0.057 mm) against all pathogenic fungal strains, respectively (Table no: 11. Fig no: 10. (A-D)).

Determination of MIC, MBC and MFC values of various plant extracts[19].

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The MBC and MFC was determined by sub culturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 h. The concentration of plant extract that completely killed the bacteria and fungi was taken as MBC and MFC respectively.

Determination of MBC for antibacterial activity

Ethyl acetate extracts of *Anthocephalus cadamba* and *Ocimum sanctum* showed least MIC values 0.9 µg/ml and 0.96 µg/ml against *S. aureus* and *E. coli* while hexane extract 1.5 µg/ml and 2.36 µg/ml against *S. aureus* and *E. coli* showed comparatively efficient MIC values of hexane 1.16 µg/ml and 2.53 µg/ml and hexane extracts of *Origanum vulgare* and *Allium sativum*, showed least MIC values 0.5 µg/ml and 0.76 µg/ml against *S. aureus* and *E. coli* while ethyl acetate extracts 0.93 µg/ml and 0.9 µg/ml against *S. aureus* and *E. coli* showed comparatively efficient MIC value of ethyl acetate are 1.63 µg/ml and 1.2 µg/ml and in ethanol extract showed least MIC value 0.3 µg/ml comparing to the hexane and ethyl acetate extracts respectively (Table no: 12. Fig no: 11. (A-D)).

Determination of MFC for antifungal activity

A. niger was proved to have highest activity 1.2 µg/ml and 0.7 µg/ml in hexane and Ethyl acetate extract respectively. The least MBC and MFC value 0.3 µg/ml and 0.4 µg/ml was observed in ethanolic extracts against *A. niger* respectively (Table no:12. Fig no: 11. (A-D)).

Table 1: Antimicrobial activity (zone of inhibition, mm) of various plant extracts Anthocephalus cadamba, Allium sativum, Origanum vulgare and Ocimum sanctum against clinical pathogens.

S.NO	Plant extract	Micro. Org	Hexane	E.A	Ethanol
Bacteria					
1	A.C	*S.aureus*	2.03 ± 0.416	1.36 ± 0.152	0.36 ± 0.057
2	A.C	*E.coli*	1.16 ± 1.069	0.90 ± 0.11	0.33 ± 0.057
3	Garlic	*S.aureus*	0.8 ± 1.360	0.90 ± 0.173	0.3 ± 0.1
4	Garlic	*E.coli*	0.76 ± 0.057	1.2 ± 0.1	0.36 ± 0.057
5	O.V	*S.aureus*	0.53 ± 0.057	1.63 ± 0.351	0.36 ± 0.057
6	O.V	*E.coli*	0.43 ± 0.057	0.93 ± o.152	0.26 ± 0.057
7	TULSI	*S.aureus*	2.36 ± 0.585	0.96 ± 0.115	0.36 ± 0.057
8	TULSI	*E.coli*	2.26 ± 0.493	0.96 ± 0.115	1.36 ± 0.152
Fungi					
9	A.C	*A. niger*	0.93 ± 0.251	0.8 ± 0.2	0.36 ± 0.057
10	Garlic	*A. niger*	0.33 ± 0.057	0.36 ± 0.057	0.33 ± 0.057
11	O.V	*A. niger*	0.6 ± 0.264	0.6 ± 0.2	0.33 ± 0.057
12	TULSI	*A. niger*	0.83 ± 0.351	0.83 ± 0.416	0.36 ± 0.057

Table 2: MIC (µg / ml), MBC and MFC performance of different extracts of Anthocephalus cadamba, Allium sativum, Origanum vulgare and Ocimum sanctum against pathogenic organisms

S.NO	Plant extract	Micro. Org	Hexane	E.A	Ethanol
Bacteria					
1	A.C	*S.aureus*	1.5 ± 0.7	1.36 ± 0.152	0.36 ± 0.057
2	A.C	*E.coli*	1.16 ± 1.069	0.9 ± 0.1	0.36 ± 0.057
3	Garlic	*S.aureus*	0.76 ± 0.057	1.2 ± 0.1	0.36 ± 0.057
4	Garlic	*E.coli*	0.8 ± 1.359	0.90 ± 0.173	0.3 ± 0.1
5	O.V	*S.aureus*	1.06 ± 0.461	1.63 ± 0.351	0.36 ± 0.057
6	O.V	*E.coli*	0.5 ± 0.1	0.93 ± 0.152	0.30 ± 0.001
7	TULSI	*S.aureus*	2.36 ± 0.585	0.96 ± 0.115	0.36 ± 0.057
8	TULSI	*E.coli*	2.53 ± 1.154	0.96 ± 0.115	0.33 ± 0.057
Fungi					
9	A.C	*A. niger*	0.63 ± 0.208	0.43 ± 0.057	0.3 ± 0.115
10	Garlic	*A. niger*	0.96 ± 0.115	0.4 ± 0.1	0.3 ± 0.1
11	O.V	*A. niger*	0.93 ± 0.152	0.5 ± 0.1	0.4 ± 0.1
12	TULSI	*A. niger*	1.16 ± 0.251	0.73 ± 0.115	0.5 ± 0.1

A.C = *Anthocephalus cadamba*, O.V = *Origanum vulgare*

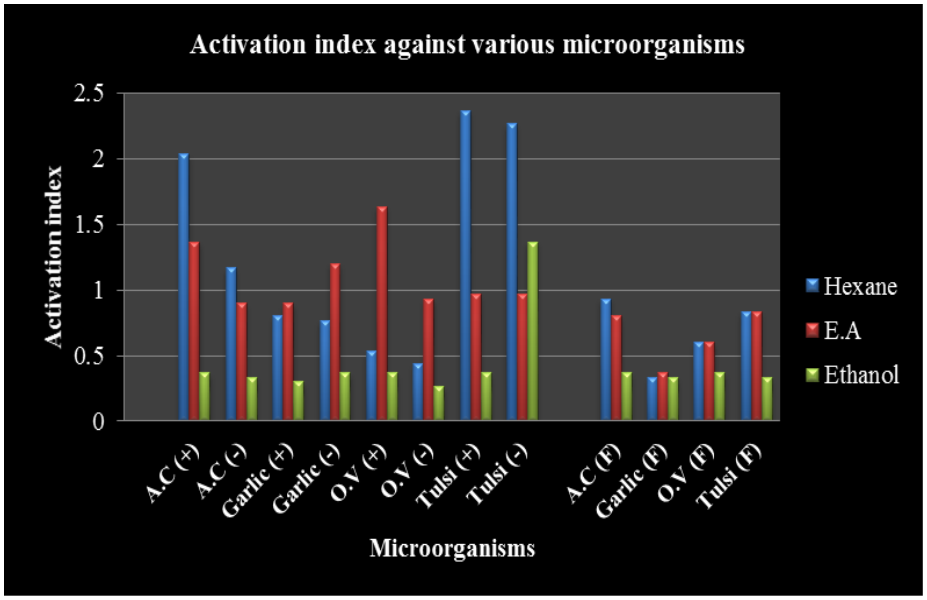


Fig no: 8. Activation index against various microorganisms

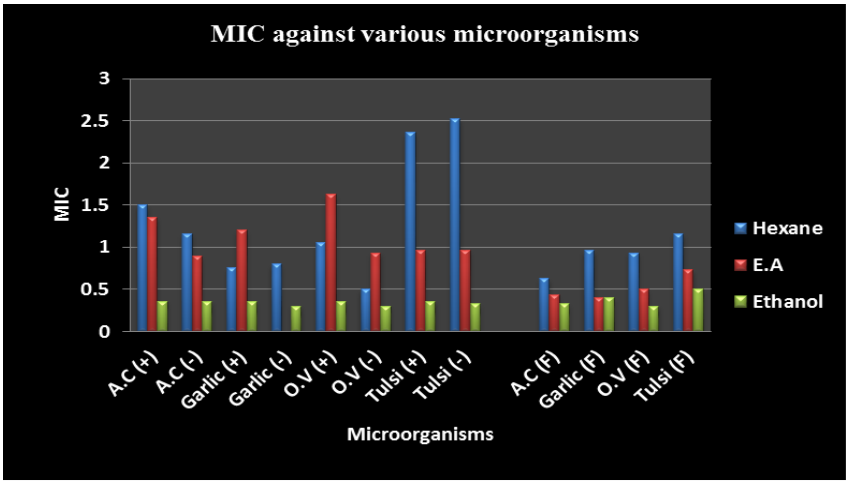


Fig no: 9. MIC against various microorganisms

A.C = *Anthocephalus cadamba,* Garlic = *Allium sativum,* O.V = *Origanum vulgare,* Tulsi = *Ocimum sanctum,* (+) = *Staphylococcus aureus,* (-) = *Escherichia coli* and F = Fungi (*Aspergillus niger*), MIC = Minimum Inhibitory Concentration, E.A = Ethyl acetate

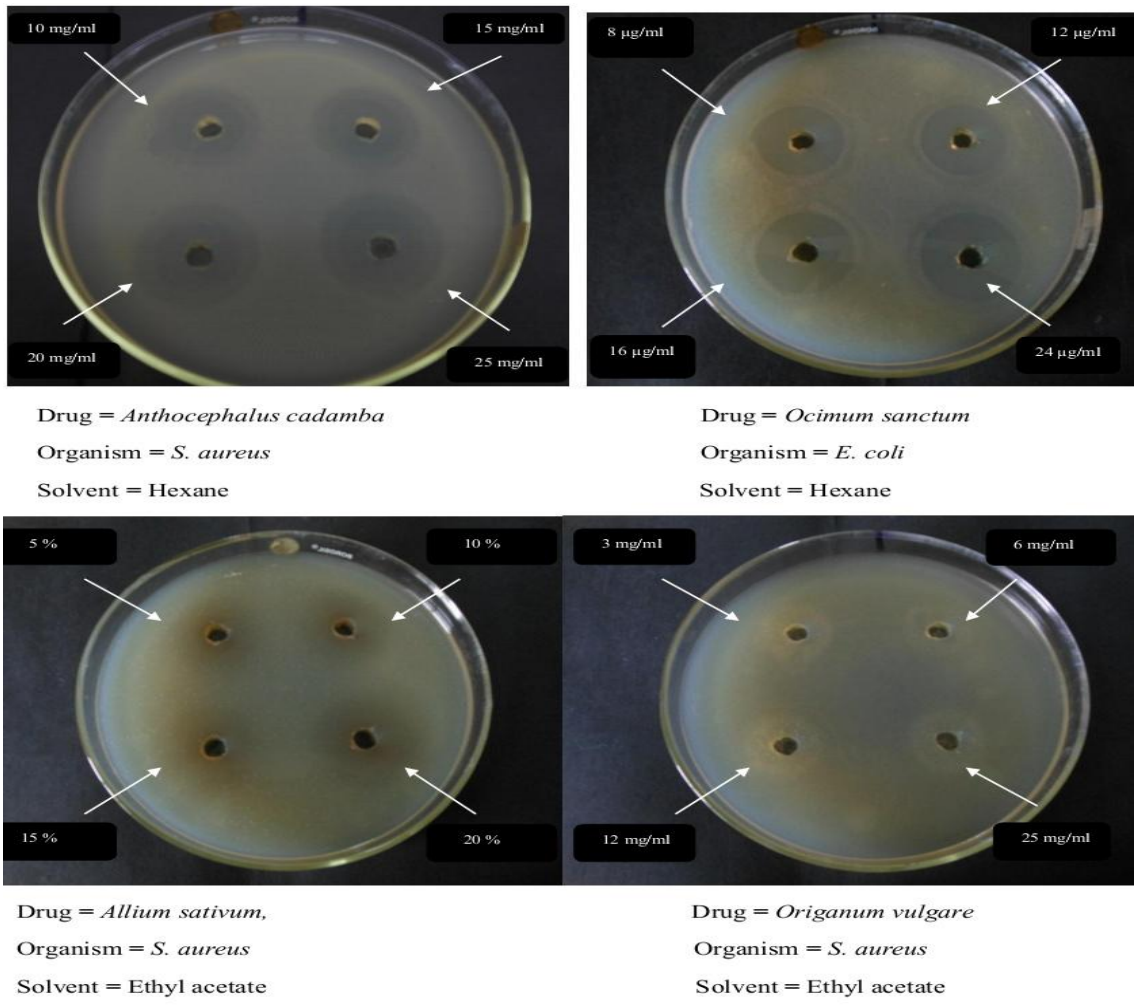


Fig no: 10. Antimicrobial activity (zone of inhibition, mm) of various plant extracts (A-D).

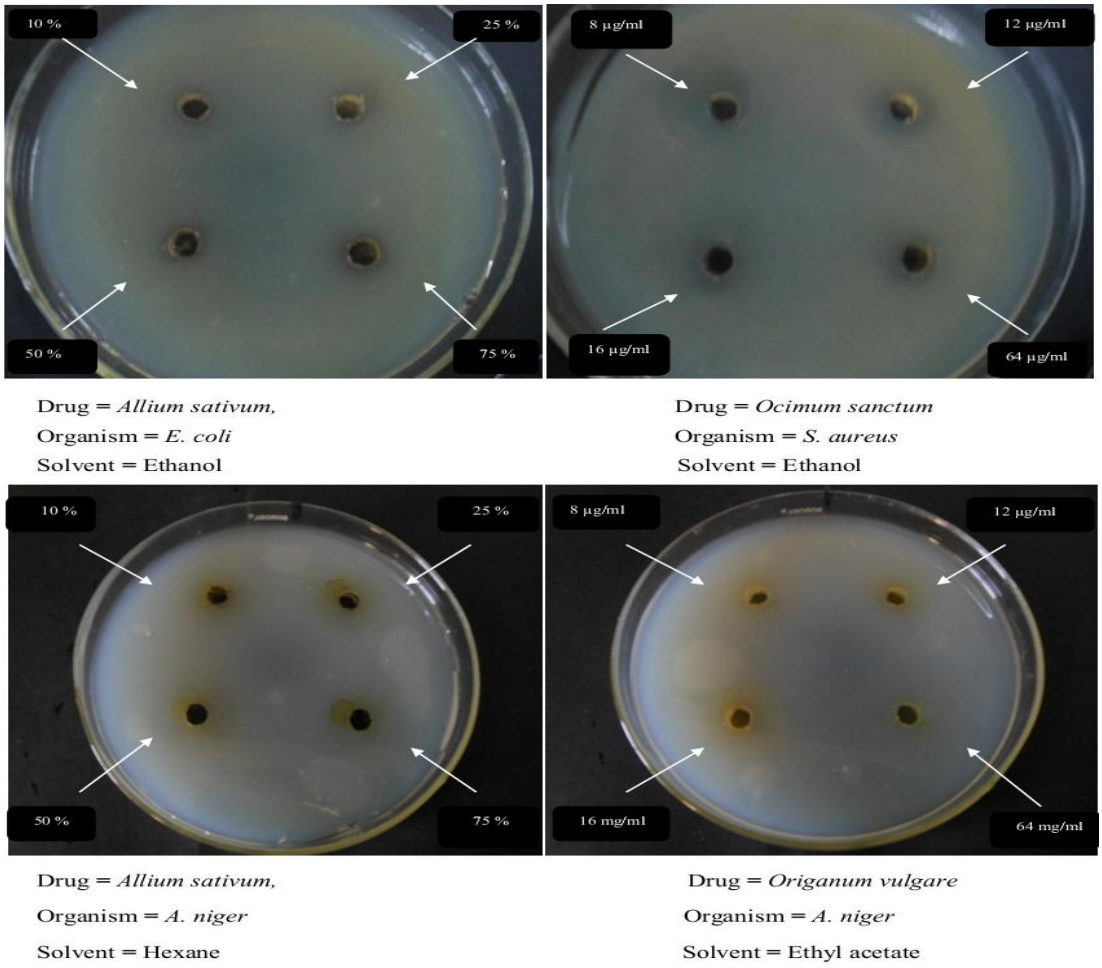


Fig no: 11. MIC (µg/ml), MBC and MFC performance of different extracts (A-D).

Evaluation of ointment[11]

Table no: 13. Evaluation of ointment

Formulation code	Acid value	Saponification value	Viscosity at 60 rpm	Spreadability	pH
F1	10.65	54.69	5670	10.8	5.61
F2	7.29	36.46	5448.4	9.64	5.58
F3	6.45	29.45	6132.6	10.06	5.20
F4	10.09	65.91	4889.6	10.45	6.14
F5	8.13	63.11	4685.8	9.96	5.05
F6	8.97	46.28	5033	11.14	6.10

Comparision of antimicrobial studies for prepared ointment formulation with marketed antiobiotic discs

Determination of MIC, MBC and MFC values

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the ointment that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The MBC and MFC was determined by sub culturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 h. The concentration of ointment that completely killed the bacteria and fungi was taken as MBC and MFC, respectively[16,17].

Determination of MBC for antibacterial activity

The F_1 to F_6 (Except F_3, F_2) formulations showed least MIC values 0.33 µg/ml and 0.33 µg/ml against *S. aureus* and *E. coli* while F_3 and F_2 formulations 0.4 µg/ml and 0.36 µg/ml against *S. aureus* and *E. coli* showed comparatively efficient MIC values of F_1 to F_6 formulations respectively (Table no: 14. Fig no: 14. (A-D)).

Determination of MFC for antifungal activity

A. niger was proved to have highest activity 0.73 µg/ml and 0.63 µg/ml in F_6 and F_3 respectively. The least MBC and MFC value 0.4 µg/ml was observed in F_5 against *A. niger* respectively (Table no: 14. Fig no: 14. (A-D)).

Comparision of antimicrobial studies for prepared ointment formulation with marketed herbal ointment formulation

determination of mic

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the ointment that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The MBC and MFC was determined by sub culturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 h. The concentration of ointment that completely killed the bacteria and fungi was taken as MBC and MFC, respectively[16,17].

Determination of MBC for antibacterial activity

The F_3 to F_6 formulations showed least MIC values 0.23 µg/ml and 0.23 µg/ml against *S. aureus* and *E. coli* while the combination of F_3+F_6 formulation 0.33 µg/ml and 0.43 µg/ml against *S. aureus* and *E. coli* showed comparatively efficient MIC values of F_3 to F_6 formulations respectively (Table no:15. Fig no: 15. (A-D)).

Determination of MFC for antifungal activity

A. niger was proved to have highest activity 0.73 µg/ml and 0.63 µg/ml in the combination of F_3+F_6 formulation respectively. The least MBC and MFC value 0.23 µg/ml was observed in F_5 against *A. niger* respectively (Table no: 15. Fig no: 15. (A-D)).

Table no: 14. Comparative antimicrobial studies (zone of inhibition, mm) for prepared ointment formulation with marked antibiotic discs (ciprofloxacin and fluconazole) against clinical pathogens.

S.NO	Formulations	GRAM (+)	GRAM (-)	Fungal
1	F_1	0.33 ± 0.057	0.33 ± 0.057	0.63 ± 0.057
2	F_2	0.3 ± 0.1	0.26 ± 0.057	0.56 ± 0.115
3	F_3	0.4 ± 0.1	0.36 ± 0.057	0.53 ± 0.057
4	F_4	0.33 ± 0.057	0.33 ± 0.057	0.56 ± 0.057
5	F_5	0.33 ± 0.057	0.33 ± 0.057	0.4 ± 0.1
6	F_6	0.33 ± 0.057	0.33 ± 0.057	0.73 ± 0.057

Table no: 15. MIC (µg/ml), MBC and MFC performance of different formulation against pathogenic organisms. Comparative antimicrobial studies (zone of inhibition, mm) for prepared ointment formulation with marked herbal ointment (vetex) formulation against clinical pathogens.

S.NO	Formulations	GRAM (+)	GRAM (-)	Fungal
1	F_3	0.23 ± 0.057	0.4 ± 0.1	0.23 ± 0.057
2	F_6	0.23 ± 0.057	0.33 ± 0.057	0.23 ± 0.057
3	$F_3 \pm F_6$	0.33 ± 0.057	0.43 ± 0.057	0.26 ± 0.057

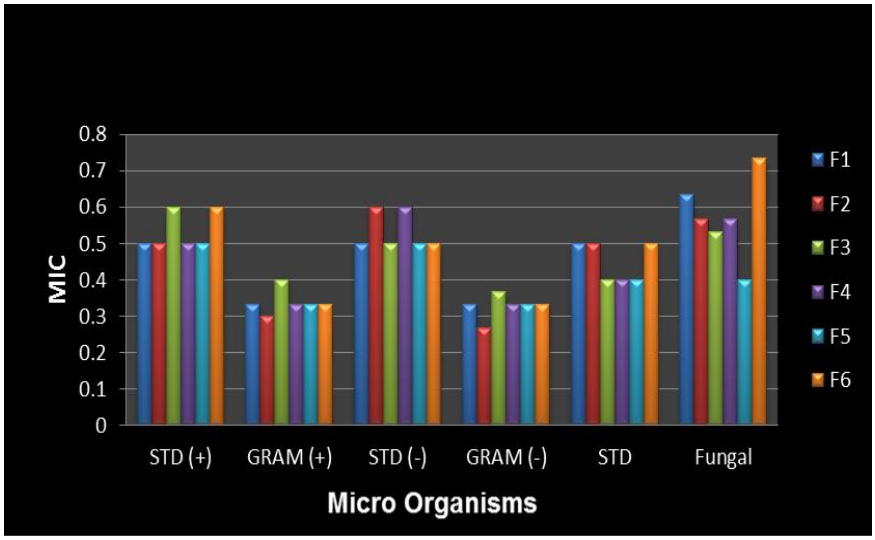


Fig no: 12. Comparative antimicrobial studies of prepared ointment formulation with marked antibiotic discs against clinical pathogens.

MIC = Minimum Inhibitory Concentration, STD for bacterial = Ciprofloxacin, STD for fungal = Fluconazole, (+) = *Staphylococcus aureus*, (-) = *Escherichia coli* and Fungi =*Aspergillus niger*.

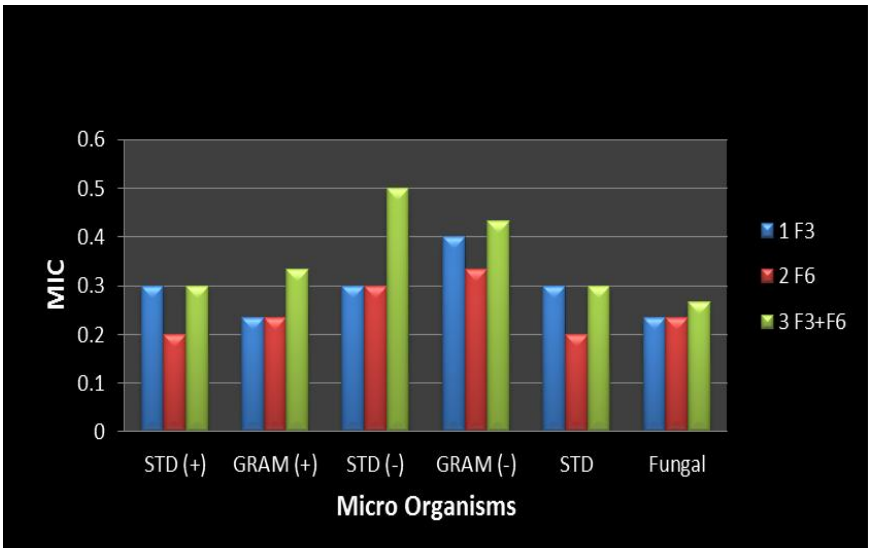
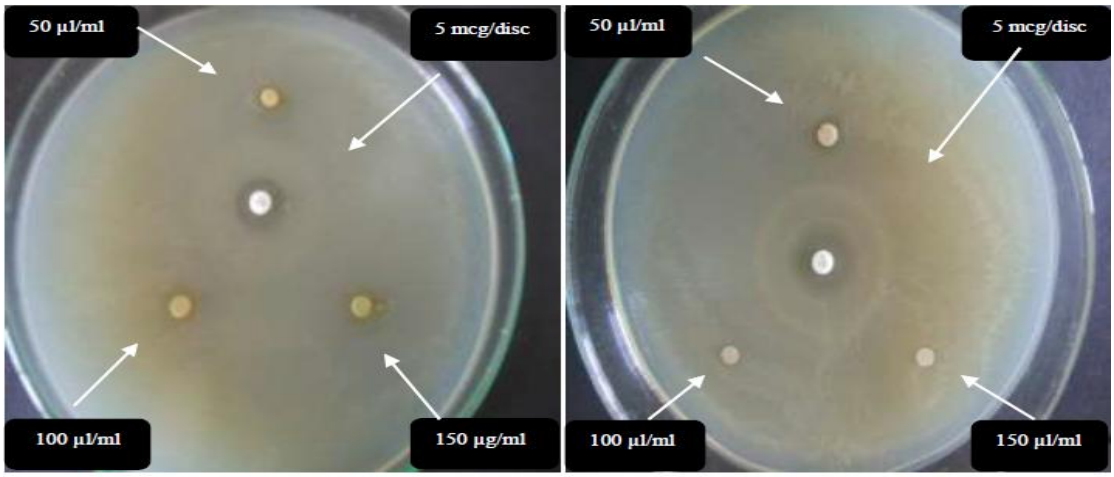


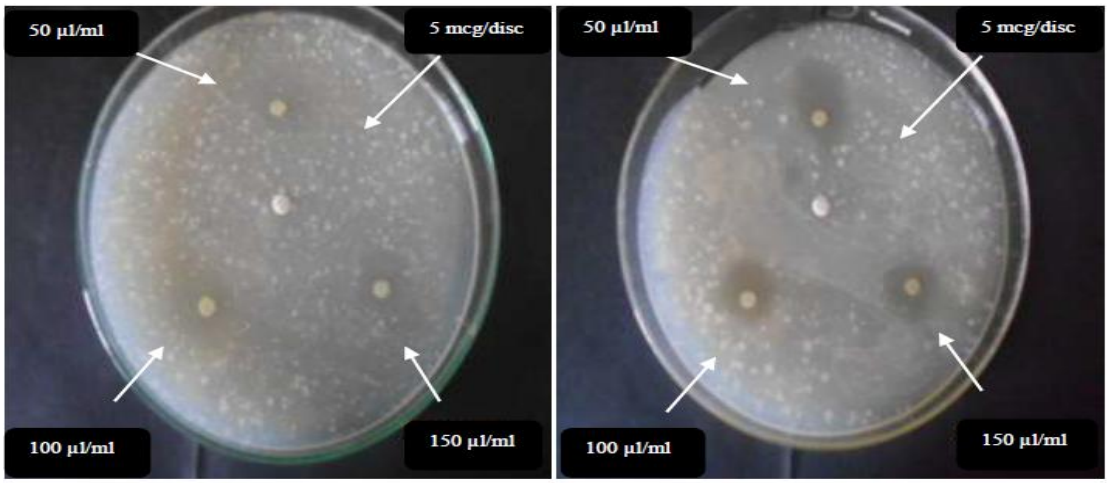
Fig no: 13. Comparative antimicrobial studies of prepared ointment formulation with marked herbal ointment against clinical pathogens

MIC = Minimum Inhibitory Concentration, STD for both bacterial and fungal = vetex herbal ointment, Gram (+) = *Staphylococcus aureus*, Gram (-) = *Escherichia coli* and Fungi = *Aspergillus niger*.



STD Disk = Ciprofloxin disk
Formulation = F_3
Organism = *S. aureus*

STD Disk = Ciprofloxin disk
Formulation = F_3
Organism = *E. coli*



STD Disk = Fluconazole disk
Formulation = F_6
Organism = *A. niger*

STD Disk = Fluconazole disk
Formulation = F_6
Organism = *A. niger*

Fig no: 14. Comparative antimicrobial studies (zone of inhibition, mm) for prepared ointment formulation with marked antibiotic disc against clinical pathogens (A-D).

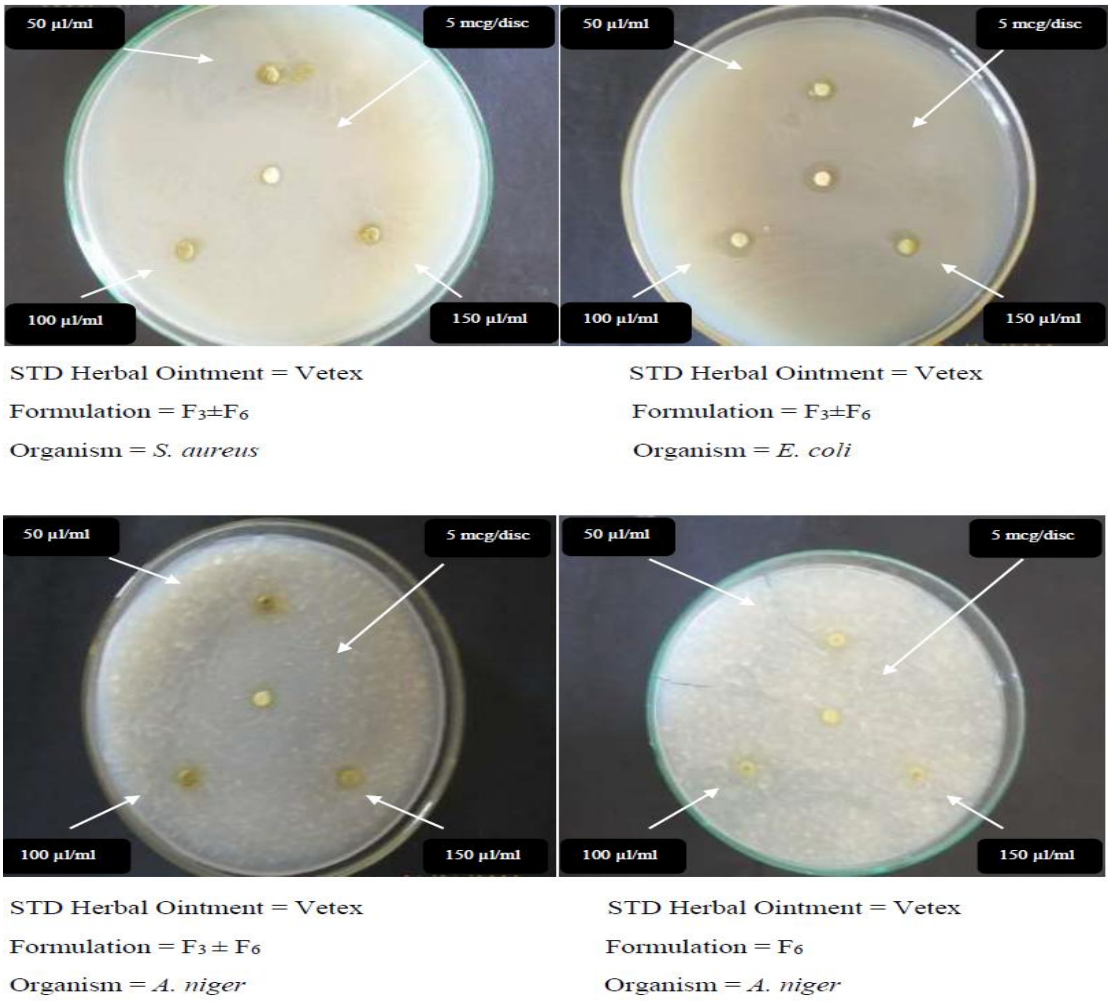


Fig no: 15. Comparative antimicrobial studies (zone of inhibition, mm) for prepared ointment formulation with marked herbal formulation against clinical pathogens. (A-D)

Stability studies[18]

Days/ Parameters	F_3 at 40°C ± 2°C			
	0	15	30	45
pH	6.62	6. 51	6.38	6.5
Viscosity (cps)	6764	6548	6246	6029
Spreadability (g.cm/sec)	10.06	10.31	10.29	10.61
Colour	Brownish Green	No change	No change	No change

Spindle no: 7. at 60 rpm

Days/ Parameters	F_6 at 40ºC ± 2°C			
	0	15	30	45
pH	6.90	6. 61	6.42	6.25
Viscosity (cps)	6920	6607	6389	6248
Spreadability (g.cm/sec)	10.97	10.64	11.13	11.44
Colour	Brownish Green	No change	No change	No change

Spindle no: 7. at 60 rpm

Days/ Parameters	$F_3 + F_6$ at 40ºC ± 2°C			
	0	15	30	45
pH	6.64	6. 41	6.34	6.28
Viscosity (cps)	6936	6692	6389	6233
Spreadability (g.cm/sec)	10.36	10.49	10.34	10.57
Colour	Brownish Green	No change	No change	No change

Spindle no: 7. at 60 rpm (Table no: 16. Stability studies)

DISCUSSION

The search for antimicrobials from natural sources has received much attention and efforts have been put in to identify compounds that can act as suitable antimicrobials agent to replace synthetic ones. Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism[20, 21]. These compounds have significant therapeutic application against human pathogens including bacteria, fungi or virus. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds[22, 23]. Therefore, medicinal plants are finding their way into pharmaceuticals, neutralceuticals and food Supplements.

In the present investigation, different solvent extracts of Anthocephalus *cadamba*, *Allium sativum*, *Origanum vulgare* and *Ocimum sanctum* was evaluated for exploration of their antimicrobial activity against certain Gram negative and Gram positive bacteria, fungus which was regarded as human pathogenic microorganism. Susceptibility of each plant extract was tested by serial microdilution method (MIC) and agar well diffusion method was determined.

Our preliminary investigation showed that all Hexane, Ethyl acetate and Ethanolic extracts of *Anthocephalus cadamba, Allium sativum, Origanum vulgare and Ocimum sanctum were active against the locally isolated human* pathogens like *Escherichia coli* and *Staphylococcus aureus*, This analysis of using several extracts so as to study the efficacy of plant for antimicrobial activity has also been realize by many scientist in many plant species like Adhatoda zeylanica, Medic[24], Trianthema decandra L.[25], Argemone mexicana L.[26], Tinospora cordifolia and Cassia fistula [27].

The alcoholic extracts of herbal plant showed significant antimicrobial activity against multi-drug resistant clinically isolated microorganisms (Graph Fig no: 8 and 9). Though, the mechanism of the action of these plant constituents is not yet fully known it is clear that the effectiveness of the extracts largely depends on the type of solvent used. This observation clearly indicates that the existence of non-polar residues in the extracts which have higher both bactericidal and bacteristatic abilities. Cowan[28] mentioned that most of the antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily solubilized in organic solvents. Similar results showing that the alcoholic extract having the best antimicrobial activity is also reported by Preethi [29] in Leucas aspera, Holarrhena antidysenterica. Suree[30] also studied the antibacterial activity of crude ethanolic extracts and essential oils of spices against salmonellae and other enterobacteria and observed that this difference in the activity between different plant extracts is due to the difference between extract compounds.

The study also revealed that ethanolic extract shows minimum antimicrobial activity. However, Ali Mirzaei[31], showed that ethanolic extract of plant *Origanum Vulgare* shows significant antimicrobial activity. Furthermore, Chloroform and acetone extract from the leaves of *Anthocephalous cadamba* had been reported to have prominent antimicrobial activity against several gram positive and gram negative human pathogenic bacteria[32]. The antimicrobial analysis using the agar well diffusion method and MIC value is been used by many researchers [33, 34, 35]. In the present study the MIC value of the active plant extracts obtained in this study were lower than the MBC values (Table no: 11-12) Graph (Fig no: 8 and 9) suggesting that the plant extracts were bacterio static at lower concentration but bactericidal at higher concentration[36]. Evaluation parameters of ointments: The results of acid value, saponification value, viscosity, spreadability and pH of all formulations of ointments are presented in table no: 13, and showed the satisfactorily values.

Comparative antimicrobial studies were done for prepared ointment formulation with marketed antibiotic discs (ciprofloxacin and fluconazole) and herbal ointment (vetex) formulation.

All the ointment were screened for their antibacterial and antifungal activities against the *Escherichia coli, Staphylococcus aureus* and the fungi *Aspergillus niger*, were investigated by the agar disk diffusion method. It was observed that F_3 and F_6 have the maximum zone of inhibition against all the microbial species. The test formulations were also compared with available marketed preparation. The comparison showed that the formulation F_3 and F_6 is comparable with available synthetic marketed antimicrobial and antifungal discs preparations and herbal ointment (vetex) preparation. The results are as shown in table no: 14 and 15 and in the graph (fig no: 12 and 13). The zone of inhibition against selected microbes are shown in fig no: 14 and 15.

Stability studies were done for the all the formulations and were stored at $40^oC \pm 2^oC$ for 60 days and evaluated regularly at an interval of 15 days were evaluated for different physico-chemical parameters. No major changes was found during entire testing period and formulations remained stable when stored at $5^oC - 40^oC$ in 24 h cycle for two weeks. The formulations for F_3, F_6 and F_3+F_6 results are shown in the table no: 16 respectively.

In conclusion, of the present investigation of these herbal plants contain potential antimicrobial components that may be of great use for the development of pharmaceutical industries as a therapy against various diseases. The hexane, ethyl acetate, ethanolic extracts of *Anthocephalus cadamba, Allium sativum, Origanum vulgare and Ocimum sanctum* possess significant inhibitory effect against tested pathogens.

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