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Full Length Research Paper

Development and evaluation of antimicrobial herbal cosmetic preparation

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This study was conducted to formulate a polyherbal cosmetic cream comprising plant extracts such as *Glycyrrhiza glabra* root, *Piper betle* leaves and *Azadirachta indica* leaves and to check their antimicrobial potential which can be used in the treatment of infectious skin diseases. Stability studies and patch test were also performed to check the efficacy of the formulations in comparison to base (control). Four types of different herbal cream formulations, namely A, B, C and D were prepared by incorporating different concentrations of herbal extracts in combination. These cosmetic preparations were evaluated at storage conditions (8 and 40°C with relative humidity 75%) on different parameters like pH, viscosity, acid value, peroxide value, total fatty matter, centrifugation, stability studies, and patch test for one month. Antimicrobial activity of the formulations was also checked by well diffusion method. Formulation D was found to be the best and A was better among all the other preparations and base. Formulations A and D showed good spreadibility, pH, appearance, viscosity, good antimicrobial potential and no evidence of phase separation. Formulations A and D showed no redness, inflammation and irritation during patch test. These formulations are safe to use for skin. Thus, the result showed that formulation D containing minimum amount of herb extracts (0.1% each) exhibited good stability during storage, antimicrobial activity and also no major changes was observed during the entire study as compared to other formulations and base.

Key words: *Azadirachta indica*, *Piper betle*, *Glycyrrhiza glabra*, antimicrobial, cream formulation, extract, cosmetics, pH, viscosity, acid value, peroxide value, total fatty matter, centrifugation, stability study, well diffusion method, patch test.

INTRODUCTION

The cosmetic and toiletry formulation market is growing based on herbs globally. Apart from traditionally documented applications, some modern trials have also established the utility of herbs in personal care products. Herbal cosmetics, referred as products, are formulated, using various permissible cosmetic ingredients to form the base in which one or more herbal ingredients are used to provide defined cosmetic benefits only, shall be called “Herbal Cosmetics”. The demand of herbal medicines is increasing rapidly due to their lack of side effects (Gediya et al., 2011). World Health Organization (WHO) notes that 74% of the plant derived medicines are used in modern medicine, in such a way that their modern application directly correlates with their traditional

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use as herbal medicines by native cultures (Kumar et al., 2003). The natural content in the herbs does not have any side effects on the human body; instead enrich the body with nutrients and other useful minerals. The plants possess a vast and complex arsenal of active ingredients (photochemical) not only able to calm or smooth the skin, but also actively restore, heal and protect the skin as it is obvious by scientific literatures (Patel et al., 2013).

There are large numbers of medicinal plants which are widely used in the treatment of skin diseases and also possessed antimicrobial activity. However, plants are very complex in their compositions and their therapeutic activity depends on their major active chemical constituents. Also, improper authentication of herbs, adulterations by microorganism, and pesticide residue, has made standardization of herbal drug of primary importance. Thus, before using these medicinal herbs in any formulation, their authentication is necessary.

This study was made to develop herbal skin care formulation for antimicrobial action against various selected microorganisms like *Staphylococcus aureus* which are associated with localized skin infection, *Escherichia coli, Bacillus subtilis, Aspergillus niger* and *Penicillium chrysogenum*. The skin care formulation consists of *Azadirachta indica* leaves, *Glycyrrhiza glabra* roots and *Piper betle* leaves and these herbs have been selected on the basis of a traditional system and scientific justification with modern uses. This study was expanded by obtaining ethanolic extracts of selected herbs, to test these plants for their main active constituents and then to incorporate these extracts into cosmetic formulations for skin care. The formulations were also checked for their antimicrobial activities. Endeavors were also made to determine the physicochemical stabilities of formulations by assessing their organoleptic characteristics over time, thereby verifying the antimicrobial efficacy of these extracts against selected microbial strains. This herbal skin care formulation (semisolid cosmetic cream) can give effective protection to skin and free from any toxicity (paraben free, alcohol free) or toxic residue or any irritation when regularly used and should also be cosmetically acceptable.

MATERIALS AND METHODS

Chemicals and glass wares

All the chemicals and reagents used were laboratory grade. Glass wares used were from Borosil. The solid media and broth used for microbial culture were from Hi-Media Pvt. Limited, Bombay, India.

Collection of plant

A. indica leaves were obtained from Biotech Park, Lucknow, India, while *G. glabra* roots and *P. betle* leaves were purchased from the local market of Lucknow, India. The samples were kept for formation of herbarium sheet and also authenticated from Biotech Park Laboratory, Lucknow, India.

Preparation of extracts

Leaves of *A. indica*, *P. betle* and roots of *G. glabra* were dried in dryer at 40 ± 1°C for 2 days. The dried samples were then powdered by grinder and stored in air tight bags till extraction. Dried powdered material was extracted using absolute ethanol as solvents by using Polytron homogenizer (Ultra Turrax® T50 Basic from IKA®-WERKE). 25 g of dried powder was dissolved in 150 ml of solvent and left for overnight. Next day, extraction was performed using Polytron homogenizer (Ultra Turrax® T50 Basic from IKA®-WERKE). The extraction procedure was repeated three times and the filtered solvent was removed under vacuum using rotatory evaporator (PERFIT, India). The dried crude extracts were stored at 4°C.

HPLC analysis of extracts

Equipment

Analysis was carried out using a Schimadzu HPLC 20A series with manual injection. The system comprises a LC-SPD-M20A VP diode array detector, CBM-20 interface AD pumps, a model CBM-20 interface, a model 7725i, manual injector (Rheodyne), 20 µl sample loop, and a PDA detector (SPD-M20A). The HPLC column used was a Phenomenex Reversed-phase C18 (250 × 4.6 mm, 5 µm, ODS). Data acquisition was done with Class VP software.

Chemicals and reagents

All solvents were HPLC grade, and were supplied by Merck, India. Deionized water was used in all procedures, and deionization was carried out by means of a Milli-Q Water Purification system. All solutions were filtered through 0.45 µm membrane filter (Fisher Scientific, USA) before HPLC analysis. Standard stock solution of rutin, glycyrhizic acid and hydroxychavicol, purchased from Sigma Aldrich, and were prepared at concentration of 1.0 mg/ml in ethanol.

HPLC analysis of A. indica

The mobile phase was acetonitrile:0.5% formic acid in water (30:70), the flow rate was 1 ml/min, detection wavelength was 340 nm. Amount of sample injected was 20 µl. The concentration of *A. indica leaves* extract and rutin standard were and 0.1 mg/ml, respectively (Figure 1a and b) (Indian Pharmacopoeia, 2010).

HPLC analysis of G. glabra

The mobile phase was glacial acetic acid:acetonitrile:water (1:35:32), the flow rate was 1.5 ml/min, detection wavelength was 254 nm. The amount of sample injected was 20 µl. The concentration of *G. glabra* root extract and glycyrrhizic acid standard were 10 and 0.1 mg/ml, respectively (Figure 2a and b) (Indian Pharmacopoeia, 2010).

HPLC analysis of P. betle

The mobile phase was acetonitrile (Solvent B):0.1% orthophosphoric acid in water (Solvent A). The Analysis was performed on gradient elution mode (Table 1). The changes of mobile phase content are shown in Table 1. The flow rate was 1.5 ml/min, detection wavelength was 200 nm. The amount of sample injected was 20 µl. The concentration of *P. betle* leaves extract and

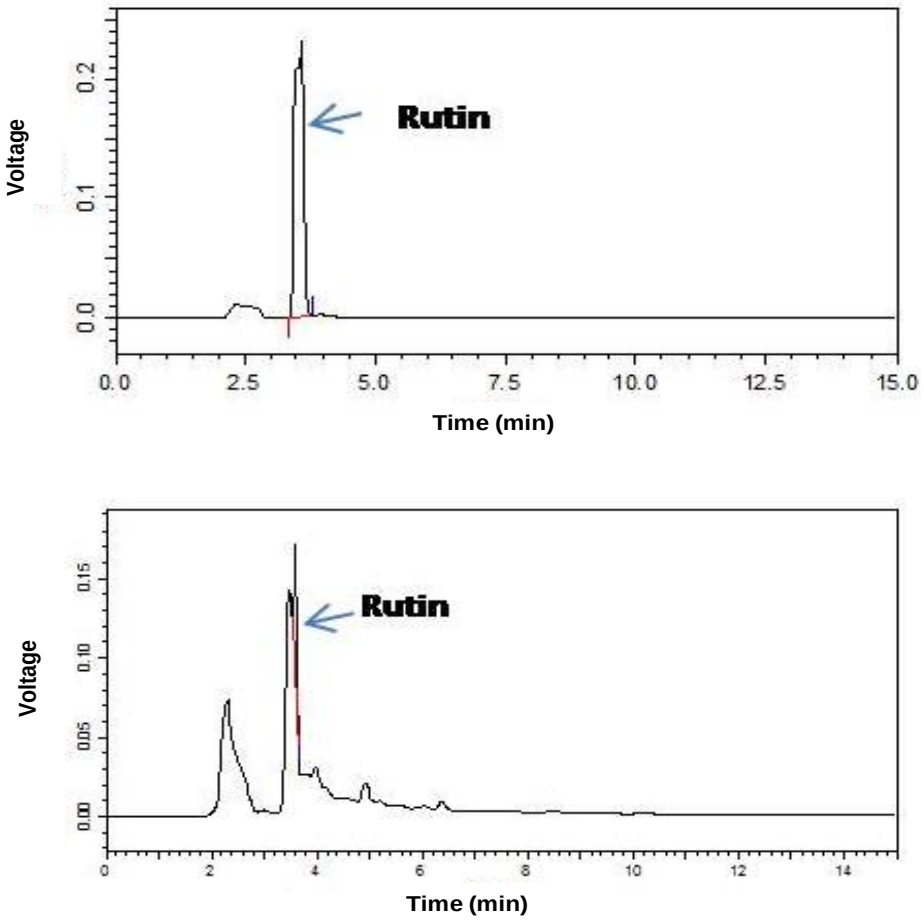

Figure 1. Chromatogram of (a) rutin and (b) *Azadirachta indica* leaf extract.

Table 1. Gradiant elution mode for hydroxychavicol in *Piper betle*.

Time	Solvent A (%)	Solvent B (%)
0	35	65
1	45	55
10	45	55
15	35	65

methoxychavicol standard were 10 and 0.1 mg/ml, respectively (Figure 3a and b) (Pin et al., 2006).

Extracts added to cosmetic skin care formulation

All the components of the formulation were denominated according to the International Nomenclature of Cosmetic Ingredients (INCI) (Table 2).

Formulation development

In this study, oil in water (O/W) emulsion based cream (semisolid formulation) was prepared. The emulsifying wax and stearic acid and other oil soluble components (Cocoa butter, cetyl alcohol, palmitic acid, lanolin, isopropyl myristate, CCTG, olive oil) were dissolved in the oil phase (part A) and heated to 75°C. The preservatives (2-phenoxyethanol, sodium benzoate) and other water soluble components (glycerin, allantoin, hyaluronic acid, herbs alcoholic extracts) were dissolved in the aqueous phase (part B) and heated to 75°C. \

After heating, the aqueous phase was added in portions to the oil phase with continuous stirring until cooling of emulsifier took place (Gidwani et al., 2010). The formula for the cream is as shown in Table 2.

By varying the ingredients and their amounts taken, different samples formed were checked to confirm that pH, colour, odour and product texture were within the specification necessary for skin care creams. The compositions of different sample made are shown in Tables 2.

Efficacy test

Efficacy analysis is an essential step to verify the claim produced by finished products. In this study, efficacy of herbal skin care antimicrobial formulation cream has been determined by antimicrobial test.

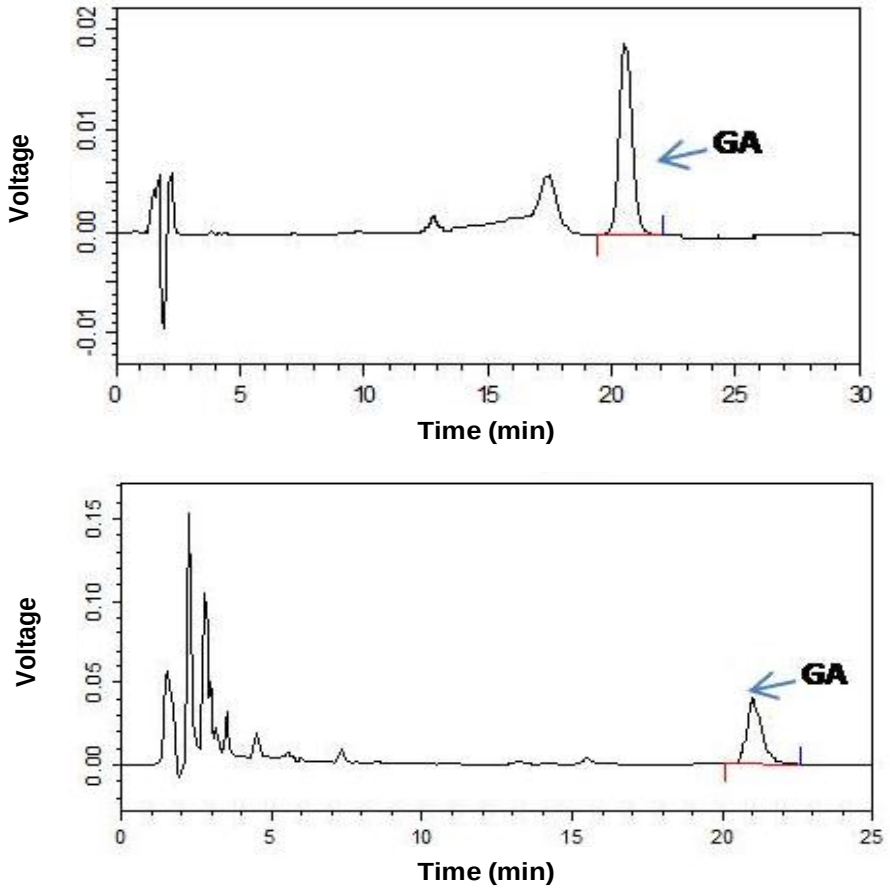

Figure 2. Chromatogram of (a) Glycyrrhizic acid and (b) *Glycyrrhiza gabra* root extract.

Table 2. Composition of the formulations (A to D) along with control (base) (without herb) under study.

Component (INCI)	Percentage of components in each formulation				
	Control	A	B	C	D
Distilled water	81.7	81	80.6	80.2	80.4
Olive oil	3.0	3.0	3.0	3.0	3.0
Vegetable glycerin	4.5	4.5	4.5	4.5	4.5
Emulsifying wax	2.5	2.5	2.5	2.5	2.5
Cocoa butter	0.5	0.5	0.5	0.5	0.5
Stearic acid	2.5	2.5	2.5	2.5	2.5
Palmitic acid	1.0	1.0	1.0	1.0	1.0
Cetyl alcohol	1.0	1.0	1.0	1.0	1.0
Lanolin	0.5	0.5	0.5	0.5	0.5
Glycyrrhiza glabra root extract	-	0.1	0.5	0.5	0.1
Piper betle leaves extract	-	0.5	0.1	0.5	0.1
Azadirachta indica leaves extract	-	0.1	0.5	0.5	0.1
Isopropyl myristate	0.5	0.5	0.5	0.5	0.5
Capric caprylic tri glycerides	0.5	0.5	0.5	0.5	0.5
Hyaluronic acid	0.5	0.5	0.5	0.5	0.5
Allantoin	0.5	0.5	0.5	0.5	0.5
2-phenoxyethanol	0.5	0.5	0.5	0.5	0.5
Sodium benzoate	0.3	0.3	0.3	0.3	0.3

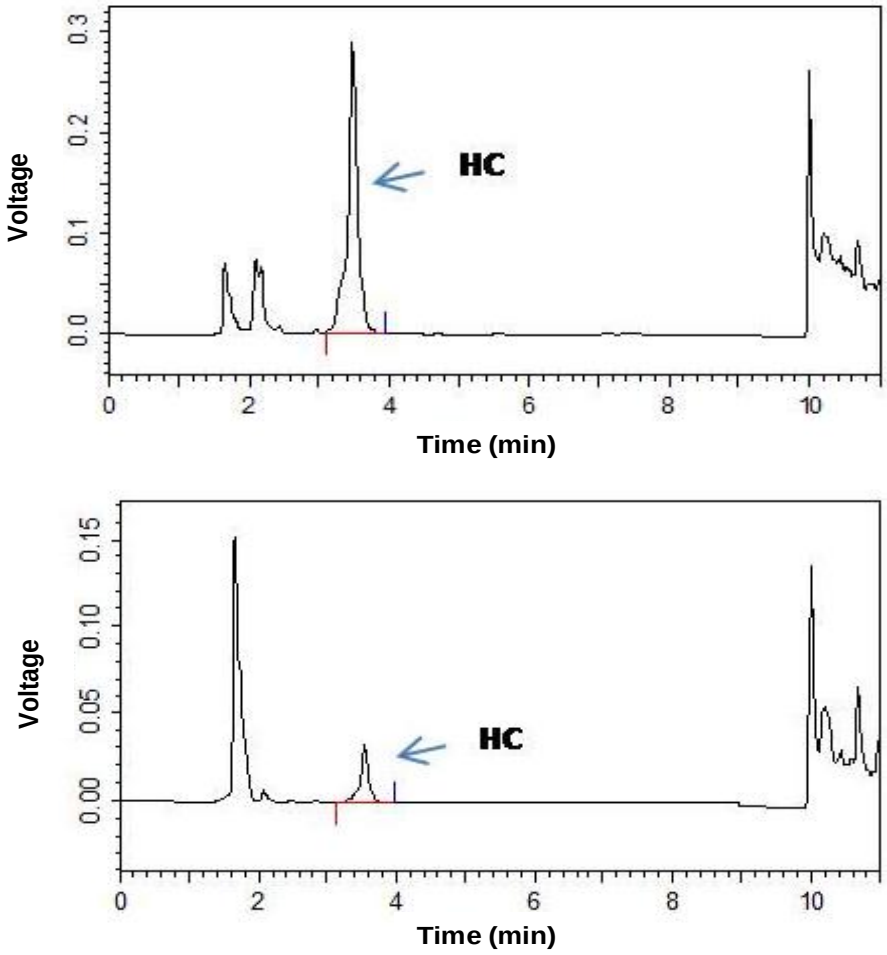

Figure 3. Chromatogram of (a) hydroxychavicol and (b) *Piper betel* leaf extract.

Well diffusion method

The antimicrobial activity of the extracts in formulated cream was screened by well diffusion method (Reller et al., 2009) in petri plates containing nutrient agar for bacteria and potato dextrose agar medium for fungi (20 ml media/plate).

Antimicrobial action of herbal formulations

The test strains [*Staphylococcus aureus* (MTCC 9122), *E. coli (MTCC 1698), B. subtilis* (MTCC 7424), *A. niger* (MTCC 8652) and *P. chrysogenum* (MTCC 6477)] were collected from microbial type culture cultivation (MTCC), IMTECH, Chandigarh, India. The plates were inoculated with test cultures and were incubated at 37°C for 24 h for bacteria and at 28°C for 24 h for fungal strains. The next day, the wells (6 mm diameter) were made with help of 6 mm diameter cork borer and the wells were loaded with herbal formulations A, B, C and D along with control. Streptomycin and tetracycline were used as positive control for bacteria and fuconazole for fungi. After 24 h of incubation, the test determines the efficacy of the product in terms of zone of inhibition of the organism. The entire test was performed in triplicate. The higher the zone of inhibition, the more effective is the test product (Joshi, 2008).

Evaluation of creams (Stability study)

Stability tests were performed at 8±0.1 and 40±0.1°C (in incubator) with 75% relative humidity (RH) up to one month (Akhtar, 2011). The formulations (A to D with control) were then evaluated for the following physic-chemical parameters at 7, 15 and 30 days time interval:

Physical analysis

The cream samples were judged for their state-semisolid, colour-off-white, odour-characteristic and appearance-homogenous.

Evaluation of the pH of the samples

The pH meter was calibrated using standard buffer solution with a pH of 7 and 10. About 0.5 g of the cream was weighed and dissolved in 50 ml of distilled water and its pH was measured (Indian Pharmacopoeia, 1996).

Viscosity

Viscosity of cream was determined by Brookefield viscometer. The viscosity measurements were done using Brookefield DV-II +

viscometer using LV-4 spindle. The developed formulation was poured into the adaptor of the viscometer at 20 rpm (Draize et al., 1944).

Centrifugation test

Centrifugation tests were performed for formulations immediately after preparation. The centrifugal tests were repeated for emulsions after 24 h, 7 days, 14 days, and 30 days of preparation. The centrifugal tests were performed at 25°C and at 5000 rpm for 10 min by placing the 5 g sample in stopper centrifugal tubes (Akhtar, 2011).

Chemical tests

Acid value

Take 0.5 g of sample dissolve it in 10 times of absolute alcohol. Heat the mixed sample for 5 min on hot plate, to these 2 to 3 drops of phenolphthalein indicator added and titrated with 0.1 N KOH until faintly pink colour appears (IFRA Analytical Method, 2011).

Acid Value= 56.1 × Titre value × N of KOH/Weight of sample

Peroxide value

Weighed 5 g of sample in 250 ml flask, and added 30 ml of acetic acid and chloroform solution and swirl it to dissolve. 0.5 ml of KI solution was added with continuous shaking and 30 ml of water ID also added. Then titrate it with 0.1 M sodium thiosulfate solution with vigorous shaking until yellow is almost gone. Add 0.5 ml of 1% starch solution and continue titration with vigorous shaking to release all I_2 from chloroform layer, until blue colour disappears (Aswal et al., 2012).

Peroxide value = S × M × 1000/g sample

where S = ml of sodium thiosulfate and M = molarity of sodium thiosulfate solution.

Total fatty matter determination

Take 2 g of sample and add 20 to 25 ml of 1:1 diluted HCL, heat the content on water bath till it become clear. Draw the sample in 250 ml separating funnel and allow it to cool at room temperature. Now add 50 ml petroleum ether in the funnel, then shake the funnel and leave it for separation. Separate the organic phase and mix it twice with ether and then wash them with water. Filter the extract and add sodium sulfate in it. Filter it and dry the extract and find the content (Indian Standard, 1978).

Total fatty matter (%) by mass = $100 \times M_1/M_2$; M_1 = mass of residue; M_2 = mass of sample in gram.

Product evaluation on skin (Patch test)

Ten volunteers were selected whose ages were in between 20 and 35 years. Prior to when study consent form was filled by the volunteers. Volunteers having serious skin diseases, asthma were excluded from the study. Patch test was performed on the forearms of each volunteer to determine any possible reactions to the formulations. The formulations A to D along with base were applied on the forearms of the volunteers separately. Adhesive tape was used to fix them in place and the test sites were marked. The patches were left in place for 48 h, during which time it is important not to wash the area. After 48 h, the patches were removed and reading is taken one hour later. Examine skin for any redness, itching, or blemishes. These visible signs plus any itchy or irritable sensations indicate that there is something wrong to the product. If the skin is clear and comfortable, the product is safe to use.

Efficacy perception: Subjective analysis

To assess the effectiveness of all the formulated creams, that is, base (control) and herbal formulated creams (A, B, C, D) tested in this study, the volunteers were asked to answer a questionnaire consisting of seven parameters after application of the cream on skin. (1) Ease of application; (2) Spread ability; (3) Sense just after application; (4) Sense on long term; (5) Irritation; (6) Shine on skin; (7) Sense on softness. These are evaluated on the basis of values from 0, 1, 2, 3 and 4 indicating very bad, no effect, average and very good, respectively.

Statistical analysis

The measured values obtained for different parameters were analyzed using SPSS 20 software and results were further tested by paired sample t test.

RESULTS

HPLC analysis

HPLC analysis of plant extracts clearly indicates the presence of active constituents in the plant extracts. The percentage of active constituents for each plant is as shown in Table 3 and Figures 1a, 1b, 2a, 2b, 3a and 3b.

Antimicrobial activity

The antimicrobial activity was determined by measuring the diameter of zone of inhibition recorded. The results obtained in the evaluation of the antibacterial and antifungal activity of the different ethanolic extracts of the selected plant against selected pathogens are shown in Table 4 and Figures 4a, 4b, 5a, 5b, 6a, 6b, 7a, 7b, 8a and 8b. Formulations A, C and D showed better zone of inhibition in comparison to base. However, formulation C has maximum activity against selected strains due to high amount of herbal extracts in comparison to others, but it was not stable. Thus formulations A and D were selected for their better results as compared to other formulations. The results were statistically significant ($p < 0.05$).

Evaluation of creams

The following shows the results of different parameters for the evaluation of creams.

Stability of emulsions

Stability of base and formulations A to D kept at different

Table 3. HPLC analysis of plant extracts.

S/N	Crude extracts	Compound	Retention time	Content (%)
1	*Azadirachta indica*	Rutin	3.58	0.98
2	*Glycyrrhiza glabra*	Glycyrrhizic acid	20.90	2.31
3	*Piper betle*	Methoxychavicol	3.50	1.20

Table 4. Antimicrobial sensitivity result of the formulations A, B, C, D and control.

S/N	Test organism	Zone of inhibition (mm) at 20 mg cream				
		A**	B*	C*	D*	Control
1	*S. aureus*	10±0.48	8±0.95	14±0.73	12±0.92	10±0.19
2	*E. coli*	11±0.56	9±1.24	15±0.44	13±0.59	9±10.41
3	*B. subtilis*	9±0.43	8±0.46	12±0.36	10±0.62	11±0.58
4	*A. niger*	9 ±0.25	7±0.21	16±0.31	15±1.84	7±0.74
5	*Penicillium chrysogenum*	8±0.65	6±0.34	10±0.38	10±1.26	8±1.34

Figures are mean ± SD; Not significant**, Significant* ($p<0.05$). Confidence interval level at 95%.

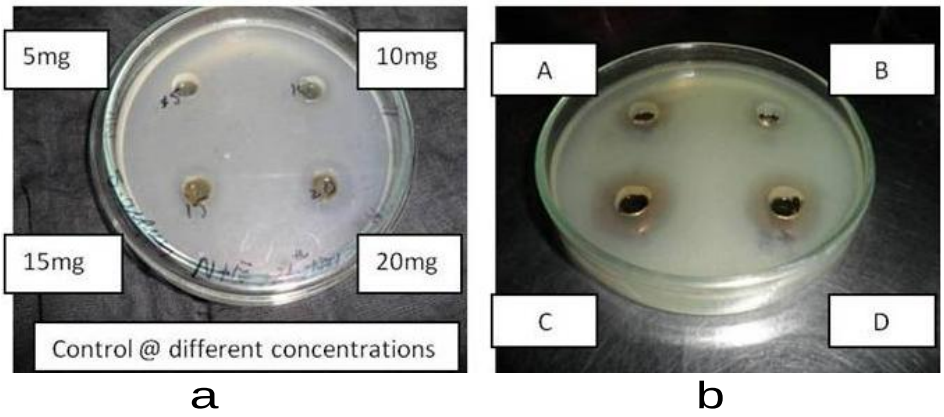

Figure 4. (A) Zone of inhibition of control; (b) Zone of inhibition of herbal formulations A, B, C, and D against *S. aureus*.

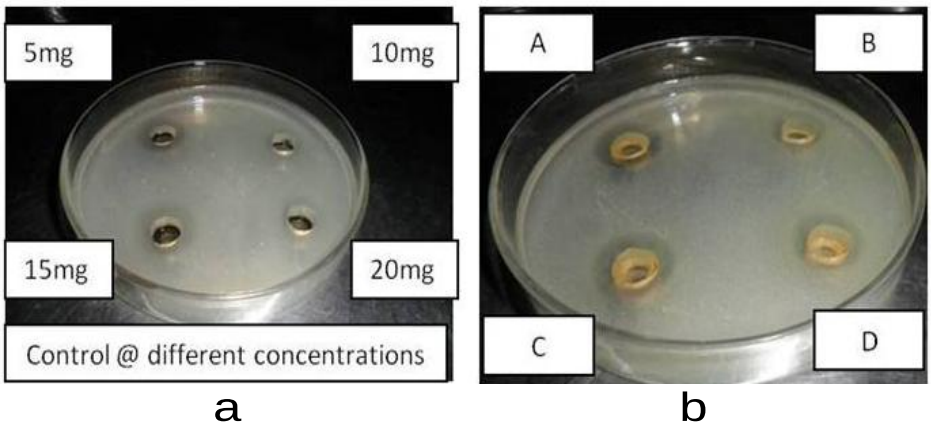

Figure 5. (a) Zone of inhibition of control; (b) Zone of inhibition of herbal formulations A, B, C, and D against *B. subtilis*.

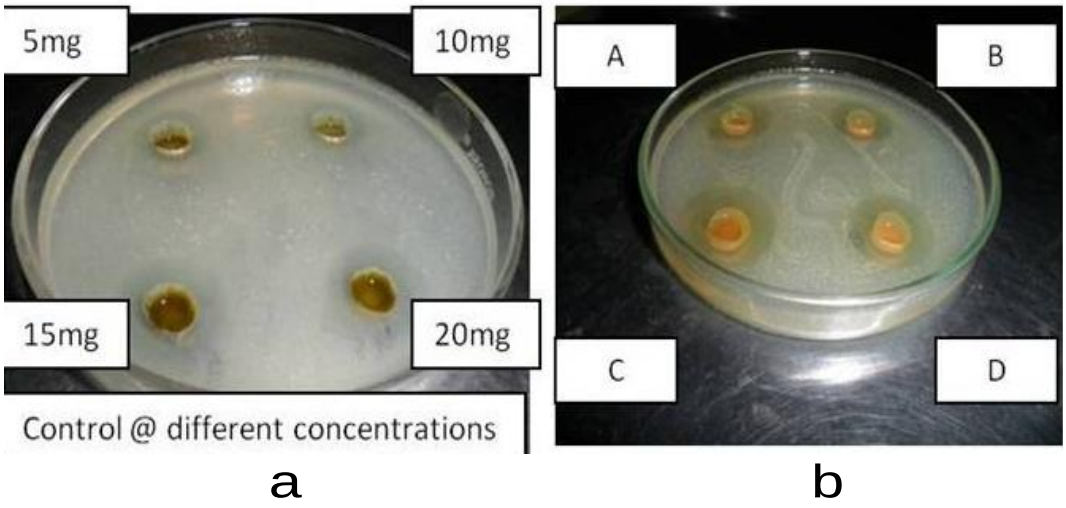

Figure 6. (a) Zone of inhibition of control; (b) Zone of inhibition of herbal formulations A, B, C, and D against *E. coli*.

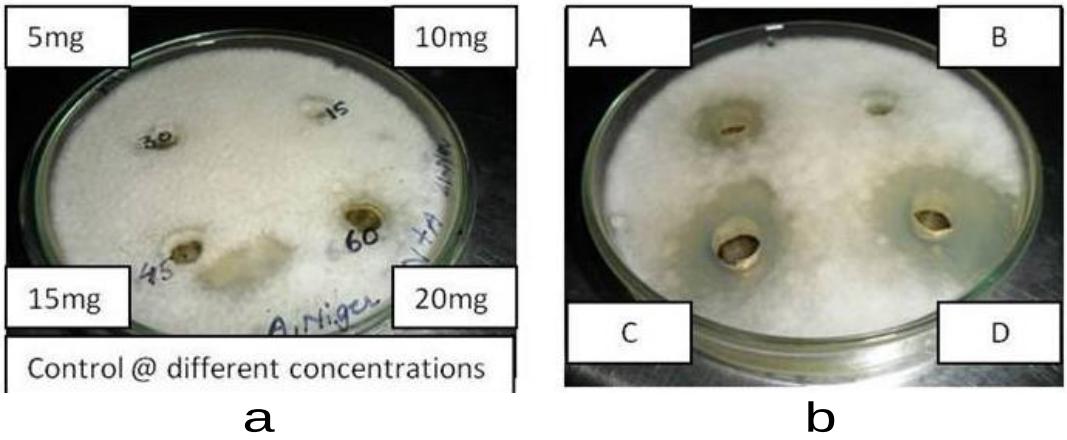

Figure 7. (a) Zone of inhibition of control; (b) Zone of inhibition of herbal formulations A, B, C, and D against *Aspergillus niger*.

storage conditions were studied and physical characteristics like color, appearance and odor were studied for 30 days (Table 5).

Physical analysis

The freshly prepared base was white and the formulations were off white to yellow in color (Table 5). Regarding the base and the formulation A and D, there was no change in color, odor and appearance up to the observation period of 30 days. This showed that emulsions A and D were stable at different storage conditions, that is, 8 and 40°C. On the other hand, in formulations C and B, there was change in odor of bad smell, dark yellow color and liquid appearance at 40°C during one month of study (Table 5).

pH of formulations

pH of cream was found to be in the range of 6 to 8, kept at different storage conditions for 30 days. pH of the formulations and base kept at 8°C for one month did not show large change and data were significant over control (base) during one month ($p < 0.05$). Interestingly at 40°C, formulation C was exhibiting elevated change in pH (7.85), while the others remained slightly stable during one month study. Data of formulations A and B at 40°C were found to be non significant and for formulations C and D data were significant over control ($p < 0.05$) (Table 6).

Viscosity test

Viscosity of the formulations, kept at storage conditions

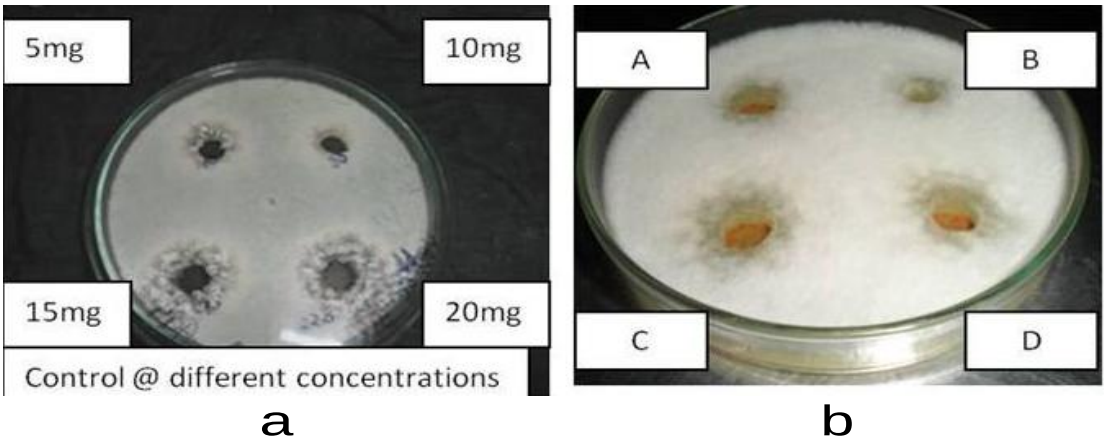

Figure 8. (a) Zone of inhibition of control; (b) Zone of inhibition of herbal formulations A, B, C, and D against *Penicillium chrysogenum*.

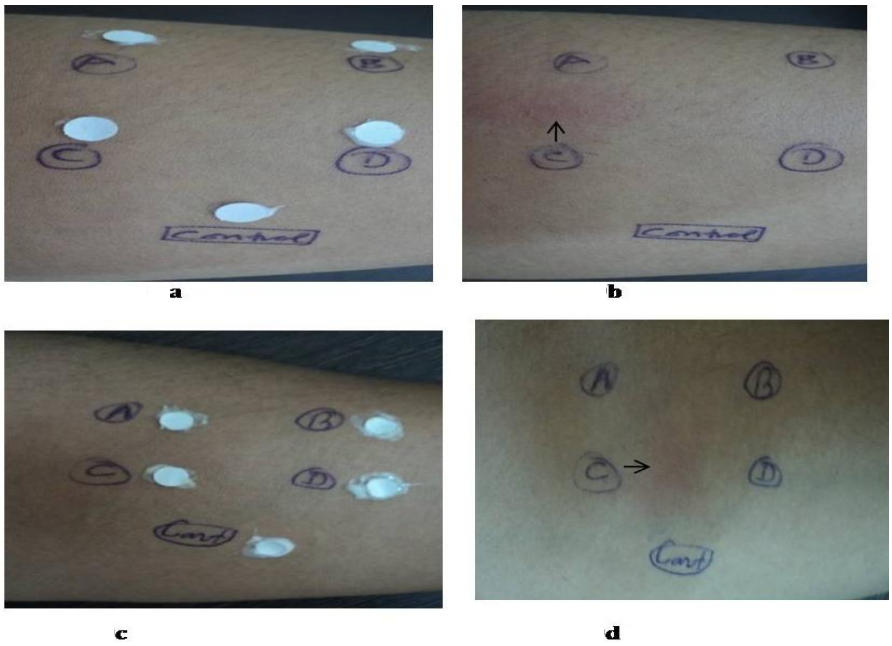

Figure 9. (a and c) Formulation and control (base) application; (b and d) Effect of all formulation and control after 48 h of application on forearms of volunteers. The arrow indicate red patches on forearms after application of formulation C.

for 30 days was found to be within the range. The data of viscosity in formulations A, B and C at 8°C and formulations A and B at 40°C were significant, while in formulation D at 8°C and formulations C and D at 40°C, the data were non significant over base during one month study (Table 6).

Centrifugation

Centrifugation test for base and formulation kept at different storage conditions were performed for 30 days. No phase separation after centrifugation was found in formulations A, D and base at 8 and 40°C during one month, while formulations B and C showed separation at 40°C at the 30th day of study (Table 5).

Acid value, peroxide value and total fatty matter determination

Acid value, peroxide value and total fatty matter for base and formulations kept at different storage conditions were observed for 30 days and values in base, formulations A, D and B were found within the range (Table 6). The acid value and peroxide value in formulation C was high (4.88

Table 5. Physical study of all formulations during one month.

Duration	7 days		15 days		30 days	
	Storage condition					
Parameter	**8°C**	**40°C**	**8°C**	**40°C**	**8°C**	**40°C**
Appearance						
A	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
B	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Slightly liquid
C	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Liquid
D	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
Control (base)	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
Colour						
A	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow
B	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow	yellow
C	Yellow	yellow	Yellow	dark yellow	dark yellow	Dark yellow
D	Off white	offwhite	Off white	Light yellow	Off white	Light yellow
Control (base)	White	White	White	White	White	White
Odour						
A	Characteristics	Characteristics	Characteristics	Characteristics	Characteristics	Characteristics
B	Characteristics	Characteristics	Characteristics	Characteristics	Characteristics	Bad smell
C	Characteristics	Characteristics	Characteristics	Characteristics	Bad smell	Bad smell
D	Characteristics	Characteristics	Characteristics	Characteristics	Characteristics	Characteristics
Control (base)	Odourless	Odourless	Odourless	Odourless	Odourless	Odourless
Centrifugation test						
A	NSL	NSL	NSL	NSL	NSL	NSL
B	NSL	NSL	NSL	NSL	NSL	SL
C	NSL	NSL	NSL	NSL	NSL	SL
D	NSL	NSL	NSL	NSL	NSL	NSL
Control (base)	NSL	NSL	NSL	NSL	NSL	NSL

NSL: No separation of layer; SL: Separation of layer.

and 2.93, respectively) in comparison to other formulations and base. Data of acid values of the formulations and base was found to be significant ($p < 0.05$) during one month of stability study. Peroxide value data in formulations A and B at 8 and 40°C as well as in formulation D at 40°C were found to be significant ($p < 0.05$). Total fatty matter data were found to be non significant in all except formulation B at 8 and 40°C and formulation D at

Table 6. Chemical study of all formulations during one month.

Duration	7 days		15 days		30 days	
Parameter	Storage condition					
	8°C	40°C	8°C	40°C	8°C	40°C
pH						
A*	6.86	6.96	6.90	6.93	6.93	6.95
B*	6.92	6.95	6.98	6.97	7.01	7.08
C* ł	7.20	7.26	7.45	7.40	7.72	7.85
D* ł	6.93	6.96	6.90	6.98	6.96	6.98
Control (base)	6.76	6.87	6.80	6.89	6.85	6.95
Viscosity at 20 rpm						
A* ł	629	621	618	610	613	605
B* ł	592	585	590	581	578	569
C*	634	622	621	615	609	602
D**	658	625	6.90	619	649	615
Control (base)	682	628	655	616	642	610
Acid value						
A*ł	4.70	4.73	4.75	4.80	4.77	4.81
B*ł	4.31	4.34	4.35	4.40	4.38	4.38
C*ł	4.54	4.50	4.70	4.75	4.86	4.88
D*ł	4.35	4.32	4.36	4.40	4.43	4.47
Control (base)	4.13	4.15	4.20	4.35	4.21	4.24
Peroxide value						
A*ł	1.63	1.66	1.65	1.67	1.66	168
B*ł	1.75	1.78	1.77	1.79	1.77	1.80
C**	1.84	1.86	2.01	2.51	2.87	2.93
D ł	1.65	1.67	1.67	1.68	1.68	1.70
Control (base)	1.64	1.69	1.67	1.70	1.69	1.74
Total fatty matter						
A**	14.5	14.3	14.3	14.1	14.1	14.0
B*ł	14.6	14.6	14.4	14.2	14.2	14.1
C**	14.5	14.2	14.3	14.1	14.1	14.0
D ł	14.5	14.7	14.5	14.7	14.7	14.5
Control (base)	14.5	14.4	14.3	14.1	14.2	14.0

*Significant at 8°C; ł Significant at 40°C ; **Not significant.

40°C (Table 6).

Patch test evaluation of volunteers

Before the application of base and formulations to human volunteer, patch test were performed to examine redness, itching, or blemishes on skin. The values obtained are shown in Table 7. Patch test was performed on forearms of volunteers for 48 h for both base and formulations, to check the safety of the formulation and base on human skin. The data showed that the parameters of ease of application, spreadibility, sense just after application and on long term, irritation as well as sense on softness on application of formulations D, A and B over forearms of volunteers, was quite good in comparison to base; while formulation C showed poor impact regarding all the parameters. With paired sample t test, it was evident that the effects of formulations and base were highly significant ($p < 0.001$) regarding all parameters of patch test. Volunteers reported there was irritation, redness in formulation C on application, while formulation D was very good in all the parameters (Table 7). Results of the patch test are shown in Figure 9.

Table 7. Patch test evaluation by volunteers.

Parameter	Volunteer	1	2	3	4	5	6	7	8	9	10	Average
Ease of application	Ct.	2	2	3	2	2	3	1	1	2	2	2
	A**	3	3	4	3	4	3	3	4	3	2	3.2
	B**	2	2	2	1	1	2	2	3	2	3	2
	C**	2	2	1	1	0	1	0	0	1	2	1
	D**	3	3	3	3	4	4	4	4	3	2	3.3
Spread ability	Ct.	3	2	2	1	2	2	3	2	2	3	2.2
	A**	3	2	3	4	3	2	2	2	2	1	2.4
	B**	2	2	2	1	1	2	1	3	3	2	1.9
	C**	2	2	1	1	0	0	1	1	2	2	1.2
	D**	3	3	4	4	3	2	2	3	3	2	2.9
Sense just after application	Ct.	3	2	2	2	2	2	2	3	3	2	2.3
	A**	3	3	2	3	3	3	2	2	3	3	2.7
	B**	2	2	1	2	2	1	2	2	3	2	1.9
	C**	2	1	1	1	0	2	2	0	2	0	1.1
	D**	4	4	3	3	4	3	3	4	4	4	3.6
Sense on Long term	Ct.	2	2	1	3	2	2	3	2	2	3	2.2
	A**	3	2	3	2	3	2	2	2	3	3	2.5
	B**	2	2	1	2	1	1	2	2	1	1	1.5
	C**	1	1	2	0	2	0	1	2	1	1	1.1
	D**	4	4	3	4	4	3	3	3	3	4	3.5
Irritation	Ct.	2	2	2	3	3	2	2	3	2	2	2.3
	A**	2	3	3	2	2	2	3	3	2	2	2.4
	B**	2	2	1	2	2	1	2	2	1	2	1.7
	C**	0	0	0	1	1	1	0	0	1	1	0.5
	D**	3	3	4	4	4	3	3	4	4	4	3.6
Sense on softness	Ct.	3	3	2	2	3	2	3	4	4	3	2.9
	A**	2	2	3	2	3	3	2	3	2	3	2.5
	B**	2	2	1	2	1	2	1	2	2	2	1.7
	C**	2	1	1	1	2	2	1	1	1	1	1.3
	D**	3	4	4	4	3	4	3	4	3	3	3.5

Highly significant data** ($p<0.001$).

This study clearly indicated that formulations which have plant extracts were more potent than the base. The possible explanation for this is the presence of active constituents of plants which are antimicrobial in action. However, access use of plant extracts in skin care cosmetic formulation can cause irritation or side effects. Out of the four formulations C has the highest antimicrobial activity but it cannot be used as a skin cream, because it is non stable and cause irritation and redness during patch test. Out of all the four formulations, formulation D was rated as best because the antimicrobial activity of this formulation was good and it passed the stability parameters and patch test.

DISCUSSION

Plants are important sources of potentially useful constituents for the development of new therapeutic agents, because most of them are safe with little side effects. A phytochemical analysis revealed that the active principle responsible for the antibacterial activity was plant's main active constituents. In the present scenario, creams have been used as vehicle for drug delivery to the body. Plants with specific medicinal properties can be used in this formulation as active ingredients in order to provide additional value (Akhtar et al., 2012).

S. aureus (Martin, 2008), *E. coli, B. subtilis* (Chaudhary

et al., 2012), *A. niger* (Ulku et al., 2013) and *Penicillium chrysogenum* (López-Martínez et al., 1999) are the pathogens that can cause skin infections. Development of microbial resistance to antibacterial is a global concern. The antimicrobial properties of *A. indica* (Nayak et al., 2011; Priscila et al., 2009), *P. betle* (Amonkar et al., 1991; Ali et al., 2010; Sharma et al., 2011; Jangala et al., 2011) and *G. glabra* (Shirazi et al., 2007; Tharkar et al., 2010; Marjan et al., 2008) plants have been previously investigated on a plant pathogens and some human pathogens. The antibacterial activity was enhanced with increase of the plant extract concentrations.

It has been previously reported that formulation of *Zataria multiflora* extract as topical cream may lead to enhancement of stability and acceptability of the active ingredient, while the antifungal activity remains considerable (Aghel et al., 2009). In another report, methanolic extract of *Eucalyptus camadulensis* has been formulated as an anti dermatophytic cream preparation (Moghimipour et al., 2009).

Every part of *A. indica* tree is used for medicinal and cosmetic purpose. It has been indicated in boils, catarrhal infections, eczema and many other skin related disorders. Cosmetically, the chemical constituents of Neem are considered to be antiseptic and natural preservatives. Rutin present in *A. indica* leaves has been reported to have antimicrobial activities. Leaf extracts of *A. indica* exhibited significant antimicrobial activity against both Gram positive and Gram negative bacteria, including *Mycobacterium tuberculosis, Streptococcus pyogenes, Vibrio cholerae, Klebsilla pneumonia, B. subtilis* (Nayak et al., 2011). *A. indica* leaves are efficient against pathogenic fungi, such as *Trichophyton, Epidermophyton, Microsporum, Trichosporon* and *Geotricum*. The activity in inhibiting the protease of *Trichophyton,* the production of aflatoxin of *Aspergillus parasiticus*, antifeedant activity and the antifungal activity against *P. expansum* have been confirmed (Priscila et al., 2009).

The major component present in *G. glabra* (Licorice) root is glycyrrhizic acid (GA). GA is the main compound present in licorice roots, found to be effective against *Helicobacter pylorei, Mycobacterial* and *Legionella* species, *S. aureus*, *Salmonella typhi, Salmonella paratyphi* B, *E. coli*. Licoricidin, a flavonoid present in licorice roots is found to be effective against *B. subtilis* and *S. aureus*. There have been some reports which show that licorice has potent antimicrobial activity against carcinogenic bacterium *Streptococcus mutans* (Shirazi et al., 2007). Some reports show that *G. glabra* is effective against *Candida albicans* and *A. niger* (Sharma et al., 2011). Glabrene present in licorice is unique compound possessing not only antimelanin production activity, but also anti-inflammatory activity. Glabrene specifically inhibits the T1 and T3 tyrosine isoenzyme activity and therefore isoliquritigenin and glabrrene serve as skin lightening agents (Marjan et al., 2008).

The phenolic compound, hydroxychavicol, found in the aqueous extract of *Piper betle* leaf is reported to exhibit useful bioactivities- anticarcinogenic and antimutagenic (Amonkar et al., 1991). It also has a tendency to act as an antioxidant and a chemopreventive agent. There have also been reports on the antimicrobial activity on hydroxychavicol (Ulku et al., 2013). Piper has been found effective against human dermatophytic fungi: *Trichophyton rubrum, Trichophyton mentagrophytes, Trichophyton tonsurans, Microsporum gypsum, C. albicans, Aspergillus flavus, A. niger, Candida tropicalis, Candida krusei* (Sharma et al., 2011). Antibacterial activity of hydroxychavicol is found against *S. aureus, S. pyrogens, E. coli, Salmonella typhi, Shigella dysentriae* (Nayak et al., 2011). Phytochemical analysis of *Piper betle* leaves revealed the presence of antioxidants. Antioxidants have a protective effect against damage to skin from UV radiation. Furthermore, it contains amide and cennamyl derivatives as well as chavicol which are responsible for its antimicrobial activity (Amonka et al., 1991).

Quality control for efficacy and safety of herbal cosmetic products is of paramount importance. So quality control test must be carried out for herbal cosmetics. It is assumed to be safe for longer periods of time. Storage at various temperatures and patch test are well known test method which can be used to know the stability and efficiency of the cosmetic herbal formulations. The result of all the formulations near to pH 6 to 8 indicates variability among formulations and base at different storage conditions for one month. The results of viscosity gives an idea about measurement of strength and the result of spread ability denote the extent of area to which the prepared formulations readily spreads on application to skin or affected part and homogeneity confirms no lumps.

In the present work, the physico-chemical parameters applied in the testing of stability of cosmetics formulations made apparent consequences that formulations A and D are much better than the other two formulations (B and C) and base. Literature survey reveals that the herbal combination used in our formulation development was not used so far. These herbs used in various topical formulations like gels and creams either in single form or with other combination of herbs.

It was concluded that formulations A and D produced no skin irritation after performing patch test of 24 h, while formulations B, C and base showed very poor impact on volunteers. It was found from the paired t test that there was significant difference between the average points of all parameters of patch test for base and formulations. So, formulations A and D can be used safely on human skin. Based on our research, it could be concluded that the plant possesses a broad spectrum of biological activities. Also, the plant is widely used in the treatment of skin diseases. The high amount of plant extracts increased the antimicrobial activity, but was unstable when kept for longer duration. On the other hand, low

amount of plant extracts showed antimicrobial activity as well and good stability for longer duration. It is suggested to use minimum amount of herb extract in cosmetic cream formulation to lower the irritation and enhance the efficacy of the cosmetic products.

Conclusions

The main ideology behind combining the plant materials is to observe the additive effect of the active constituents from different plants in the development of skin care formulation. The combination proves to be beneficial and hence it can be used in preparation of herbal anti-acne cream formulations. The herbal anti-microbial cream formulation prepared was checked for its efficacy using well diffusion method. Hence, a new way can be found to combat antibiotic resistance of pathogenic organism and provide safe and healthy living through germ free skin, although the removal is not 100%, but a major number can be reduced. From this study, it can be concluded that the formulated herbal anti-microbial cream formulations was associated with significant reduction in microbial growth which causes acne and also was found to produce moisturizing effect with no irritation and rashes on skin.

Conflict of interest

The authors declare that they have no conflict of interest.

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Abbreviations

INCI, International Nomenclature of Cosmetic Ingredients; **RH,** relative humidity; **M,** molarity; **WHO,** World Health Organization; **HPLC,** high performance liquid chromatography.

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