ORIGINAL ARTICLE e-ISSN: 2349-0659 p-ISSN; 2350-0964

Preparation of Polyherbal Cream (formulation) Against Multiple Drug Resistant Bacteria-*Staphylococcus aureus*

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ABSTRACT

The present study was done to find out the phytochemical constituents, antibacterial activity, and minimum inhibitory concentration of water extract of four medicinal plants, that is, *Murraya koenigii*, *Eucalyptus globulus*, *Dodonaea viscose,* and *Mentha spicata*. Polyherbal formulation was designed on the basis of phytochemical and antibacterial activity of medicinal plants. All plants showed antibacterial activity against Methicillin resistant *Staphylococcus aureus* in the form of zone of inhibition. The maximum zone of inhibition showed by *M. koenigii* (leaf water extract) among all plants against methicillin resistant *S. aureus,* that is, 19 mm. All plants used collectively with different concentration and found zone of inhibition, that is, 22 mm. Also determined MIC of polyherbal formulation and it was found 14mg/ml against Methicillin resistant bacteria *Staphylococcus aureus*. Cream was prepared using 14 mg/mL concentration of polyherbal formulation in Phase B (Water phase) and all physicochemical parameter of poly herbal cream was found stable at room temperature and at 45°C for treatment of multiple drug resistant bacteria (*S. aureus*) causing skin disease. This polyherbal formulation can be used as good alternative of allopathic medicine for the treatment of skin disease in future. Being herbal this formulated cream has no side effects as compare to allopathic drugs.

Keywords: Antimicrobial activity, *Murraya Koenigii*, Phytochemical

Asian Pac. J. Health Sci., (2022); DOI: 10.21276/apjhs.2022.9.2.57

Introduction

Skin is the largest organ in our body. It protects skin from bacteria and viruses and regulates our body temperature. Conditions that irritate, clog, or inflame the skin can cause symptoms such as redness, swelling, burning, and itching, allergies, irritants, your genetic makeup, and certain diseases and immune system problems can cause dermatitis, hives, and other skin conditions.[1] At present, many natural products from plants have been used by various cultures all over the world to treat skin diseases or their symptoms caused by micro-organisms.[2] *Murraya koenigii*, *Eucalyptus globulus*, *Dodonaea viscose,* and *Mentha spicata* possessed antimicrobial, antioxidant, wound, anti-inflammatory, analgesic, antispasmodic, and detoxification effects. Curry leaves are rich in calcium, phosphorous, iron, and vitamin such as C, A, B, and E. It has good medicinal qualities such as fight with infection, improve hair, and skin qualities and also help in controlling the blood sugar level at the same time improve the digestion. Herbal formulations always have attracted considerable attention due to their good activity and comparatively lesser or nil side effects with synthetic drugs. For various types of skin ailments, formulations such as skin protective, sunscreen, antiacne, anti-wrinkle, and anti-aging are designed using varieties of materials, either natural or synthetic. Cream is a polyherbal formulation that consists of extracts of *M. koenigii*, *E. globulus*, *D. viscose,* and *M. spicata*. These herbs have been selected on the basis of a traditional system and scientific justification with modern uses. An herbal cream that can give effective protection to skin and free from any toxicity or toxic residue or any irritation when regularly used and should also be cosmetically acceptable. The present study was designed to investigate the phytochemical properties of four medicinal plants, formation of polyherbal formulation, and preparation of herbal cream for treatment of multiple drug resistant bacteria (*Staphylococcus aureus*) causing skin disease.

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How to cite this article: Sharma R, Ahuja D. Preparation of Polyherbal Cream (formulation) Against Multiple Drug Resistant Bacteria-*Staphylococcus aureus*. Asian Pac. J. Health Sci., 2022;9(2):405-410.

Source of support: Nil.

Conflicts of interest: None.

Received: 02/01/2022 **Revised:** 15/02/2022 **Accepted:** 11/03/2022

Materials and Methods

Preparation of Plant Extracts

Preparation of extract

Using a Soxhlet device, 20 g of four plants, that is, *M. koenigii*, *E. globulus*, Dodonaea viscosa, and *M. spicata* powder was extracted with high polarity solvents such as water, methanol, and ethanol. The water, methanol, and ethanol extracts were dark in color, yielding 2.21, 3.61, and 2.98%, respectively. The extraction temperature of 40°C being determined to be the most effective.[3,4] Aqueous extracts were selected for further process.

Preliminary Phytochemical Analysis

The leaf powder of the study plant was dissolved in various solvents and the preliminary phytochemical tests were carried out.

Phytochemical Screening

Alkaloids (Mayer's test)

1.36 g of mercuric chloride is solved in 60 mL and 5 g of potassium iodide were dissolved in 10 mL of distilled water, respectively.

These two solvents were mixed and diluted to 100 mL using distilled water. To 1 mL of acidic aqueous solution of samples, few drops of reagent were added. Formation of white or pale precipitate showed the presence of alkaloids.

Flavonoids

In a test tube containing 0.5 mL of alcoholic extract of the samples, 5–10 drops of diluted HCl and small amount of Zn or Mg were added and the solution was boiled for few minutes. Appearance of reddish pink or dirty brown color indicated the presence of flavonoids.

Glycosides

A small amount of alcoholic extract of samples was dissolved in 1 mL water, and then, aqueous sodium hydroxide was added. Formation of a yellow color indicated the presence of glycosides.

Steroids (Salkowski's Test)

About 100 mg of dried extract was dissolved in 2 mL of chloroform. Sulfuric acid was carefully added to form a lower layer. A reddish-brown color at the inter face was an indicative of the presence of steroidal ring.

Cardiac Glycosides (Kellerkilliani's Test)

About 100 mg of extract was dissolved in 1 mL of glacial acetic acid containing one drop of ferric chloride solution and 1 ml of concentrated sulfuric acid was added. A brown ring obtained at the interface indicated the presence of a deoxy sugar characteristic of cardenolides.

Saponins

A drop of sodium bicarbonate was added in a test tube containing about 50 mL of an aqueous extract of sample. The mixture was shaken vigorously and kept for 3min. A honey comb like froth was formed and it showed the presence of saponins.

Resins

To 2 mL of chloroform or ethanolic extract 5–10 mL of acetic, an hydrite was added and dissolved by gentle heating. After cooling, 0.5 mL of H_2SO_4 was added. Bright purple color was produced. It indicated the presence of resins.

Phenols (Ferric Chloride Test)

To 1 mL of alcoholic solution of sample, 2 mL of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. Formation of blue or green color indicated the presence of phenols.

Tannins (Lead Acetate Test)

In a test tube containing about 5 mL of an aqueous extract, a few drops of 1% solution of lead acetate were added. Formation of a yellow or red precipitate indicated the presence of tannins.

Terpenoid

2 mL of chloroform and 1 mL of conc. H_2SO_4 was added to 1 mg of extract and observed for reddish-brown color that indicated the presence of terpenoid.

Test for Quinone

To 1 mL of extract, a few drops of concentrated hydrochloric acid were added. A yellowish-brown color was observed that showed the presence of quinone.

Test for Proteins Ninhydrin Test (Acetone)

Ninhydrin was dissolved in acetone. The leaf extract was treated with ninhydrin and observed for the formation of purple color.

Results and Discussion

Phytochemical analysis of four different water extracts of *M. koenigii*, *E. globulus*, *D. viscose,* and *M. spicata* was analyzed which shown in Table 1.

Collection, Enrichment, and Identification of *S. aureus*[5]

Nasal samples will be collected from Nalagarh civil hospital for the isolation of multiple drug resistant *S. aureus*. Selective media (Mannitol Salt Agar) media will be used in the isolation of *S. aureus*. Biochemical testing will be done for identification of species

Table 1: Phytochemical analysis of four different water extracts

Phytochemical constituents	*Eucalyptus globules*	*Murraya koenigii*	*Dodonaea viscosa*	*Mentha spicata*
Alkaloids	-	+	-	+
Flavonoids	+_	_+	_+	+
Glycosides	++	-	++	-
Steroids	+	-	+	+
Cardiac glycosides	+	-	+	-
Saponins	_+	_+	_++	+
Phenols	-	+	-	+
Tannins	-	+	-	+
Terpenoid	+	++	+	+

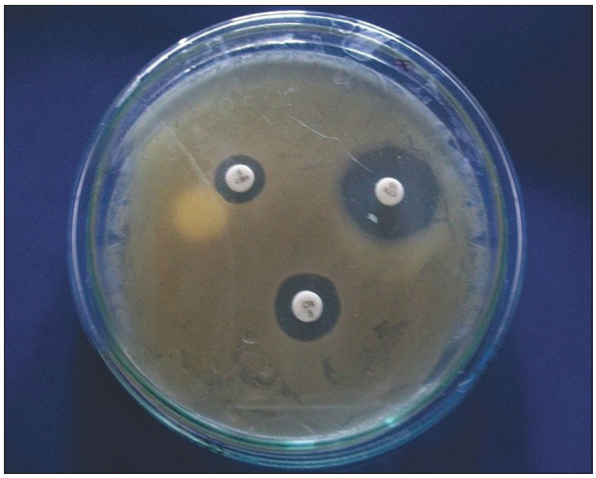


Figure 1: Different zones of inhibition shown by methicillin-resistant *Staphylococcus aureus*

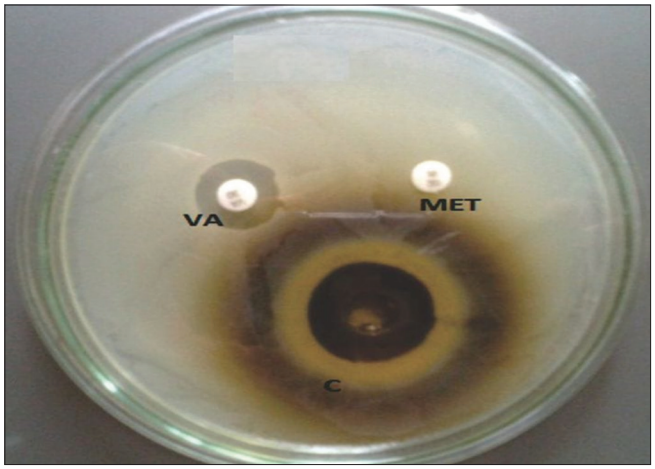


Figure 2: Antibacterial activity of polyherbal formulation against MRSA (C-extract of poly herbal formulation, METL: Methicillin, and VA: Vancomycin

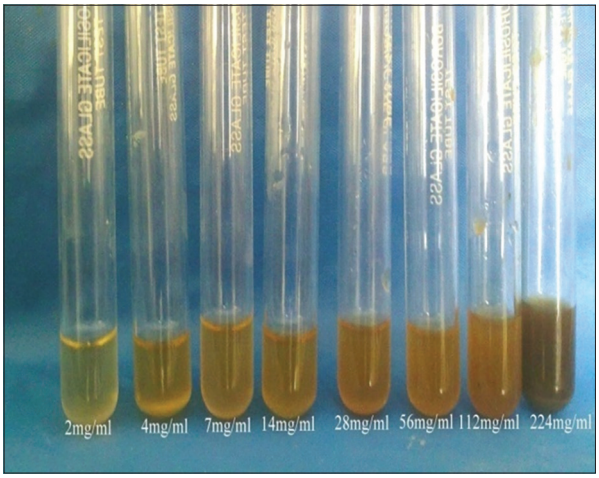


Figure 3: Before 24 h of incubation

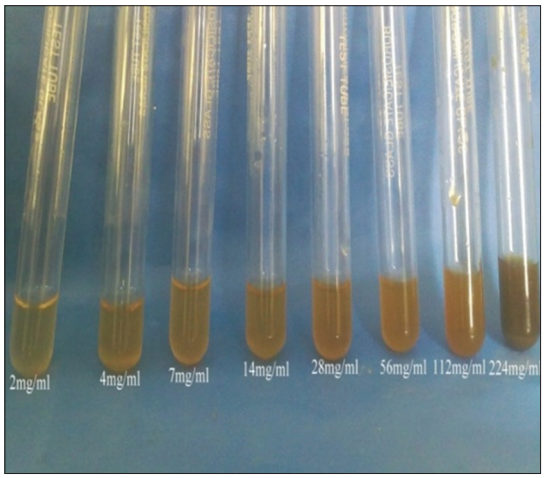


Figure 4: After 24 h of incubation

(Catalase test, DNase, Coagulase test). Hi Crome Me Re Sa agar will be used for identification of MDR *S. aureus*.

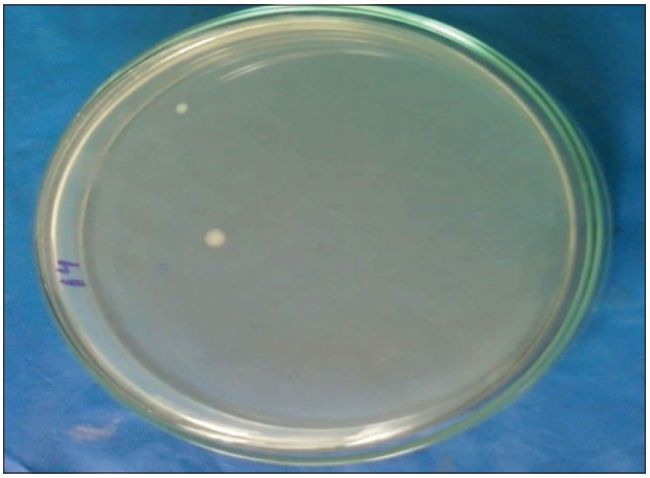


Figure 5: Minimum inhibitory concentration of polyherbal formulation (14 mg/mL)

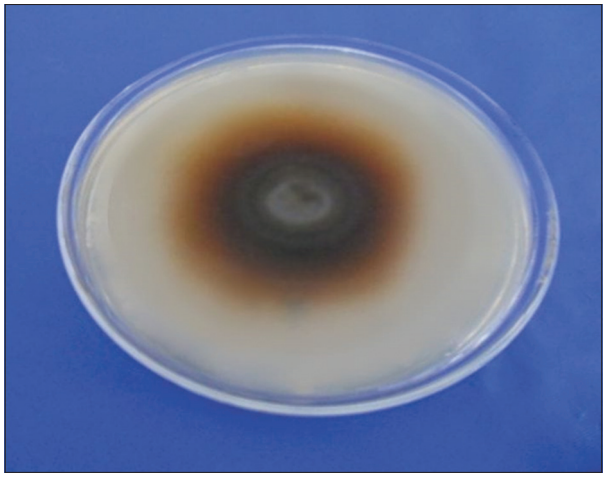


Figure 6: Zone of inhibition of 14 mg of polyherbal formulation (20 mm)

Antibiotic susceptibility testing

Antibiotic sensitivity of all isolates of *S. aureus* was determined using HiMedia antibiotic disks as per the method described by Kirby and Bauer – 1966. The zone of inhibition around the disks was measured and interpreted as sensitive, moderately sensitive, and resistant using the interpretation chart supplied by the antibiotic disk manufacturers (HiMedia, Mumbai).

Determination of the Antibacterial Activity of Medicinal Plants[6]

From the crude extract, the 500 mg/mL dilution of plant paste will be prepared for antibacterial assay. The modified agar well diffusion method will be employed to determine the antibacterial activity of plant extracts,

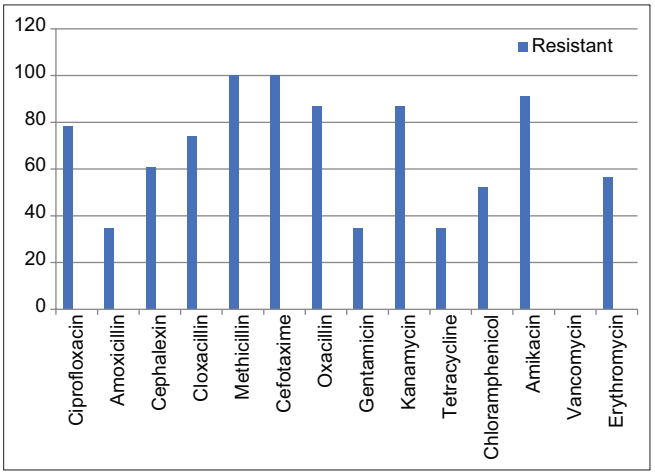
Out of 20 nasal samples, 12 *S. aureus* were recovered and were, further, subjected to biochemical testing. Only three methicillin-resistant *S. aureus* (MRSA) strain were recovered from 12 *S. aureus*. The prevalence of MRSA from nasal samples of Nalagarh hospital was 25%.

Antibiogram

We have tested 14 different types of antibiotics for the susceptibility pattern of methicillin resistant *S. aureus* isolates on Mueller–Hinton agar (MHA) plates.[3] The drug resistance patterns of MRSA isolated from clinical specimens and carrier screening samples were found to be highly variable. Almost all the MRSA strains screened from nasal samples were 100% resistant to amikacin, 86.95% to kanamycin and cloxacillin, 78.26% to ciprofloxacin, 56.52% to erythromycin, 52.17% to chloramphenicol, and 34.78% to both tetracycline and gentamycin. In general, all MRSA provided were multidrug resistant [Table 2 and Graph 1].

Antibacterial Activity of Polyherbal Formulation against MRSA

Different zone if inhibition were determined of the antibiotics Merhicillin, oxycyllin and vancomysin against methicillin resistant *Staphylococcus aureus* bacteria. Vancomycin showed high zone of inhibition as compared tp other antibiotics as ashown in Figure 1. Further, antibacterial activity of polyherbal formula was also determined in coparison with vancomycin and methicillin bacteria. Out of which Polyherbal formula showed good results as shown in Figure 2.



Graph 1: Different antibiotic resistant pattern of methicillin-resistant *Staphylococcus aureus*

Table 2: Antibiogram of MRSA

Antimicrobial agent	*Disk potency (μg)*	*Zone diameter (mm)*		
		Resistant	*Intermediate*	*Susceptible*
Ciprofloxacin	10	78.26%	21.73%	0.01%
Amoxicillin	10	34.78%	4.34%	60.86%
Cephalexin	30	60.86%	4.34%	34.78%
Cloxacillin	5	73.91%	17.39%	8.69%
Methicillin	5	100%	-	-
Cefotaxime	10	100%	-	-
Oxacillin	5	86.95%	-	13.05
Gentamicin	50	34.78%	39.13%	26.09%
Kanamycin	5	86.95%	13.04%	0.01%
Tetracycline	10	34.78%	52.17%	13.04%
Chloramphenicol	10	52.17%	43.47%	4.36%
Amikacin	10	91.30%	4.34%	4.36%
Erythromycin	15	56.52%	43.47%	-

MRSA: Methicillin-resistant *Staphylococcus aureus*

Determination of Minimum Inhibitory Concentration (MIC) of MDR Bacteria using Polyherbal Formulation

MIC is defined as the lowest concentration of extracts that completely inhibit the growth of the microorganism in 24 h (Thongson C, 2004). Growth of MRSA bacteria inhibited at 14 mg/mL concentration of polyherbal formulation was the minimum concentration required to kill bacteria shown in Figures 3-5. Final zone if inhibition of the above said concentration that is 14 mg/ml showed 20 mm zone of inhibition as shown in Figure 6.

Preparation of Anti-microbial Polyherbal Cream Formulation

Various dummy formulations were prepared and out of which the optimized formulation was selected for further studies.[7]

Cream

Preparation of cream formulation

Water phase consists of carbopol 980 and xanthan gum which were weighed accurately into separate vessel. It was dispersed in demineralized water under gentle mixing using stirrer in water bath. Similarly, Oil phase which comprises of Cetyl alcohol, Cetostearyl alcohol, steric acid, triglycerides, Isopropyl myristate, Glyceryl monostearate and were weighed accurately in separate vessel. followed by heating of both phases in seperately. Both the phases were heated at 80°C in water bath and when both the temperature were attained at 80°C that oil phase was transferred to water phase and homogenized for 30 min at 100 rpm. It was cooled at room temperature. Antioxidant, vitamin E and preservative, and phenoxyethanol were added to the cream formulation. Cream was mixed well for 10 min. The five different formulas were selected to optimize viscosity, pH, and rheological studies such as emulsion

Table 3: Formulation of polyherbal cream

Content	*Concentration (%)*
Drug extract	19.2
Oil phase	
Stearic acid	4
Cetyl alcohol	3
Cetostearyl alcohol	5
triglycerides	2
Isopropyl myristate	2
Glycerol monostearate	2
Aqueous phase	
Carbopol 980	4
Xanthan gum	3
Methyl paraben	0.2
DM Water	Q.S

Table 4: Parameters for cream

Type of formulation	*Parameters*			
	Clarity and appearance	*Presence of fiber and particles*	*Viscosity (cps)*	*Hardness (kg/cm²)*
Cream	Good	Clear	6665±95	-

Table 5: Parameter of polyherbal cream

Formulation	*Parameter*		*Adverse effect*			
	Acid value	*Saponification value*	*Irritant*	*Erythema*	*Edema*	*pH*
Poly herbal cream	6.8	30.1	NIL	NIL	NIL	6.8

Table 6: Physical parameter of polyherbal cream at room and accelerated temperature

Formulation	*Homogeneity*	*Appearance*	*Spreadibility*	*After feel*	*Type of smear*	*Removal*	*pH*
Poly herbal cream	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.8
At room temperature							
1st day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.8
5th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.6
10th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.6
15th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.5
20th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.5
At 40°C+1°C							
1st day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.8
5th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.4
10th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.6
15th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.7
20th day	Good	NCC	Good	Emollient	Non-Greasy	Easy	6.4

NCC: No change in color

strength, thixotropicity and elasticity from lowest to highest concentration of emulsifiers, and gelling agents. Five different permutation combinations helped to optimize a single stable formula which was studied at 30°C and 45% RH and 45°C and 75% RH which are mentioned in Table 3 followed by evaluation of cream as discussed in Table 4.

The organoleptic properties, including physical appearance, color, texture, phase separation, homogeneity, and immediate skin feel of the cream formulation. Results discussed in Table 4, showed that the formulation has a good appealing appearance and smooth texture, and it was homogenous with no signs of phase separation. The formulation was brownish in color and characteristic odor.

pH of dosage form formulation was found from 6.94 ± 0.2 and 7.0 ± 0.4 that is within the range and compatible with skin pH. The pH of all formulation lies in the normal pH range of the skin.

Hardness test is indicative of strength cream and the results are found to be 121 ± 2.35 kg/cm^2, which indicating that cream has adequate strength of hardness which is compatible with skin surface and produce the best result.

Rheological property of the semisolid formulations gel can be assessed by spread ability. Spread ability test is a qualitative tool to evaluate physical state as well as the bioavailability of the formulation. The spread ability value was found in the range from 6.99 ± 0.12 to 7.3 ± 0.14 (g. cm/s) which indicates the better spread ability of the formulations.

Viscosity is another most important aspect of any semisolid dosage form which indicates the maximum retention time of formulation over the skin surface. In all these formulations, the viscosity was found between 6505 ± 110 and 6862 ± 98, which almost same and indicating the good retention time over the skin surface.

While evaluation cream for adverse effects it was fount to have no adverse effect on the skin as discussed in Table 5.

Physical Parameter of polyherbal cream at room and accelerated temperature was also found to be satisfactory as shown in Table 6.

Discussion

The present study was carried out to evaluate the phytochemicals present in the *M. koenigii*, *E. globulus*, *D. viscose,* and *M. spicata*. Water extract of each plants showed the presence of alkaloids, glycosides, tannins, and terpenoids. These plants possessed best antibacterial activity against multiple drug resistant bacteria, that is, *S. aureus*. Polyherbal formulation prepared using the concentration of plant extract as per the results of antibacterial activity of the plants. Instead of single plants, we found more effective results when we used combined plant extract in the form of polyherbal formulation. Cream was prepared using polyherbal formulation and was safe in respect to skin irritation and allergic sensitization. The polyherbal formulation has antiseptic activity, anti-inflammatory activity, and also increases whitening of skin. Hence, all these properties are beneficial to normal human skin and it is safe and stable too.

Our results of cream are found similar to study conducted by Bauer *et al.*[8] He has prepared polyherbal formulation using Alovera and turmeric extract and cream found effective against skin disease.[9] He has prepared moistening, nourishing, lightening cream, and treatment of various diseases of the skin. However, they have used more than ten plants for polyherbal formulation, but we have used only four plants. From costing point of view, our herbal cream is much better than others. Our cream results are safe and effective as study done by Shankar *et al.*[10] He has prepared the skin care cream using three different plants and found safe and effective.

Conclusion

The herbal cream was successfully developed that met the relevant pharmaceutical characteristics form. The cream possessed definite antibacterial activity against the multiple drug resistant bacteria, that is, *S. aureus* reported to be major cause for various skin manifestations and should be effective *in vivo*. The developed herbal cream is a potential candidate for treatment of multidrug resistant bacteria causing skin disease.

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