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Original Article

Evaluation of Synergistic Anti-microbial efficacy of plant extracts and their formulation as Topical Agents

Zeeshan Afsar*[1], Salma Khanam[2] and Aravind Ram A.S. [3]

[1]Department of Pharmacognosy,[3]Department of Pharmaceutics,Farooqia College of Pharmacy, Mysore.
[2]Department of Pharmacognosy, Al Ameen College of Pharmacy, Bangalore., India

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ABSTRACT: The objective of the present study was to evaluate the antimicrobial activity of *Cassia fistula, Ficusreligiosa, Milletiapinnata*and *Wendlandiathyrsoidea*and to check the synergistic efficacy of these extracts when combined. The individual extracts and their combinations were evaluated against *E coli* (MTCC-1698), *S aureus* (MTCC-1143)and *P aeruginosa* (MTCC-2453) by agar diffusion method. The percentage growth inhibition of the combined extracts was determined by ditch plate method. The most active extract combinations were formulated as cream and gel including citronella oil and without oil in it. The prepared formulations were evaluated for their antimicrobial effect against two acne causing organisms viz; - *Propionibacterium acnes* and *Staphylococcus epidermidis* at various concentrations. The extracts exhibited significant antimicrobial effect in combined form when compared to individual extracts in terms of zones of inhibition as well as percentage inhibition. The prepared cream and gel also exhibited significant antimicrobial effect against the selected strains. The physicochemical parameters of the prepared cream and gel also exhibited satisfactory results.

⇑ Corresponding author at:
Zeeshan Afsar, Department of Pharmacognosy, Farooqia College of Pharmacy, Mysore, India
E-mail address:zeeshanafsar@gmail.com

INTRODUCTION

Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines [1]. Plant extracts and products have been used for centuries in traditional medicine, functional food, natural dyes, cosmetics, as detergents and in the treatment of diseases [2]. Essential oils and extracts from several plant species can control microorganisms related to skin, dental caries and food spoilage, including Gram-negative and Gram-positive bacteria, fungi and viruses.

The essential oils are very well known for their bactericidal and bacteriostatic activities [3]. Most of the antimicrobial activity in essential oils is found in the oxygenated terpenoids (e.g., alcohols and phenolic terpenes), while some hydrocarbons also exhibit antimicrobial effects. Interactions between these components may lead to antagonistic, additive or synergistic effects [4].

Herbal medicinal products are of global importance both medicinally and economically. Although usage of these herbal medicines has increased, their quality, safety and efficiency are serious concerns in industrialized and developing countries [5].

WHO report 80% of the world population relies on the drug from natural origin, hence there is a need to work in the field of development, evaluation and standardization of herbal formulations [6, 7].

Cassia fistula Linn, belonging to the family *Leguminosae,* is a deciduous medium sized tree; it is cultivated throughout India, the parts of which are used in the Ayurvedic system of medicine for various disorders.Literature survey of this plant shows that it is reported to possess good antibacterial and antifungal properties [8, 9].

*Ficusreligiosa*belonging to the family Moraceaeis a very common tree in India. It is commonly known as people's tree or sacred fig. *Ficusreligiosa*is used in traditional system of medicines to treat about 50 types of disorders; it is also a common ingredient in various ayurvedic formulations such as Nalpamaraditailam, Chandanasavam, Nyagrodhadichurna, and Saribadyasavam[10]. The methanolic bark extract of *Ficusreligiosa*is reported to poses good antimicrobial properties [11]. *Milletiapinnata* is a species of tree belonging to family *Fabaceae*, it is a native in tropical and temperate Asia including India, China, Japan, Malaysia, Australia and pacific islands [12].

The fruits and sprouts of *Milletiapinnata* are used in folk remedies to treat cold, coughs, gonorrhea, and leprosy. The roots are used for cleaning gums and teeth, the oil is used as antiseptic [13]. Different parts of the plant *Milletiapinnata*are also reported to poses antibacterial properties [14]. *Wendlandiathyrsoidea* belonging to the family Rubiaceae is a small tree or large shrub distributed in south India and Sri Lanka. Different parts of the plant are used in treatment of skin cuts and infections in traditional systems [15]. Different parts of *Wendlandiathyrsoidea*have also been reported to poses antimicrobial properties [16].

Hence, these plants were selected and their antimicrobial efficacy confirmed and their extracts were formulated into topical antiseptic formulations.

MATERIALS AND METHODS

Plant material

The leaves and bark of plants *Cassia fistula, Ficusreligiosa, Milletiapinnata*and *Wendlandiathyrsoidea*were collected from Mysore and Coorg districts, the specimens were authenticated at RRL, Bangalore.

Preparation of extracts

The leaves and bark of *Cassia fistula, Ficusreligiosa, Milletiapinnata*and *Wendlandiathyrsoidea*were dried in hot air oven at 35^{o}C, powdered to a mesh size of # 40 and stored. The powder was then extracted successively by refluxation for eight hours using five different solvents with increasing polarity. Viz: - Petroleum ether, Chloroform, Ethyl acetate, Methanol, and 40% methanol. All the extracts were evaporated in rotary flash evaporator to the get the semisolid residues which were dried and stored in refrigerator. 40 extracts were prepared in the concentration of 0.5mg/ml in DMSO and were used for antimicrobial screening.

Antimicrobial screening of individual extracts

All the leaf & bark extracts of *Cassia fistula, Ficusreligiosa, Milletiapinnata*and *Wendlandiathyrsoidea*were subjected to antimicrobial screening by agar diffusion method. Microorganisms used were *E coli* (MTCC-1698), *S aureus* (MTCC-1143)and *P aeruginosa* (MTCC-2453), the inoculums were prepared at 0.5 Mc Farland concentration and spread over the agar plates by sterile cotton swab. Media used was Mueller-Hinton agar medium. The plates were incubated at 37^{o}C for 24 hours and the zones of inhibition were recorded in mm.

Preparation of extract combinations

Among the twenty extracts obtained from four plants, eleven extracts were found to poses antimicrobial activity. By using these extracts, six different combinations were prepared with each extract having a concentration of 0.25 mg/ml. The combinations were selected based on same parts of different plants, same solvents of different plants and different parts of the same plant. Following is the list of combinations prepared.

Combination no: 1= CB-MEOH+WB-ETH+FB-MEOH.

Combination no: 2= CB-ETH+FB-ETH+CB-MEOH.

Combination no: 3= CL-MEOH+ML-MEOH+FL-MEOH.

Combination no: 4= ML-ETH+WL-ETH+FL40MOH.

Combination no: 5= CB-MEOH+CL-MEOH+FL-MEOH.

Combination no: 6= CB-ETH+ML-ETH+WL-ETH.

Note: CB= *Cassia fistula* bark, CL= *Cassia fistula* leaf, MB= *Milletiapinnata*bark, ML= *Milletiapinnata*leaf, WB=*Wendlandiathyrsoidea*bark. WL=*Wendlandiathyrsoidea*leaf, FB= *Ficusreligiosa*bark, FL=*Ficusreligiosa*leaf. ETH= Ethyl acetate, MeOH= Methanol, 40MOH= 40% Methanol.

Evaluation of synergistic antimicrobial efficacy of the extract combinations

Different combinations of extracts were prepared with each extract having a concentration of 0.25 mg/ml. 1ml each extract was mixed and tested against the microorganisms, *E coli* (MTCC-1698), *S aureus* (MTCC-1143)and *P aeruginosa* (MTCC-2453) by agar diffusion method. The inoculums were prepared at 0.5 Mc Farland concentration and spread over the agar plates by sterile cotton swab. Media used was Mueller-Hinton agar medium. The plates were incubated at 37^{o}C for 24 hours and the zones of inhibition were recorded in mm.

Determination of percentage growth inhibition by ditch plate method

The most active extract combinations were evaluated for their percentage microbial growth inhibition by ditch plate method. Ditches were made in petri plates containing Mueller Hinton agar medium by using a punch. These ditches were filled with three concentrations of the combinations i.e. 0.375, 0.75 and 1.5 mg/ml was used. Six microorganisms' i.e. *S aureus, P aeruginosa, E coli, Listeria, Bacillus cereus* and *Salmonella* were used. Three organisms were streaked on each side of the petri plate, the length of streak (LOS) was recorded and the plates incubated at 37^{o}C for 24 hrs, after which the length of inhibition (LOI) and percentage inhibition was calculated.

Formulation of cream and gel using the extract combinations

Among the prepared extract combinations, the combination no. 2 consisting the ethyl acetate and methanolic bark extracts of *Cassia fistula* and *Ficusreligiosa* exhibited maximum activity, this was formulated into cream and gel by using the following formulas.

Each extract was incorporated in the concentration of 0.15grams to produce a total extract concentration of 0.45 grams in each formulation.

Formula of cream formulation

Ingredient	Quantity taken (10g)
Extract combination	0.45g
White bees wax	2g
Liquid paraffin	6g
Borax	0.1g
Purified water	1.9ml
Rose oil	QS

Formula of gel formulation

Ingredient	Quantity taken(10g)
Extract combination	0.45g
Sodium alginate	1.2g
Glycerine	0.2g
Methylhydroxyl benzoate	0.02g
Calcium gluconate	0.005g
Purified water	QSP 10g

Antimicrobial screening of the cream and gel formulations against acne producing organisms

The *in-vitro* antimicrobial activity of the formulated gel and cream were evaluated alone and combined with citronella oil against acne producing microorganisms. Two microorganisms were used for the study they were *Propionibacterium acnes* and *Staphylococcus epidermidis*. Three concentrations, viz: - 0.25g, 0.5g and 1.0 g of the formulation diluted with 10 ml sterile water and in citronella oil combined formulations, the oil was added in 1:1 ratio, these two formulations were used for antimicrobial screening. The antimicrobial activity was evaluated by agar well diffusion method and the zones of inhibition were recorded.

Evaluation of various physicochemical parameters of the prepared formulations

Various physicochemical parameters such as color, odour, appearance, pH, viscosity, homogenicity and spreadability were performed on the prepared formulations which are as follows: -

Determination of clarity and colour: It was done with naked eyes against white background.

Determination of odour: It was done by mixing in water and smelling it.

pH: The pH of all the prepared formulations was determined by using Digital pH Meter. A 50% aqueous suspension of the prepared formulation was stored for two hours. The measurement of pH was done in previously calibrated pH meter.

Viscosity: About 50 ml of hand wash gel was taken into a 100ml beaker and placed under a Brookfield viscometer. The tip of the Brookfield viscometer was dipped in the beaker and the viscosity measured.

Determination of homogenicity: The formulated hand wash was examined for its homogenicity and observed for any phase separation by visual inspection.

Determination of spreadability: The Spreadability was expressed in terms of time in seconds taken by two slides to slip off from the gel and cream, placed in between the slides, under certain load. Lesser the time taken for separation of the two slides, better the Spreadability. Two sets of glass slides of standard dimensions were taken. The formulations were placed over one of the slides. The other slide was placed on the top of the formulation, such that the formulation was sandwiched between the two slides weight was placed upon the upper slides so that the gel and cream between the two slides was pressed uniformly to form a thin layer. The weight was removed and the excess of formulation adhering to the slides was scrapped off. The upper slide allowed slipping off freely by the force of weight tied to it. The time taken for the upper slide was noted.

RESULTS AND DISCUSSION

Antimicrobial screening of the individual extracts

All the leaf and bark extracts of four plants were prepared in the concentration of 0.5mg/ml in DMSO and subjected to antimicrobial screening by agar well diffusion method against the microorganisms'*E coli* (MTCC-1698), *S aureus* (MTCC-1143)and *P aeruginosa* (MTCC-2453). Among the 40 extracts, 25 extracts exhibited good antimicrobial activity against the selected strains of microorganisms with zones of inhibition above 10mm.

Maximum activity was observed in the methanolic bark extracts of *Cassia fistula* (ZOI= 18mm), which was followed by the methanolic leaf and bark extracts of *Ficusreligiosa,Milletiapinnata*and *Wendlandiathyrsoidea* (ZOI= 14 mm). The results are tabulated in Table1.

Evaluation of synergistic antimicrobial efficacy of the extract combinations

Different combinations of extracts were prepared with each extract having a concentration of 0.25 mg/ml. 1ml each extract was mixed and tested against the strains of *E coli* (MTCC-1698), *S aureus* (MTCC-1143)and *P aeruginosa* (MTCC-2453) by agar diffusion method. Maximum antimicrobial activity was seen in combination no. 2 followed by fifth, third and first combinations respectively. The combination no. 4 and 6 showed very less activity. The zones of inhibition of the combinations were better when compared to the individual screened extracts, this may be attributed to the synergistic effect of the combined extracts. The results of the antimicrobial activity in terms of zones of inhibition are tabulated in Table 2.

Determination of percentage growth inhibition by ditch plate method

The extract combinations no. 1, 2, 5 and 6, which exhibited maximum antimicrobial activity were evaluated for its percentage growth inhibition by ditch plate method against six microorganisms' i.e. *S aureus, P aeruginosa, E coli, Listeria, Bacillus cereus* and *Salmonella*nn at concentrations of 0.30, 0.75 and 1.5mg/ml.

Results revealed that maximum percentage inhibition was observed with combination no. 1 which exhibited 100% growth inhibition which was followed by combination no. 6 and 5 which exhibited inhibition of 96.4 and 92.5% respectively at various concentrations against the selected strains of microorganisms. The results are tabulated in Table 3 and Figures 3 and 4.

Table 1: Zones of inhibition (in mm) diameter of the extracts.

S. No.	Extract	Zones of inhibition(mm)		
		P aeruginosa	*E coli*	*S aureus*
1	CB-PET	--	10.0	9.0
2	CB-CHL	--	10.0	--
3	CB-ETH	16.0	12.0	14.0
4	CB-MEOH	14.0	16.0	18.0
5	CB-40MOH	--	10.0	9.0
6	CL-PET	--	10.0	9.0
7	CL-CHL	--	10.0	9.0
8	CL-ETH	--	--	11.0
9	CL-MEOH	--	14.0	14.0
10	CL-40MOH	--	--	--
11	FL-PET	--	--	--
12	FL-CHL	--	--	--
13	FL-ETH	--	--	--
14	FL-MEOH	14.0	8.0	14.0
15	FL-40MOH	15.0	8.0	12.0
16	FB-PET	--	--	--
17	FB-CHL	--	--	--
18	FB-ETH	6.0	12.0	14.0
19	FB-MEOH	--	10.0	14.0
20	FB-40MOH	8.0	10.0	12.0
21	MB-PET	--	10.0	10.0
22	MB-CHL	--	10.0	9.0
23	MB-ETH	16.0	12.0	--
24	MB-MEOH	--	14.0	12.0
25	MB-40MOH	--	13.0	--
26	ML-PET	--	9.0	--
27	ML-CHL	--	9.0	--
28	ML-ETH	14.0	16.0	14.0
29	ML-MEOH	15.0	18.0	18.0
30	ML-40MOH	--	12.0	--
31	WB-PET	--	--	--
32	WB-CHL	--	--	--
33	WB-ETH	14.0	10.0	13.0
34	WB-MEOH	--	14.0	10.0
35	WB-40MOH	--	9.0	9.0
36	WL-PET	--	--	--
37	WL-CHL	--	--	--
38	WL-ETH	12.0	14.0	12.0
39	WL-MEOH	--	9.0	8.0

40	WL-40MOH	--	--	--

Note: CB= *Cassia fistula* bark.CL= *Cassia fistula* leaf. FL= *Ficusreligiosa*leaf. FB= *Ficusreligiosa*bark. MB=*Milletiapinnata*bark. ML=*Milletiapinnata*leaf. WB= *Wendlandiathyrsoidea*bark. WL= *Wendlandiathyrsoidea*leaf. PET= Petroleum ether. CHL= Chloroform. ETH= Ethyl acetate. MEOH= Methanol. 40MOH= 40% Methanol.

Table 2: Zones of inhibition (mm) in diameter of combined extracts

S. No.	Extracts	Zones of inhibition (mm)	
		E coli	*S aureus*
1	CB-MEOH+WB-ETH+FB-MEOH	14.0	20.0
2	CB-ETH+FB-ETH+CB-MEOH	14.0	18.0
3	CL-MEOH+ML-MEOH+FL-MEOH	12.0	12.0
4	ML-ETH+WL-ETH+FL-40MOH	6.0	6.0
5	CB-MEOH+CL-MEOH+FL-MEOH	14.0	16.0
6	CB-ETH+ML-ETH+WL-ETH	12.0	12.0

Table 3: Percentage microbial growth inhibition by the extract combinations by ditch plate method.

Organism	Conc. (mg/ml)	Combination-01			Combination-02			Combination-05			Combination-06		
		LOS (mm)	LOI (mm)	%INH	LOS (mm)	LOI (mm)	%INH	LOS (mm)	LOI (mm)	%INH	LOS (mm)	LOI (mm)	%INH
S aureus	0.30	27	24	89.0	28	06	21.4	27	08	29.6	27	16	22.2
	0.75	32	29	90.6	30	12	40	29	22	75.8	30	20	66.6
	1.5	29	28	96.5	27	14	51.8	26	24	92.3	28	22	78.5
P. aeruginosa	0.30	29	26	89.6	26	04	15.3	28	10	35.7	28	12	42.8
	0.75	33	30	91.0	31	12	38.7	31	20	64.5	31	25	80.6
	1.5	28	27	96.4	26	16	61.5	27	25	92.5	28	27	96.4
B. cereus	0.30	32	27	84.3	27	08	29.6	26	12	46.1	29	10	34.4
	0.75	33	30	90.9	33	14	42.4	32	21	65.6	31	27	87.0
	1.5	32	32	100.0	26	18	69.2	27	24	88.8	27	24	88.8
E. coli	0.37	28	24	85.7	29	08	27.5	28	11	39.2	26	04	15.3
	0.75	30	27	90.0	32	16	50.0	30	18	60.0	38	12	31.5
	1.5	27	26	96.2	27	18	66.6	28	24	85.7	25	20	80.0
Listeria	0.37	27	24	88.8	28	01	3.5	28	10	35.7	26	00	00
	0.75	29	26	89.6	30	08	26.6	32	12	37.5	29	00	00
	1.5	26	26	100.0	28	12	42.8	30	18	60.0	27	00	00
Salmonella	0.37	26	22	84.6	27	06	22.2	27	14	51.8	28	00	00
	0.75	29	27	93.1	29	08	27.5	29	18	62.0	29	08	27.5
	1.5	27	27	100.0	28	12	42.8	26	24	92.3	27	14	51.8

Note: LOS= Length of streak. LOI= Length of inhibition. %INH= Percentage inhibition.

Antimicrobial screening of the cream and gel formulations against acne producing organisms

The *in-vitro* antimicrobial activity of the formulated gel and cream were evaluated against microorganisms *Propioni-bacterium acnes* and *Staphylococcus epidermidis*. The cream and gel formulations with citronella oil exhibited slight more activity when compared to formulations without oil.

Maximum zone of inhibition was observed in gel formulation combined with citronella oil in the concentration of 0.5g and 1.0g, which exhibited zones of inhibition of 16 and 24mm respectively. The lowest zones of inhibition were observed with cream formulation without oil which exhibited zones of inhibition of 6, 10 and 16mm at concentrations of 0.25, 0.5 and 1.0g respectively. The results are tabulated in table4andfigures1 & 2.

Table4: Zones of inhibition of the cream and gel formulations alone and combined with citronella oil

Formulation	Concentration(g)	Zone of Inhibition (mm)			
		P. acnes		*S. epidermidis*	
		Without oil	With oil	Without oil	With oil
Cream	0.25	8.0	14.0	6.0	12.0
	0.5	12.0	16.0	10.0	14.0
	1.0	18.0	18.0	16.0	22.0

Gel					
	0.25	6.0	10.0	4.0	10.0
	0.5	15.0	16.0	12.0	20.0
	1.0	24.0	24.0	22.0	22.0

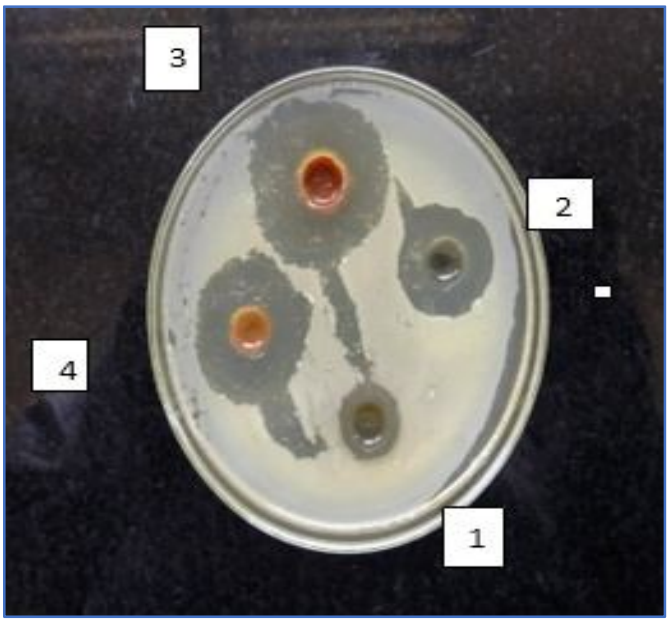

Fig. 1: 1=gel(0.25g). 2=gel(0.5g). 3=gel(1.0g). 4=cream (1.0g)

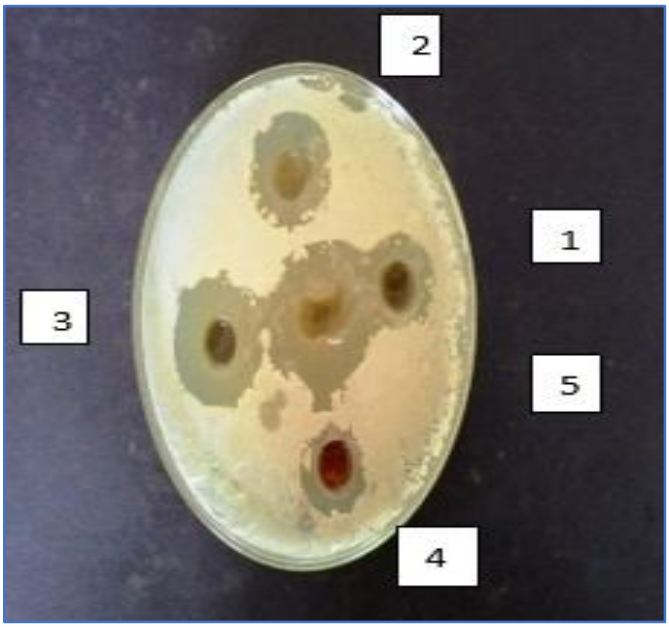

Fig. 2: 1=gel(0.25g). 2=cream(0.5g). 3=cream(1.0g). 4=cream(0.25g). 5=gel (1.0g)

Figures1&2: Zones of inhibition of the formulated gel and cream against *P acnes&S epidermidis* combined with citronella oil and without oil respectively.

Evaluation of physicochemical parameters of the prepared formulations

Various physicochemical parameters such as color, odour, appearance, pH, spreadability and extrudability as mentioned in the methodology were performed to establish quality of the prepared formulations. All the four-prepared gel and cream formulations exhibited good and satisfactory characters; the results are tabulated in Table 5.

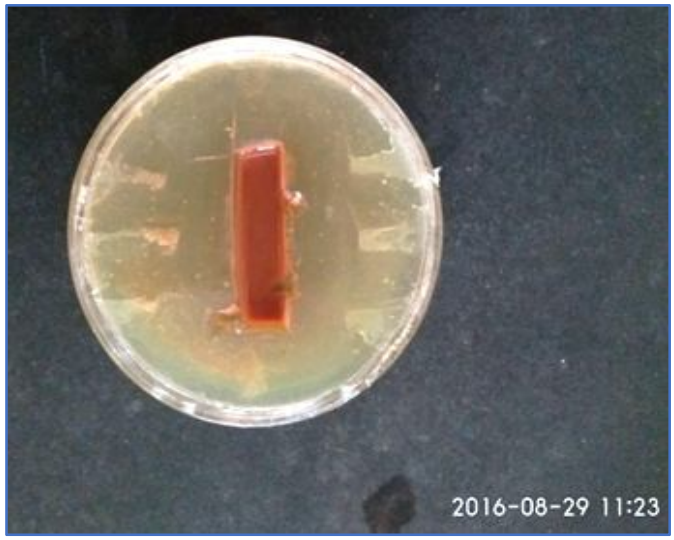

Fig. 3: Antimicrobial activity of creamby "Ditch plate" method

Fig.4: Antimicrobial activity of gel by "Ditch plate" method

Table5: Physicochemical parameters of the prepared cream and gel formulations

Formulation	Color	Odor	Appearance	pH	Spreadability	Extrudability
Cream	Light brown	Fragrant	Good	6.5	Easily spreadable	Good
Gel	Light brown	Fragrant	Good	6.0	Spreadable	Hard

CONCLUSION

It was finally concluded that the results obtained from this research work clearly indicated a promising antiseptic and antimicrobial efficacy of the prepared topical formulations and can be used after further standardization.

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