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## Full Length Research Paper

## Development and evaluation of antimicrobial herbal cosmetic preparation

Sonika Pandey\*, Akanksha Seth, Rajesh Tiwari, Sunita Singh, H. M. Behl and Suman Singh

Biotech Park, Lucknow, India.

Received 3 December, 2013; Accepted 29 April, 2014

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 spreadability, 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  $40 \pm 1^\circ\text{C}$  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  $4^\circ\text{C}$ .

### HPLC analysis of extracts

#### Equipment

Analysis was carried out using a Shimadzu 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  $\mu\text{l}$  sample loop, and a PDA detector (SPD-M20A). The HPLC column used was a Phenomenex Reversed-phase C18 (250  $\times$  4.6 mm, 5  $\mu\text{m}$ , 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  $\mu\text{m}$  membrane filter (Fisher Scientific, USA) before HPLC analysis. Standard stock solution of rutin, glycyrrhizic 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  $\mu\text{l}$ . The concentration of *A. indica* leaves extract and rutin standard were 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  $\mu\text{l}$ . 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  $\mu\text{l}$ . The concentration of *P. betle* leaves extract and

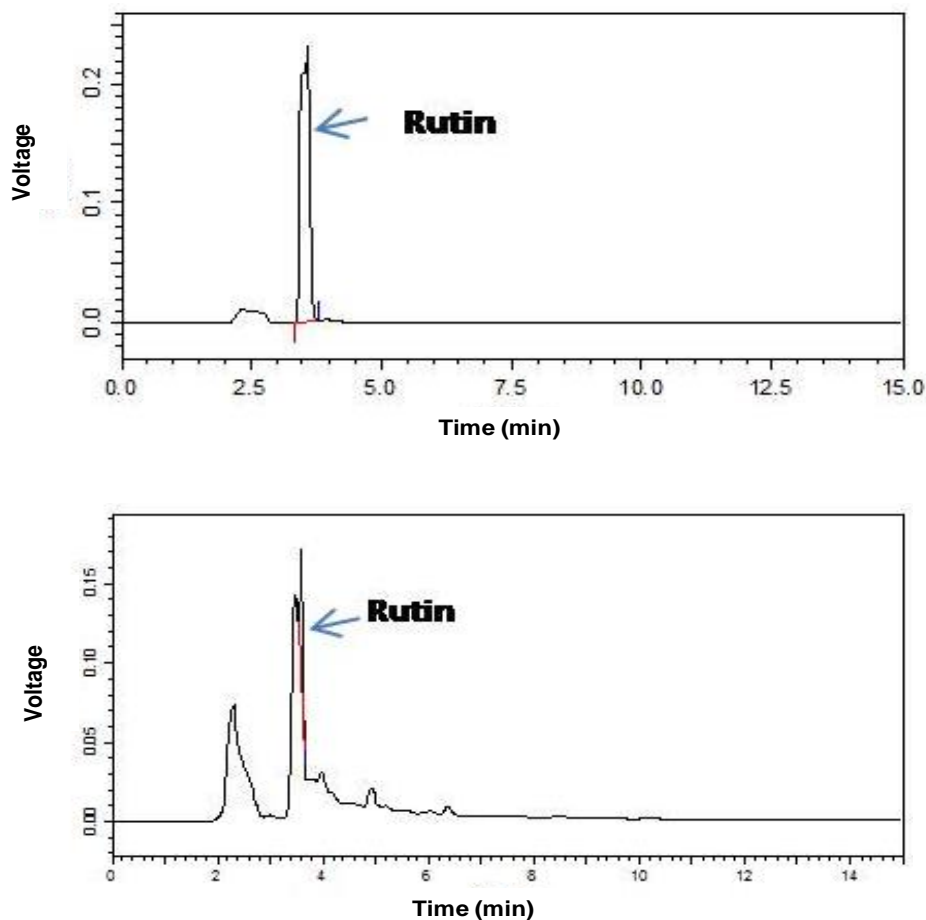


Figure 1. Chromatogram of (a) rutin and (b) *Azadirachta indica* leaf extract.

Table 1. Gradient 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.

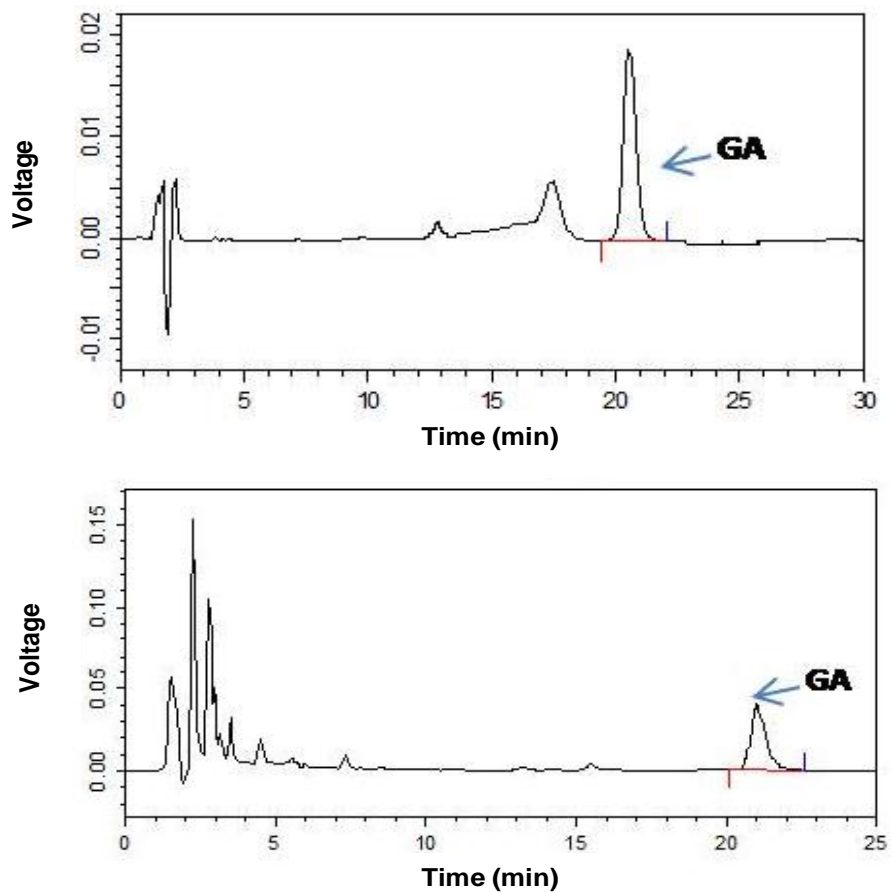
After heating, the aqueous phase was added in portions to the oil phase with continuous stirring until cooling of emulsifier took place (Gidwani et al., 2010). The formula for the cream is as shown in Table 2.

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 glabra* 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
<i>Glycyrrhiza glabra</i> root extract	-	0.1	0.5	0.5	0.1
<i>Piper betle</i> leaves extract	-	0.5	0.1	0.5	0.1
<i>Azadirachta indica</i> 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

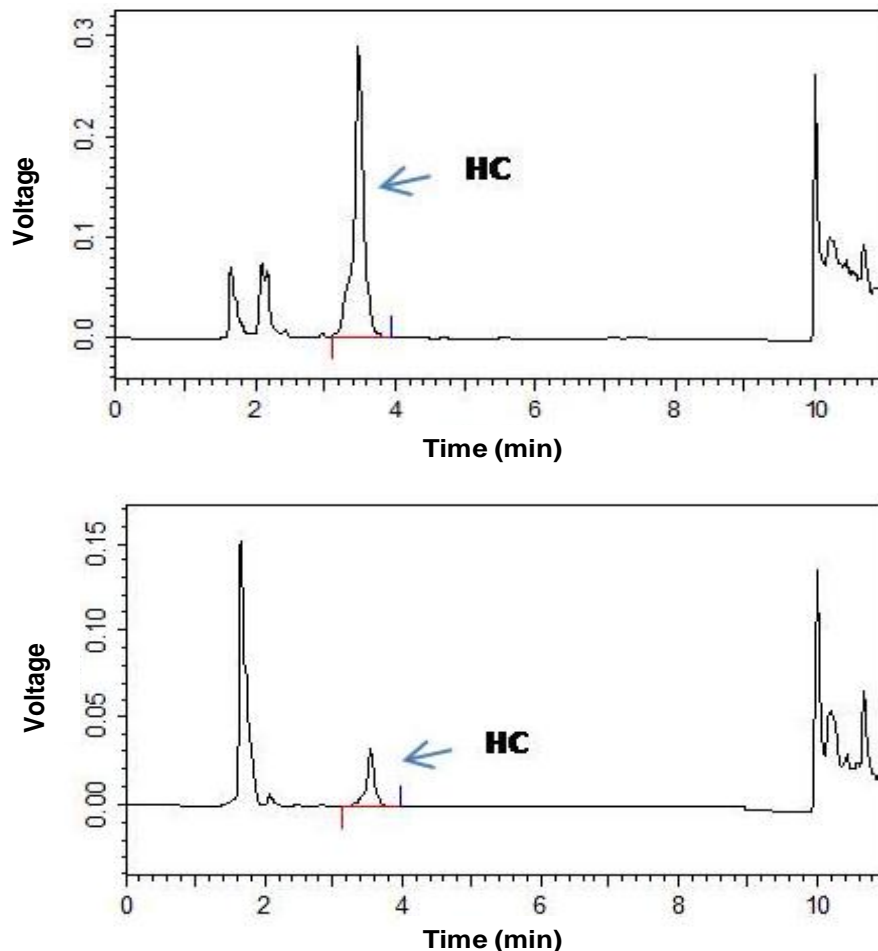


Figure 3. Chromatogram of (a) hydroxychavicol and (b) *Piper betel* leaf extract.

#### 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  $8\pm 0.1$  and  $40\pm 0.1^\circ\text{C}$  (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 Brookfield viscometer. The viscosity measurements were done using Brookfield 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 =  $56.1 \times \text{Titre value} \times \text{N of KOH/Weight of sample}$

#### **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<sub>2</sub> from chloroform layer, until blue colour disappears (Aswal et al., 2012).

Peroxide value =  $S \times M \times 1000/\text{g sample}$

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<sub>1</sub> = mass of residue; M<sub>2</sub> = 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

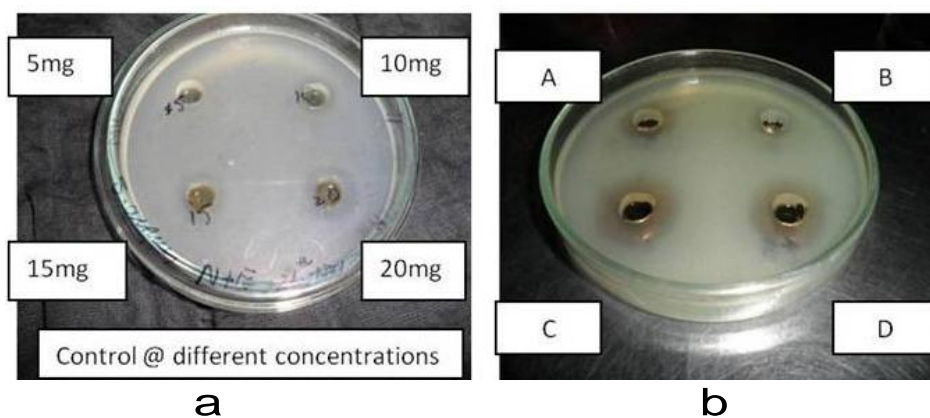
**Table 3.** HPLC analysis of plant extracts.

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

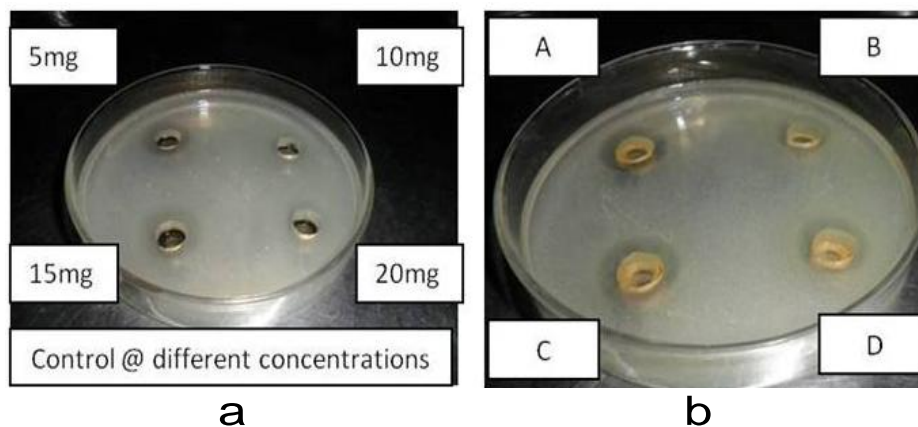
**Table 4.** Antimicrobial sensitivity result of the formulations A, B, C, D and control.

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

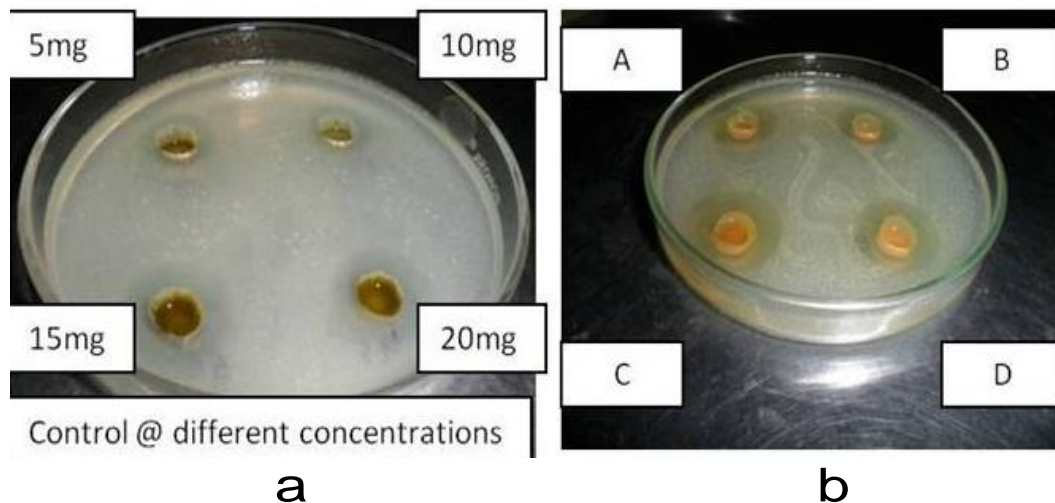
Figures are mean ± SD; Not significant\*\*, Significant\* ( $p < 0.05$ ). Confidence interval level at 95%.



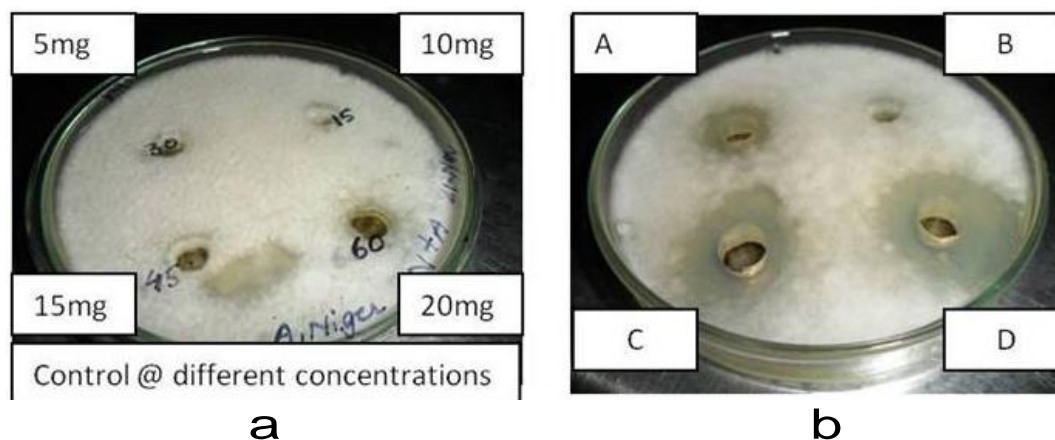
**Figure 4.** (A) Zone of inhibition of control; (b) Zone of inhibition of herbal formulations A, B, C, and D against *S. aureus*.



**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**

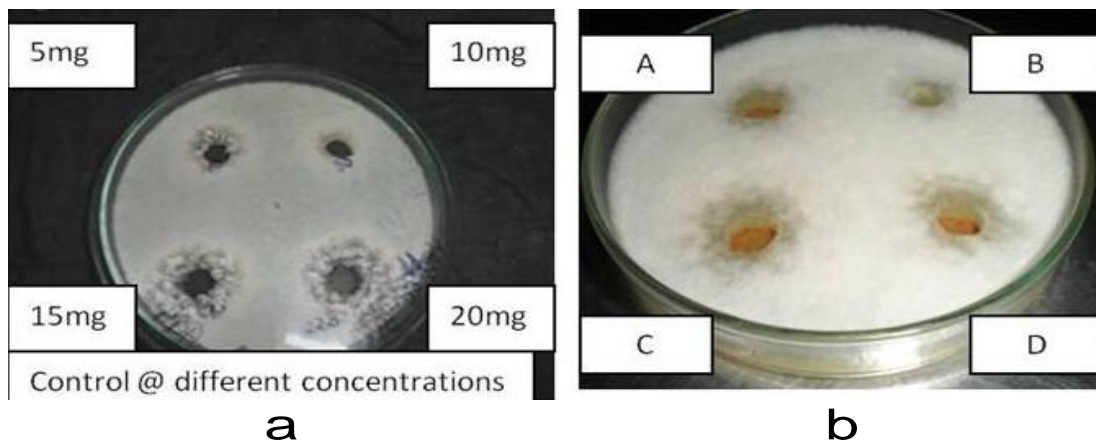
The freshly prepared base was white and the formulations were off white to yellow in color (Table 5). Regarding the base and the formulation A and D, there was no change in color, odor and appearance up to the observation period of 30 days. This showed that emulsions A and D were stable at different 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**

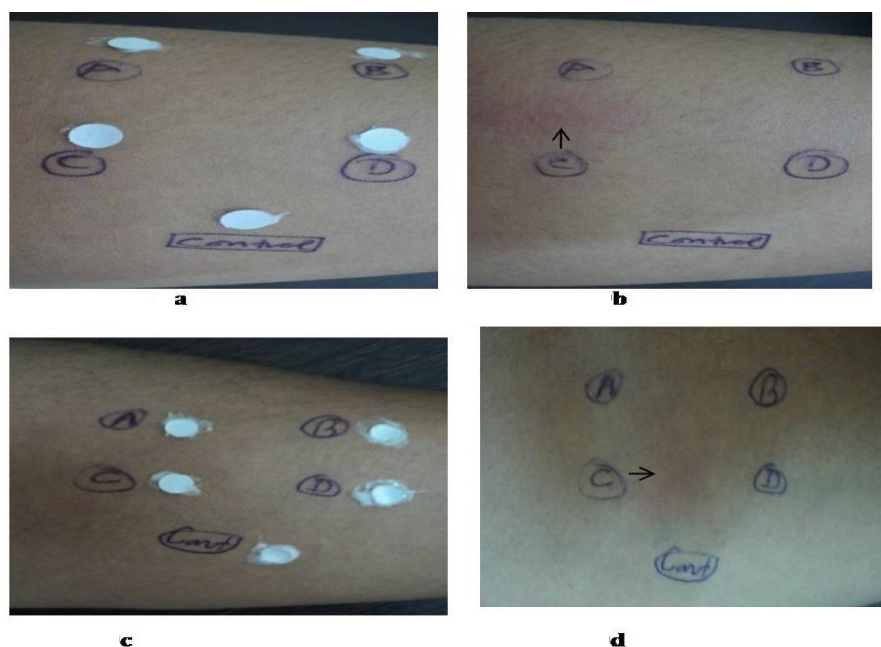
pH of cream was found to be in the range of 6 to 8, kept at different storage conditions for 30 days. pH of the formulations and base kept at 8°C for one month did not show large change and data were significant over control (base) during one month ( $p < 0.05$ ). Interestingly at 40°C, formulation C 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
<b>Appearance</b>						
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
<b>Colour</b>						
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
<b>Odour</b>						
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
<b>Centrifugation test</b>						
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	
	Storage condition					
Parameter	8°C	40°C	8°C	40°C	8°C	40°C
<b>pH</b>						
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* †	7.20	7.26	7.45	7.40	7.72	7.85
D* †	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
<b>Viscosity at 20 rpm</b>						
A* †	629	621	618	610	613	605
B* †	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
<b>Acid value</b>						
A* †	4.70	4.73	4.75	4.80	4.77	4.81
B* †	4.31	4.34	4.35	4.40	4.38	4.38
C* †	4.54	4.50	4.70	4.75	4.86	4.88
D* †	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
<b>Peroxide value</b>						
A* †	1.63	1.66	1.65	1.67	1.66	1.68
B* †	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 †	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
<b>Total fatty matter</b>						
A**	14.5	14.3	14.3	14.1	14.1	14.0
B* †	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 †	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; †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, spreadability, 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	4	3	2
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 (Moghimpour 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 pylori*, *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 isoliquiritigenin and glabrene 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 dysenteriae* (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.

## ACKNOWLEDGEMENTS

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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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## Full Length Research Paper

## Antimicrobial and enzyme inhibitory assay of constituents of *Lonicera lanceolata*

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The phytochemical study on the chloroform fraction of *Lonicera lanceolata* led to the first time isolation of four known compounds (1 to 4). The antimicrobial and enzyme inhibition assay of four compounds isolated from *L. lanceolata* are presented in this study. These compounds were screened against two human Gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*) and four Gram-negative ones (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Klebsiella Pneumoniae*) by performing Agar well diffusion method for antibacterial and antifungal activities. Minimum inhibitory concentration (MIC) was determined by Agar well dilution method. Minimum bactericidal concentration (MBC) was carried out by viable cell count method. Compound 1 showed maximum antimicrobial activities, while the other compounds also showed significant antimicrobial activities. In addition, the isolated compounds were assayed for their acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. Moreover, the IC<sub>50</sub> (50% inhibitory effect) values of compounds 1 and 2 against AChE were determined to be 1.99 and 1.75 μM, while the values obtained against BChE were 3.65 and 4.90 μM, respectively.

**Key words:** *Lonicera lanceolata*, antimicrobial activity, cholinesterase inhibition, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC).

### INTRODUCTION

*Lonicera lanceolata* is a member of genus *Lonicera* usually found in Bhutan, Nepal, and China and mountains area of Pakistan. The genus *Lonicera* is a member of the family Caprifoliaceae, which comprise about 12 genera and 450 species (Mabberley, 1997), distributed mostly in moderate region of Northern Hemisphere. Many plants of genus *Lonicera* are used for the treatment of a variety of diseases like acute fever, headache, respiratory infections (Houghton et al., 1993), antibacterial (Puupponen-

Pimia et al., 2001), antioxidant (Ali et al., 2013), cytoprotective (Chang and Hsu, 1992), hepatoprotective (Ya-Ping et al., 1992; Shi et al., 1999), antiviral (Chang et al., 1995), antitumor (Wang et al., 2009; Yip et al., 2006) and anti-inflammatory activities (Yoo et al., 2008).

Microbial assay is a principal means of deterioration of foods and is frequently responsible for damage of quality and safety of the foods. The pathogenic and spoilage microorganisms in foods are increasing with the increase

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in occurrence of food-borne diseases. *Staphylococcus aureus* is mostly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Mylotte et al., 1987). *Listeria monocytogenes* is mainly responsible for the harsh food-borne illness, listeriosis, which has been one of the rising zoonotic diseases during the last two decades (Farber et al., 2000). *Escherichia coli* is found in human intestines and is responsible for urinary tract infection, coleocystitis or septicaemia (Singh et al., 2000).

Many compounds were isolated previously from genus *Lonicera* including iridoids, bisiridoids, sulphur containing monoterpenoids, alkaloidal glycosides, triterpenoids, saponins, coumarin glycosides and flavones glycosides (Machida et al., 1995; Bailleul et al., 1981; Souzu et al., 1969; Souzuet al., 1970). The significant medicinal applications of this genus have encouraged us to investigate the compounds of *L. lanceolata*.

In this study, we are going to isolate and investigate the antimicrobial activity and enzyme inhibitory assay of the compounds which are first time isolated from *L. lanceolata*, namely, apigenin 7-O-gentiobioside (compound 1), 3'-O-methyl lanoflavone [5',5'',7',7''-tetrahydroxy 3'-methoxy 4',4''-biflavonyl ether ] (compound 2), 7-O-ethyl sweroside (compound 3), secoxyloganin (compound 4) (Masao AND Noriko, 1995; Neeraj et al., 2005; Yue et al., 2006). These compounds of *Lonicera lanceolata* were screened for antimicrobial and enzyme inhibitory activities.

## MATERIALS AND METHODS

### Chemicals and culture media

Thin layer chromatography (TLC) plates which are made up of aluminium (20 × 20, 0.6 mm thick) pre-coated with silica gel 60 F<sub>254</sub> (20 × 20 cm, 0.2 mm thick; E. Merck, Darmstadt, Germany) were prepared in order to check the purity and isolation of the compounds. The support used in column chromatography (CC) was silica gel of 230 to 400 mesh (E. Merck, Darmstadt, Germany). Ceric sulphate and potassium permanganate solutions were applied as visualization reagents. UV spectra ( $\lambda_{\max}$  nm) were recorded on Shimadzu UV-2700 spectrophotometer (Shimadzu, Japan) in EtOH. Mass spectra were taken on Bruker TOF Mass Spectrometers (Billerica, USA) using electrospray ionisation (ESI). <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of the compounds were recorded on a Bruker DPX-400 NMR Spectrometer (Billerica, USA) (400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C-NMR), using CDCl<sub>3</sub> as solvents.

### Plant

The plant materials of *L. lanceolata* were collected from Hazara Division, District Mansehra, in May 2012. The plant was identified by Professor Manzoor Ahmad, Plant Taxonomist on the basis of its morphology and the database present in the library at the Department of Botany, Government Degree College Abbotabad, Pakistan, where a voucher specimen were deposited in herbarium (Accession No. D-056).

### Extraction and isolation

The shade dried powdered material of *L. lanceolata* (5 kg) was extracted with methanol (40 L) at 25°C for 7 days (3 × 40 L). The methanol of the methanolic extract was evaporated with the help of rotary evaporator to obtain dark greenish gummy crude (98 g). Four fractionations of methanolic crude were taken with *n*-hexane (F1, 18 g), chloroform (F2, 21 g), ethyl acetate (F3, 21.6 g), and *n*-butanol (F4, 23 g).

Chloroform fraction was transferred to column chromatography over silica gel (70 to 230 mesh) eluting with the percentage polarity of the solvent system that is, *n*-hexane (100%), *n*-hexane:chloroform (2:18 to 18:2), chloroform (100%), chloroform:MeOH (2:18 to 18:2), MeOH (100%), with increasing polarity to obtain 14 fractions A-N.

Fraction D (6 g) was again introduced to a series of silica gel column chromatography eluting with *n*-hexane, *n*-hexane:chloroform and chloroform with increasing polarity give compound 1 with 100% CHCl<sub>3</sub> and to a preparative TLC using *n*-hexane:chloroform (2:3) as solvent system and isolated compounds 2 and 3, respectively.

Fraction F (5 g) was again transferred over silica gel eluting with *n*-hexane, *n*-hexane:chloroform and chloroform in increasing order of polarity followed by preparative TLC eluted with *n*-hexane:CHCl<sub>3</sub> (4:1) and isolated compound 4.

### Microorganisms

To check the antibacterial activity of these compounds, two human Gram-positive bacteria and four Gram-negative bacteria, that is, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* (clinical strain/PIMS), Enterobacteriaceae (clinical strain/PIMS), *S. aureus* (MRSA, clinical strain/PIMS) and *Micrococcus luteus* (clinical strain/PIMS) were selected for the antibacterial test. Strains were gotten from Microbiology Research Laboratory, Beijing University of Chemical Technology (BUCT), China where their identification and characterization took place. These strains were placed on Agar slants at 4°C for antimicrobial screening. Microorganisms were kept in incubator overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.2. Ofloxacin (10 µg) and ampicillin (10 µg) (Oxoid) were used as reference antibiotics (Table 1).

### Screening for antibacterial activity by Agar well diffusion method

For determination of antibacterial activity of the isolated compounds, Agar well diffusion method (Hadacek et al., 2000) was carried out. All bacterial strains were first grown in Nutrient Broth at 37°C for 24 h incubated till turbidity became equal to McFarland 0.5 turbidity standard. Using a sterile swab, the inocula of the respective bacteria were streaked on to the Muller Hinton Agar (Oxoid) plates in order to make sure a uniform thick lawn of growth following incubation. Using sterile cork borer, wells of 5 mm in diameter were formed on to Nutrient Agar plates. The wells were filled with 100 µl of compounds 1 to 4 and the plates were then kept to stay for 2 h at 25°C. At last, the plates were incubated at 37°C for 24 h and the resultant diameters of zones of inhibition were measured carefully.

### Determination of minimum inhibitory concentration (MIC)

#### Agar dilution method

Agar well dilution protocol was followed to carry out MIC of

**Table 1.** Zone of inhibition of reference antibiotics.

Reference/Antibiotic	Microorganisms and their zone of inhibition (mm)					
	Ec	Kp	Ps	Ent	MI	Sta
Ofloxacin	15.1(±0.02)	14(±0.01)	11.9(±0.01)	13(±0)	10(±0.11)	9.6(±0.05)
Ampicillin	14.1(±0.05)	12.3(±0.5)	11(±0)	14(±0.11)	13(±0.05)	14(±0.05)

Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Ps: *Pseudomonas aeruginosa*; Ent: *Enterobacter cloacae*; MI: *Micrococcus luteus*; Sta: *Staphylococcus aureus* (methicillin resistant); mm: millimeter.

**Table 2.** Inhibition zones of compounds 1-4.

Compound	Zone of inhibition (mm)					
	Ec	Kp	Ent	Ps	MI	Sta
1	16(±0.4)	16(±0.3)	15(±0)	14(±0.3)	16(±0.7)	15(±0.4)
2	13(±0.4)	13(±0.6)	14(±0.4)	12(±0.4)	14(±0.5)	14(±0.2)
3	7(±0.3)	7(±0.4)	5(±0.2)	7(±0.2)	6(±0.5)	6(±0.5)
4	2(±0.2)	4(±0.1)	6(±0.2)	4(±0.6)	4(±0.3)	3(±0.5)

Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Ps: *Pseudomonas aeruginosa*; Ent: *Enterobacter cloacae*; MI: *Micrococcus luteus*; Sta: *Staphylococcus aureus* (methicillin resistant); mm: millimeter.

compound 1 (EUCAST Definitive Document, 2000; Mukherjee, 2002; WHO, 2002). After sterilizing, Muller Hinton Agar (oxid) was allowed to cool to 50°C. With 1 ml of different concentrations of compound 1 in sterilized test tubes, about 19 ml of Muller Hinton Agar was mixed. After mixing carefully, this mixture was poured into pre-labelled sterile Petri dishes. Petri dishes having only growth media were prepared in the same way so as to serve for assessment with Petri plate containing compound. 2000 to 0.156 µg/ml concentrations of the compound were used in this assay. The density of the suspensions of the respective microorganisms was adjusted to 0.5 McFarland turbidity standards. By using sterilized standard loop, these were inoculated onto the series of Agar plates. The incubation of these plates took place at 37°C for 24 h. The minimum concentration of the compound, which inhibited the growth of the respective organisms, was considered as MIC. The assay was carried out in triplicate.

#### Determination of minimum bactericidal concentration (MBC)

##### Viable cell count method

For the determination of MBC of the compound 1, the viable cell count method was followed (Toda et al., 1989; Rodríguez-Tudela et al., 2003), and the results were shown as number of viable cells as a percentage of the control.

##### Screening for antifungal activity

By using sterilized standard loop the required amount of fungal strain was suspended in 2 ml of Sabouraud Dextrose Broth. This suspension was uniformly streaked on Petri plates having Sabouraud Dextrose Agar media by using sterilized cotton swabs. Compounds were poured into wells using same technique for bacteria, but these were incubated at 25°C for 72 h, that is, 3 days. The plates were then examined for the presence of zones of inhibition and the results were measured and recorded. Itraconazole was used as a standard or positive control which is a potent antifungal.

#### Cholinesterase inhibition assay and determination of IC<sub>50</sub> values

Acetylcholinesterase (EC 3.1.1.7), acetylthiocholine iodide, butyrylthiocholine chloride, butyrylcholinesterase (E.C 3.1.1.8), galanthamine and 5, 5-dithiobis [2-nitrobenzoic-acid] (DTNB) were purchased from Sigma. All other chemicals used in this assay were of analytical grade. By performing the customized spectrophotometric assay used by Ellman et al. (1961), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibiting activities were performed. The protocol conditions were the same as described by Rocha et al. (1993).

Butyrylthiocholine chloride and acetylthiocholine iodide were chosen as substrates to assay AChE and BChE, respectively. DTNB was used for the measurement of cholinesterase activity. 0.2 mM DTNB in 62 mM sodium phosphate buffer (pH 8.0, 880 µl), test compounds solution (40 µl) and AChE or BChE solution (40 µl) were thoroughly mixed and incubated for 15 min at 25°C. The reaction was initiated by the addition of acetylthiocholine or butyrylthiocholine (40 µl), respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored, when yellow 5-thio-2-nitrobenzoate anion was formed as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). All the reactions were carried out in triplicate in a BMS spectrophotometer (USA). The concentrations of test compounds that repressed the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC<sub>50</sub>) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The final DMSO concentration in the reaction mixture was 6% (Figure 1).

## RESULTS

Compound 1 has high antibacterial activity as compared to other compounds as shown in Table 2. Thus, it was further considered for determination of MIC and MBC,

**Table 3.** MIC and MBC of *Lonicera lanceolata* compound 1.

Microorganism	MIC ( $\mu\text{g/ml}$ )	MBC ( $\mu\text{g/ml}$ )
<i>E. coli</i>	>10	15.5
<i>K. pneumoniae</i>	>10	N.d
<i>P. aeruginosa</i>	>10	12
<i>E. cloacae</i>	5	15
<i>M. luteus</i>	0.625	1.877
<i>S. aureus</i>	0.156	1.260

N.d: Not detected.

**Table 4.** Antifungal activities of compounds 1 to 4.

Compound	Zone of inhibition (mm)
1	7( $\pm$ 0.5)
2	7( $\pm$ 0.11)
3	2( $\pm$ 0.17)
4	3( $\pm$ 0.14)
Standard	8(0)

**Table 5.** AChE and BChE inhibitory activities of compound 1 to 4 *Lonicera lanceolata* ( $\text{IC}_{50}$ ,  $\mu\text{M}$ ).

Compound	AChE $\pm$ SEM <sup>a</sup>	BChE $\pm$ SEM <sup>a</sup>
1	Nil	Nil
2	1.99 $\pm$ 0.07	3.65 $\pm$ 0.079
3	1.75 $\pm$ 0.03	4.90 $\pm$ 0.079
4	4.27 $\pm$ 0.04	12.76 $\pm$ 0.087
Allanzanthane <sup>b</sup>	2.94 $\pm$ 0.45	12.96 $\pm$ 0.053
Galanthamine <sup>b</sup>	1.79 $\pm$ 0.061	7.98 $\pm$ 0.01

<sup>a</sup>Standard error of mean of five assays. <sup>b</sup>Positive control used in the assays. Data shown are values from triplicate experiments.

respectively. The MIC values ranged from 0.156 to >10  $\mu\text{g/ml}$  for all tested strains, while the MBC values reported were many times higher than MIC (Table 3). The MBC value for *K. pneumoniae* was not detected.

In the same way, nearly similar pattern of defencelessness was reported against fungal strain *Aspergillus niger*. The widest zones of inhibition (maximum antifungal activity) were presented by Compounds 1 and 2. Compounds 3 have reasonable antifungal, while compound 4 has the lowest antifungal activity as shown in Table 4.

The interested of this study was to identify AChE and BChE inhibiting compounds from herbal medicinal plants, performed bioassay-guided search for AChE and BChE inhibitors from this medicinal plant. Compounds 1 to 4 isolated from *L. lanceolata* were tested against AChE and BChE, which show the most attention-grabbing target for drug treatment of neurone design and innovation of mechanism-based inhibitors for the degenerative disorders

such as Alzheimer's disease (Zhang, 2004). The percentage of inhibition was first determined at 0.1 mM. Those compounds which showed greater than 50% enzyme inhibition, were consequently assayed for  $\text{IC}_{50}$  (50% inhibitory effect) determination. Among the isolated compounds, compounds 2 and 3 showed the most effective inhibition activity against AChE and BChE as compared to standard drugs: allanzanthane and galanthamine in a dose dependent manner. The  $\text{IC}_{50}$  values of compounds 2 and 3 against AChE were determined to be 1.99 and 1.75  $\mu\text{M}$ , while against BChE, were measured as 3.65 and 4.90  $\mu\text{M}$ , respectively. Compound 1 has no inhibition while compound 4 showed weak inhibition profile against AChE and BChE (Table 5).

## DISCUSSION

The antimicrobial activities of four compounds isolated from chloroform fraction were tested against six bacteria species: *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Enterobacter cloacae*, *S. aureus*, and *Micrococcus luteus*. Nearly all compounds exhibited more or less antimicrobial activity against the test strains. Compound 1 exhibited the best activity against these bacteria. Besides that, compounds 2 and 3 have moderate and compound 4 have lowest activity against these bacteria. Nearly all the constituents from *Lonicera lanceolata* were primarily reported as active against the *A. niger*. Compounds 1 and 2 showed the highest antifungal activities, while compound 4 showed least antifungal activity.

The MIC of compound 1 was taken as 0.156 mg/ml. It is important that the MIC value is too high to be taken in susceptible ranges (Paul et al., 2006). The MBC value of compound 1 is many times higher than MIC. The antibacterial and antifungal assays were done by Agar well diffusion method. MIC was carried out by using Agar well dilution method, while MBC was performed by viable cell count method. The MBC values for *K. pneumoniae* were not detected.

All the four compounds isolated from the chloroform fraction of *L. lanceolata* are polar and were primarily tested as antimicrobial reagents. Compounds 2 and 3 have the highest acetyl cholinesterase and butyryl cholinesterase inhibitory effects. This investigation is probably the first to exhibit the antimicrobial and enzyme inhibitory activities of compounds 1 to 4 of *L. lanceolata*, as a comprehensive literature review to the best of our knowledge; there is no information about the antimicrobial and enzyme inhibitory activities of these isolated compounds from this plant.

## Conclusion

From the present studies, it is clear that four compounds isolated from chloroform fraction of *L. lanceolata* show



**Figure 1.** Structures of compounds 1 to 4 from *Lonicera lanceolata*.

prominent antimicrobial and enzyme inhibitory activities. Compound 1 exhibited the highest antibacterial activity. The MIC of compound 1 was taken as 0.156 mg/ml. These constituents of *L. lanceolata* can be used for enzyme inhibition and antimicrobial preparations. Therefore, other fractions, that is, *n*-hexane, ethyl acetate and *n*-butanol are further recommended for investigations to explore the potential medicinal compounds.

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*Full length research paper*

# Prevention and treatment of skin infections and disorders: A novel water-based topical antidermatitic body lotion

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Poor nutrition is an issue in Nigeria, with the average daily calorie intake per capita of 2,000. This dictates unhealthy skin. To meet local demands, Nigeria imports, under different trade names, water-free dermatitic ointments indicated for the prevention and treatment of skin infections and disorders. The oily products are preferred in harmattan season and are unpopular with ladies who consciously avoid oily creams that procure sweat and attract dust. This study was aimed at formulating a topical water-based antidermatitic body lotion for the prevention and treatment of most skin disorders and infections, using the conventional body cream raw materials. The novel body lotion showed a negligible 0.03% cases of skin irritation and hypersensitivity reactions. It prevents and treats eczema, dandruff, psoriasis, acne, ringworm, after-shave rashes, heat rashes, napkin and urinary rashes, dermatitis, seborrhea, African beauty spots and crawl-crawl. It also treats bruises, burns and cuts, proving antipyretic, anti-inflammatory and preventive of post-healing skin discolouration. It does not treat pimples. It is more acceptable all-year round as a cosmetic moisturizing body lotion than the water-free antidermatitic ointment, which is preferred only in the harmattan period and is unpopular with ladies.

**Key words:** Water-based, topical, antidermatitis, lotion, prevention, treatment, skin, infections, disorders.

## INTRODUCTION

Malnutrition, poor health and skin issues are common indicators of the low living standards in Nigeria, with an average daily calorie intake per capita of 2,000 (Eneh, 2011). To meet local demands, Nigeria imports, under different trade names, water-free antidermatitic ointments indicated for the prevention and treatment of skin infections and disorders. This dictates enormous demand on foreign exchange. To address this economic challenge, an earlier study formulated a topical antidermatitis ointment with a wider-spectrum of and additional

activities than familiar ointments, using over 90% local substitutes for foreign raw materials. The water-free oily cream product, which contains benzoic acid, salicylic acid and sulphur as active ingredients, paraffins as vehicle and body and solidifier, as well as colourant and perfume additives, prevented and treated eczema, dandruff, psoriasis, acne, ringworm, after-shave rashes, heat rashes, napkin and urinary rashes, dermatitis, seborrhea, African beauty spots and crawl-crawl. It also treated bruises, burns and cuts, proving antipyretic, anti-inflammatory

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**Table 1.** Tests for benzoic acid, salicylic acid and precipitated sulphur powder.

S/No.	Material	Physical inspections	Chemical reactions
1	Benzoic acid	White crystalline solid Melting point: 121°C Sparingly soluble in cold water, but fairly soluble in hot water, alcohol and ether Faint aromatic odour Readily sublimates Volatile in steam	Dissolves in hot water, but separates as shining whites flakes on cooling  Its natural solution gives a white precipitate with silver nitrate, AgNO <sub>3</sub>  Gives effervescence with sodium bicarbonate, NaHCO <sub>3</sub>  Gives a buff-colour precipitate with ferric chloride, FeCl <sub>3</sub>
2	Salicylic acid	White crystalline solid Melting point: 155°C Sparingly soluble in cold water, but fairly soluble in hot water, alcohol and ether Poisonous in nature Readily sublimates Volatile in steam	Dissolves in hot water, but separates as shining whites flakes on cooling Gives effervescence with sodium carbonate, Na <sub>2</sub> CO <sub>3</sub> , or sodium bicarbonate, NaHCO <sub>3</sub> , releasing carbon dioxide, CO <sub>2</sub> , to form sodium salt, sodium salicylate  Gives a violet colour with ferric chloride, FeCl <sub>3</sub> , in aqueous solution  Gives 2,4,6-tribromophenol with bromine water, this reaction forming the basis of quantitative estimation of the acid in medicinal preparations
3	Sulphur	Yellow powder solid Melting point: 113-119°C	Gives characteristic sulphide odour with Mg as MgS is formed Vapour from melted sample gives pale yellow solid on contact with a cold surface

and preventive of post-healing skin discolouration. It showed 0.02% cases of skin irritation and hypersensitivity reactions (Eneh, 2007a). A further study improved the antibiotic properties of the oil-based antidermatitic ointment by incorporating neem powder which shortened the therapeutic periods by as much as half in some of the tested cases. The efficacy and local content were both further improved in another study by substituting the imported beeswax with local beeswax (2009). The oily product in each of the studies is more accepted in harmattan season and is unpopular with ladies who consciously avoid oily creams that procure sweat and attract dust. This work was, therefore, aimed at formulating a water-based moisturising body lotion for all seasons that prevents and treats these and other common skin

disorders and infections, using the same raw materials.

#### MATERIALS AND METHODS

The pharmaceutical grade chemicals and cans (glass or plastic) used for this study were purchased from Headbridge Market, Onitsha. Distilled deionized water was obtained from the Soil Science Laboratory of the University of Nigeria, Nsukka. Various equipment were available in science and engineering laboratories of the same university.

#### Procedure

Physical and chemical quality control tests for the active ingredients (benzoic acid, salicylic acid and sulphur) were

designed as outlined in Table 1 after consulting the literature. The quality of the petroleum jelly was ascertained following NIS 371: 1997 (SON, 1997). A common method for preparing body lotion was used. Varied concentrations of the active ingredients drawn from preparations earlier reported (British Medical Association (BMA) and TPSGB, 2011), were used to get three (3) preparations which were applied topically on different portions of approximately the same size of eczema of a patient. Consent of the patient had been sought and obtained. Application in this preliminary test was done twice daily (after morning and night baths) for 21 consecutive days. Results from the preliminary test informed which of the three (3) preparations had the best concentrations and proportions of active ingredients for further investigations. This sample was sent to a public analyst for analysis and examination for suitability as cosmetic product. The sample was next issued to about 10,000 students and parents (staff) of some secondary schools for girls and boys in Enugu metropolis, with a wide

**Table 2.** Summary of sample users' report.

S/NO.	Skin Infection/disorder	Number of users	Number that reported effective and satisfactory performance	Post-healing skin discolouration reported	Number that reported skin irritation	Anti-inflammatory effect reported	Smoothing effect reported	Number that reported compatibility	Average healing period (Days)
1	Acne	48	45	-	-	n.a.	n.a.	46	23.1
2	After-shave rashes	55	52	-	3	47	49	54	3.3
3	Napkin/Urinary Rashes	302	296	-	-	n.a.	266	286	3.9
4	Heat Rashes	412	387	-	-	n.a.	318	394	4.1
5	Craw-craw	994	869	-	-	838	888	946	26.2
6	Dandruff	1,996	1,761	-	-	n.a.	n.a.	1,934	3.8
7	Eczema	1,971	1,863	-	-	n.a.	n.a.	1,964	14.3
8	Psoriasis	11	8	-	-	7	8	8	29.7
9	Ringworm	1,898	1,770	-	-	1,761	1,666	1,716	13.5
10	Seborrhea	13	9	-	-	9	11	8	26.4
11	African beauty spots	28	21	-	-	18	26	23	42.6
12	Dermatitis	29	21	-	-	18	28	28	28.3
13	Pimples	2,333	-	-	-	1,856	-	2,236	-
14	Burns	34	26	-	-	26	31	29	12.7
15	Bruises	912	884	-	-	834	909	906	11.2
16	Cuts	444	392	-	-	382	404	396	13.9

Secondary schools involved: 1. Metropolitan Girls' Secondary School, Ogui-Enugu; 2. Queens School, Enugu; 3. Union Boys' Secondary School, Awkunanaw-Enugu; 4. Government Technical College, Enugu; 5. National Grammar School, Nike-Enugu; 6. City Girls' Secondary School, Ogui-Enugu; 7. Urban Girls' Secondary School, Ogui-Enugu. Number of Sample Users: 10,000 (male, female, youth, children, and infants).

range of dermatological differences assumed among them. Their consent had been sought and obtained. Sixteen most common skin disorders and infections were clearly explained to the literate sample recipients. They were instructed to apply the preparation after morning and evening baths daily for ten (10) weeks. They were to carry out daily observations of the prevention and treatment of eczema, dandruff, psoriasis, acne, ringworm, after-shave rashes, heat rashes, napkin and urinary rashes, dermatitis, seborrhea, African beauty spots, pimples and craw-craw. Also to be noted were its effects on cuts, bruises and burns, its effects on and compatibility with the skin during and after treatment of a skin disorder or infection as well as its prevention of post-healing skin discolouration. Report sheets were given out together with the samples. The preparation was stored on the shelf for observation/analysis and used for treatment at intervals for two (2) years.

## RESULTS AND DISCUSSION

Although, not all recipients of samples turned in reports, some recipients turned in multiple reports from using the samples on households with various disorders and infections. These made up for those who failed to return their reports. The use of samples on households also brought babies and children into the tests, besides the youth and adult male and female people with age variations who received the samples. A summary of the first 10,000 reports turned in is given in Table 2, where a factor not applicable to a particular skin infection or disorder is marked "n.a". The novel moisturising antidermatitic body

lotion had smoothing, pain-relieving and anti-inflammatory effects on cuts, bruises and burns, resulting in their healing within 11.2 to 13.9 days. This agreed with earlier reports that topical body creams incorporating benzoic acid and/or salicylic acid have a mild anti-inflammatory effects, soothe and smoothen the skin and are indicated for dry scaling disorders, such as eczematous disorders and psoriasis (BMA and TPSGB, 2011). Skin problems treated with nine similar products healed between 8.2 and 15.7 days (Tkac, 1990).

Benzoic acid, salicylic acid and sulphur have disinfecting effect, while salicylic acid has, in addition, antipyretic and analgesic effects (Tewari et al., 2007; Tedder et al., 2005). This disinfecting

**Table 3.** Public analyst's report.

Parameter	Sample	Universal standard
<b>Physical inspection</b>		
Appearance	Pink coloured water-based lotion with tiny air pores	White to any chosen-coloured water-based lotion with tiny air pores
Odour	Perfumed	Perfumed
Specific Gravity	0.9246	0.8190-0.9998
Colour	Pink	Pink
Texture	Soft to touch	Soft to touch
<b>Chemical composition (%)</b>		
Benzoic acid	6.00	4.5-7.5
Salicylic acid	3.00	2.5-6.0
Sulphur	3.00	2.5-6.0
Mineral oil	0.5	0.25-1.0
Emulsifying wax	6.00	3.0-6.5
Petroleum jelly	7.50	5.0-12.0
Cetyl alcohol	1.75	1.5-2.5
Citric acid	1.75	1.5-2.5
Glycerine	2.50	1.5-4.5
Propylene glycol	2.50	1.25-3.5
Lanoline	1.50	1.3-2.5
Stearic acid	0.75	0.35-1.5
Triethanolamine	0.75	0.35-1.5
Fragrance	2.25	1.5-3.5
Colourant	0.25	0.15-3.5
Water	60.00	48-82
<b>Remarks</b>		
The product is deemed suitable to be sold as cosmetic.		

Source: Public Analyst.

effect must have been responsible for the enhanced healing of cuts, bruises and burns (Leydon, 1990), as well as the prevention of post-healing skin discolouration. These findings support other reports on the medical and medicinal uses of the active ingredients in the preparation (Kirk and Othmer, 2002; Large, 2000).

Psoriasis, ringworm, napkin and urinary rashes, crawl-craw, heat rashes and acne cleared between 3.9 and 29.7 days. This agreed with earlier reports (Auld, 1986; Lucky, 1987). Dandruff on shaven scalp cleared within 3.8 days, but returned when scalp was overgrown with hair such that contact with novel topical preparation was difficult. Topical preparations are not expected to have a therapeutic effect (BMA and TPSGB, 2011) on dandruff and eczema, among others. Eczema cleared within 14.3 days but re-appeared about 3 months after the application was discontinued. It cleared again when treatment was restored for 14 to 23 days. This was not only cheaper than, but saved the discomforting side-effect of most systemic treatments of skin disorders and infections (Tedder et al., 2005; Burger, 2000; Liprot, 2008; Wolverton, 1991). Applying the preparation after

shaving prevented the development of after-shave rashes. Continued application till the next shaving exercise might be necessary. The application on the developed rashes got them cleared within 3.3 days. African beauty spots took longest duration, 42.6 days, to be treated and there was no report of successful treatment of pimples.

Products containing the same active ingredients at the chosen concentrations are non-toxic and the skin irritation and hypersensitivity reactions reported by three (3) out of 10,000 users is acceptable (BMA and TPSGB, 2011). There was no change in the concentration, colour and potency of the preparation for the two (2) years of investigation. Expiry or "best before" or "use before" date of two (2) years from date of manufacture was, therefore, recommended. The preparation was found compatible, confirming the report of earlier workers (Tkac, 2000; Tewari et al, 2007). The report of the Public Analyst is shown in Table 3. The characteristic of the sample is shown side-by-side with that of the universal standard. Both had tiny air pores, were perfumed and had similar texture (soft to touch).

The specific gravity of the sample of the water-

**Table 4.** Assessment (%) of sample's performance.

<b>Skin infection/ disorder</b>	<b>% Effective and satisfactory performance</b>	<b>% Compatibility</b>	<b>% Smoothing effect</b>	<b>% Anti-inflammatory effect</b>
Acne	93.75	95.83	n.a	n.a
After-shave rashes	94.55	98.18	89.09	85.45
Napkin/urinary rashes	98.01	94.70	88.08	n.a
Heat Rashes	93.93	95.63	77.18	n.a
Craw-craw	87.42	95.17	89.34	84.31
Dandruff	88.23	96.89	n.a	n.a
Eczema	94.52	99.64	n.a	n.a
Psoriasis	72.73	72.72	72.73	63.64
Ringworm	93.26	94.41	87.78	92.78
Seborrhea	69.23	61.54	84.62	69.23
African beauty spots	75.00	82.14	92.86	64.29
Dermatitis	72.41	96.55	96.55	62.07
Pimples	-	95.84	-	79.55
Burns	76.47	85.29	91.18	76.47
Bruises	96.93	99.34	99.67	91.45
Cuts	88.29	89.19	90.99	86.04

free antidermatitic preparation was 0.9246, whereas the universal range was 0.8190 to 0.9998. The sample contained 6% benzoic acid, against the universal range of 4.5 to 7.5%; 3% salicylic acid, whereas the universal range was 2.5 to 6.0%; 3% sulphur, against the universal range of 2.5 to 6.0%; 0.5% mineral oil, whereas the universal range of 0.25 to 1.0%; 6% emulsifying wax, against the universal range was 3.0 to 6.5%; 7.5% petroleum jelly, whereas the universal range of 5 to 12%; 1.75% cetyl alcohol against the universal range was 1.5 to 2.5%; 1.75% citric acid, whereas the universal range was 1.5 to 2.5%; 1.75% cetyl alcohol, against the universal range of 1.5 to 2.5%; 2.5% glycerine, whereas the universal range was 1.5 to 4.5%; 2.5% propylene glycol,

against the universal range of 1.25 to 3.5%; 1.5% lanolne, whereas the universal range was 1.3 to 2.5%; 0.75% stearic acid, against the universal range of 1.3 to 2.5%; 0.75% triethanolamine, whereas the universal range was 1.3 to 2.5%; 2.25% fragrance, against the universal range of 1.3 to 3.5%; 0.25% colour, whereas the universal range was 0.15 to 2.5%; and 60% distilled deionised water, against the universal range of 48 to 82%. The analyst also remarked that the product was deemed suitable to be sold as cosmetic.

Table 4 contains the data on percentage assessment of the performance of the novel topical moisturising antidermatitic body lotion. The product rating ranged between satisfactory

(61.54%) and excellent (99.67%) for the various performance criteria assessed. The novel topical moisturising antidermatitic body lotion was even more acceptable as a cosmetic than water-free ointment, which is more desirable only in the hammattan period and for scaling disorders (BMA and PSGB, 2011). It promised to be popular among ladies, who consciously avoid oily creams that procure sweat and attract dust.

## CONCLUSION AND RECOMMENDATIONS

About sixteen (16) different skin infections and disorders are prevented and/or treated with water-free ointments containing benzoic acid, salicylic

acid and sulphur as active ingredient and other ingredients as additives. The product was more acceptable in the harmattan period and for scaling disorders, but unpopular among ladies, who consciously avoid oily creams that procure sweat and attract dust. This work has successfully formulated an all-season novel water-based topical moisturising antidermatitic body lotion for the prevention and/or treatment of all the sixteen (16) skin infections and disorders. The product promised to be popular among ladies as well, who consciously avoid oily creams that procure sweat and attract dust. From the bench-scale production, economic viability of the commercial-scale production could easily be established to make for the commercialization of the product. Further work to establish the longest shelf-life is also recommended.

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Review

# The effect of saffron (*Crocus sativus* L.) and its ingredients on the management of diabetes mellitus and dislipidemia

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The rapidly increasing incidence of diabetes mellitus is becoming a serious threat to mankind's health in all parts of the world and is in a group of metabolic disorders having hyperglycemia as a common manifestation. Implication of oxidative stress in the pathogenesis of diabetes is proposed, not only by oxygen free-radical generation, but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes, and lipid peroxides formation. Moreover, oxidative stress induces systemic inflammation, endothelial dysfunction, impaired secretion of pancreatic  $\beta$  cells and impaired glucose utilization in peripheral tissues. Nowadays, the use of antioxidants still remains a controversy, but its use as a therapy for diabetes can be considered, because it demonstrated effectiveness in lowering the risk of diabetes and its complications. Therefore, this review provides an updated overview of experimental *in vitro* and *in vivo* investigations on the biological activities of saffron (*Crocus sativus* L.) and its principal phenolic ingredients, especially focusing on their anti-diabetics effect. This data has led to the suggestion that saffron (*C. sativus* L.) and its principal phenolic ingredients might be beneficial for preserving diabetes and its complications; however, the application remains controversial. Therefore, this review highlights the antidiabetic effects of saffron and its main ingredients, related to antioxidant properties of carotenoids of saffron.

**Key words:** Antidiabetic, antioxidant, dyslipidemia, saffron.

## INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by relative or absolute deficiency of insulin secretion and/or insulin resistance that causes chronic hyperglycemia and impaired carbohydrates, lipids, and proteins metabolism (Chaturvedi et al., 2007). DM is the principal factor responsible for high prevalence of mortality

due to coronary heart disease. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications, while on the other hand, hyperglycemia engenders free radicals, and it also impairs the endogenous antioxidant defense system in many ways during diabetes (Boynes et al., 1991;

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Maritim et al., 2003). Diabetes-linked alterations in antioxidant defense system enzymes, such as catalase, glutathione peroxidase and superoxide dismutase have been demonstrated (Robertson et al., 2007), while insulin and oral anti-hyperglycemic drugs are the cornerstone of the diabetes treatment; they have important adverse effects and cannot always prevent diabetes complications significantly (Dey et al., 2002; Gilbert et al., 2009). Thus there is a continuing need for alternative anti-diabetic remedies with better risk-benefit ratios and greater patient acceptability. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease. More attention has been paid to the protective effects of natural antioxidants against chemically induced toxicities (Neelesh et al., 2010; Samini et al., 2013). Antioxidant therapy may play great role for diabetic patients; therefore, it can be considered for treatment of oxidative stress in DM and may be good choices for diabetes therapy. Phenolic compounds (e.g., phenolic acids, flavonoids, quinones, and tannins) are natural antioxidants abundant in many desert and steppic plants (Schroeter et al., 2002; Samarghandian et al., 2012). A positive linear correlation was indicated between the antioxidant activity and the total phenolic content in plants (Al-Mustafa et al., 2008; Tawaha et al., 2007), proposing that phenolic compounds contribute significantly to the antioxidant capacity of the plant. Direct correlation between the antioxidant property of medicinal plants and the latter's anti-diabetic activity was found (Sabu et al., 2002), and the relationship between the molecular structure of flavonoids and their radical-scavenging capability was found. However, there are many controversies nowadays on the role of antioxidant therapy for diabetic patients (Rahimi et al., 2005). Thus, this review provides an updated overview of experimental *in vitro* and *in vivo* investigations on the biological activities of saffron (*Crocus sativus* L.) and its principal phenolic ingredients, especially focusing on their anti-diabetics effect. Potential use of these natural agents for controlling diabetes complications is also discussed.

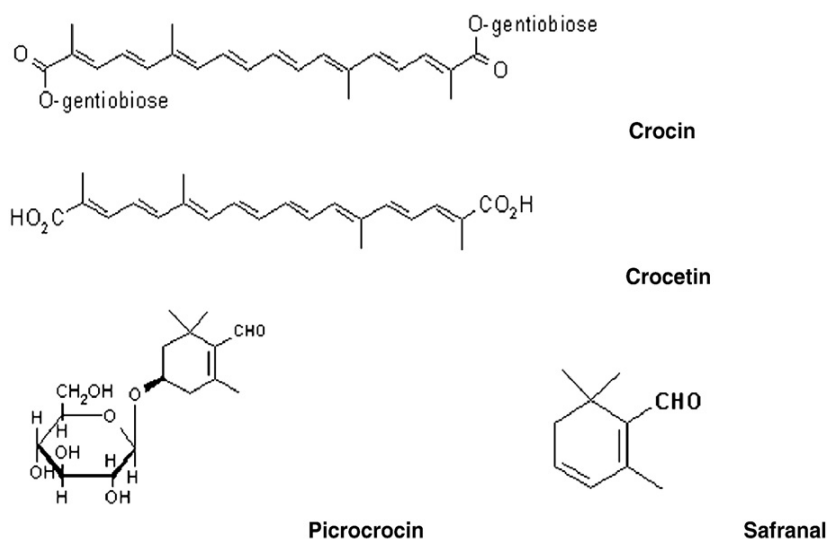
### TRADITIONAL APPLICATION OF SAFFRON

Saffron is the dried stigmas of *C. sativus* L. *C. sativus* L belongs to the family of Iridaceas, the line of liliaceas, and is mainly cultivated in several countries of mild and dry climate (Abdullaev et al., 1993). Although, the source of saffron is unknown, it apparently originated in the area of Iran, Turkey and Greece, but now it is also successfully cultivated in European countries as Spain, Italy, France, and Switzerland, as well as in Morocco, Egypt, Israel, Azerbaijan, Pakistan, India, New Zealand, Australia and Japan. While the world's total annual saffron production is estimated to be 190 tons, Iran produces about 90% of the total with a commercial cost

(Abdullaev et al., 2007; Zarinkamar et al., 2011). The main reason for its great cost is that saffron is still cultivated and harvested as it has been for millennia by hand (Hosseinpour et al., 2010). Saffron's name is derived from the Arab word for yellow, a name reflecting the high concentration of carotenoid pigments present in the saffron flowers' stigmas which contribute most to the color profile of this spice. From ancient times, the saffron is widely used as drug to promote health and fight disease and it is also valued as a food additive for tasting, flavoring and coloring, as well as for its therapeutic properties (Abdullaev et al., 2007). In the traditional medicine, saffron is used as a diaphoretic, eupeptic, tranquilizer, expectorant, aphrodisiac, abortifacient, emmenagogue and in the treatment of hepatic disorders, flatulence, spasm, vomiting, dental and gingival pain, insomnia, depression, seizures, cognitive disorders, lumbago, asthma, cough, bronchitis, colds, fever, cardiovascular disorders and cancer. Saffron is recognized as an adaptogen in Indian ayurvedic medicine (Kianbakht et al., 2009).

### CHEMISTRY OF SAFFRON AND ITS INGREDIENTS

Saffron contains more than 150 volatile, non-volatile and aroma-yielding compounds which consist of lipophilic and hydrophilic carbohydrates, proteins, amino acids, minerals, musilage, vitamins (especially riboflavin and thiamine) and pigments including crocin, anthocianin, carotene, lycopene, zigzantin, flavonoids, starch, gums, and other chemical compounds. Based on chemical analyses of dry stigma of saffron extracts, carotenoids, namely crocin and crocetin and the monoterpene aldehydes picrocrocin and safranal are the most important active carotenoid secondary metabolites of saffron. Crocin, with elementary composition  $C_{44}H_{64}O_{24}$  and molecular weight 976.96, is a hydrophilic carotenoid (8'-diapocarotene-8,8'-dioic acid), constitutes approximately 6 to 16% of saffron's total dry matter depending upon the variety, growing conditions, and processing methods (Gregory et al., 2005). This is the diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin. Deep red color of crocin produces the color of saffron. Crocin 1 (or  $\alpha$ -crocin), a digentiobioside, is the most abundant crocin with a high solubility being attributed to these sugar moieties. Crocin widely used as a natural food colorant (Lage et al., 2009). In addition to crocin, saffron contains crocetin as a free agent and small amounts of the pigment anthocianin,  $\alpha$ -carotene,  $\beta$ -carotene, and zexxantin (Lage et al., 2009). The structure of crocetin is presented as shown in Figure 1. Crocetin, with elementary composition ( $C_{20}H_{24}O_4$ ), melting point  $285^{\circ}C$  and molecular weight 328.4, is an amphiphilic low molecular natural carotenoid (8, 8'-diapo-8, 8'-carotenoic acid) and consists of a C-20 carbon chain with seven double



**Figure 1.** Chemical structures of crocin, crocetin, picrocrocin, and safranal.

bonds and a carboxylic acid group at each end of the molecule. This compound present in the central core of crocin and responsible for the color of saffron, constitutes approximately 14% of saffron's total dry matter depending upon the variety, growing conditions, and processing methods. It is a soluble in organic bases and slightly soluble in aqueous solution (20  $\mu\text{M}$  at pH 8.0) (Lage et al., 2009). The structure of crocetin is as shown in Figure 1. Picrocrocin, with elementary composition ( $\text{C}_{16}\text{H}_{26}\text{O}_7$ ) and molecular weight 330.37 g/mol is the main bitter crystalline terpene-glucoside of saffron. The actual taste of saffron is derived primarily from picrocrocin which is the second most abundant component (by weight), accounting for approximately 1% to 13% of saffron's dry matter (Alonso et al., 2001). Action of  $\beta$ -glucosidase on picrocrocin liberates the aglycone 4-hydroxy-2, 6, 6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC,  $\text{C}_{10}\text{H}_{16}\text{O}_2$ ), which is transformed to safranal by dehydration during the drying process of the plant material. Natural de-glycosylation of picrocrocin will yield another important aroma factor, safranal, ( $\text{C}_{10}\text{H}_{14}\text{O}$ ) which comprises about 60% of the volatile components of saffron. Dehydration is not only important to the preservation of saffron, but is actually critical in the release of safranal from picrocrocin via enzymatic activity, the reaction yielding D-glucose and safranal, the latter being the volatile oil in saffron. Safranal, with, elementary composition ( $\text{C}_{10}\text{H}_{14}\text{O}$ ) and molecular weight 150. 21 g/mol is the major volatile oil responsible for the aroma (Lage et al., 2009). The stability of saffron and its ingredients is also dependent upon temperature, light and humidity on degradation of potency under storage conditions. Ingredients of saffron can be stored under  $-20^\circ\text{C}$  and pharmacological activities as a supplement

remain unaltered for at least 2 years or even longer (Hosseinpour et al., 2009).

Toxicological studies have identified that the toxicity of saffron has been found to be quite low and oral  $\text{LD}_{50}$  of saffron in animal was 20.7 g/kg administered as a decoction. It has been demonstrated that oral administration of saffron extract at doses from 0.1 to 5 g/kg was non-toxic in mice. Ames/Salmonella test system revealed that crocin and dimethyl-crocetin isolated from saffron were non-mutagenic and non-toxic (Nair et al., 1995). Saffron should always be obtained from a reputable source that observes stringent quality control procedures and industry-accepted good manufacturing practices. People with chronic medical conditions should consult with their physician before taking the herb. Pregnant women should never take the herb for medicinal purposes, as saffron can stimulate uterine contractions (Abdullaev and Espinosa-Aguirre, 2004).

## BIOMEDICAL FINDING OF SAFFRON AND ITS INGREDIENTS

Biomedical findings have been demonstrated that saffron and its ingredients may be useful as a treatment for neurodegenerative disorders and related memory impairment, ischemic retinopathy and/or age-related macular degeneration, coronary artery disease, blood pressure abnormalities, acute and/or chronic inflammatory disease, mild to moderate depression, seizure, and Parkinsonism. Furthermore, antioxidant, antimutagenic, antigenotoxic, tumoricidal and antioxidant activity of saffron and its ingredient have been found (Abdullaev and Espinosa-Aguirre, 2004).

### Antioxidant activity of saffron and its ingredients and management of diabetes

Recent scientific findings have been encouraging, uniformly showing that saffron and its derivatives can affect hyperglycemia in a variety of *in-vivo* and *in-vitro* models, particularly, crocin, crocetin and safranal have significant anti-diabetic activity (Kianbakht et al., 2011). *In-vitro* and *in-vivo* studies have also been designed to evaluate the exact mechanism and effective derivate of saffron against diabetes and its complications. One of the main hypotheses for the modes of saffron and its ingredients (crocin, cocetin and safranal) is inhibitory effect on free radical chain reactions (Assimopoulou et al., 2005; Kanakis et al., 2007; Halataei et al., 2011; Hariri et al. 2010). The useful effects of saffron and its ingredients as an antioxidant in biological systems have been attributed to its capability to stabilize bio-membranes, to scavenge ROS, and to decrease the peroxidation of unsaturated membrane lipids (Papandreou et al., 2011). They have been shown to have a hydroxyl radical scavenging activity (Assimopoulou et al., 2005). It has been shown that the radical scavenging activity of the saffron methanol extract and its constituents, crocin and safranal, is significant, probably because these donate hydrogen atoms for DPPH radical stabilization (Kurechi et al., 1980). Saffron ingredients modulated antioxidant gene expression and upregulate mitochondrial antioxidant genes, leading to a lower mitochondrial oxygen radical generation, which may be responsible at least in part for the improved hyperglycemia, hyperlipidemia and oxidative stress in experimental diabetic model (Hosseinzadeh and Sadeghnia, 2005). These findings were confirmed by variety of studies in which saffron, crocin, crocetin and safranal had protective effects against oxidation induced tissue injuries due to their antioxidant properties. Experimental findings showed that saffron and its ingredients insert anti-genotoxic, tumoricidal and anti-aging through modulating oxidative stress (Premkumar et al., 2003; Samarghandian et al., 2010; Samarghandian et al., 2011; Farahmand et al., 2013). Moreover, antioxidant activities of saffron and its main ingredients played essential role in the treatment of neurodegenerative disorders and related memory impairment, ischemic retinopathy and/or age-related macular degeneration, coronary artery disease, blood pressure abnormalities, acute and/or chronic inflammatory disease, mild to moderate depression, seizure and Parkinsonism in animal modeling (Zhang et al., 1994; Pitsikas et al., 2007; Zheng et al., 2007; Schmidt et al., 2007; Papandreou et al., 2011; Kamalipour et al., 2011; Rahbani et al., 2011; Bharti et al., 2012; Samarghandian and Shabestari, 2013).

### Glycemic control and management of diabetes

The hypoglycemic effect of saffron extract seems to be

exerted by mechanisms including stimulation of glucose uptake by peripheral tissues (Yang et al., 2003), inhibition of intestinal glucose absorption (Youn et al., 2004), inhibition of insulinase activity in both liver and kidney (Achrekar et al., 1991), inhibition of endogenous glucose production (Eddouks et al., 2002), inhibition of renal glucose reabsorption (Maghrani et al., 2005) or correction of insulin resistance (Hu et al., 2003) stimulation of  $\beta$ -cells of islets of Langerhans to release more insulin (Xi et al., 2007) regeneration of  $\beta$ -cells islets of Langerhans (Elgazar et al., 2013).

However, possibilities of other mechanisms to exert hypoglycemic effect cannot be rejected. The mechanism of alloxan and streptozotocin (STZ) diabetes has been the subject of many investigations and it is now generally accepted that destruction of the  $\beta$ -cell pancreatic islets is associated strictly with the induction of oxidative and nitrosative stress, both systemically and locally. Therefore, the pancreas is especially susceptible to the action of STZ and alloxan-induced free-radical damage. Many substances have been shown to ameliorate the diabetogenicity of STZ and alloxan in animals by reacting with free radicals formed from STZ and alloxan during its interaction with the  $\beta$ -cell, or prevent radical formation (Jörns et al., 1999). Recently, it was reported that the saffron extract, crocin, crocetin and safranal insert considerable radical scavenging activity and thus antioxidant property (Assimopoulou et al., 2005). Xi et al. (2005) revealed that saffron is used in the traditional medicine in the treatment of diabetes due to its effect in insulin resistance. They also indicated that crocetin has increased insulin sensitivity and ameliorated abnormalities related to insulin resistance such as impaired glucose tolerance, hyperinsulinemia due to high-fructose diet and dexamethasone injection in rats (Xi et al., 2007). This was reported that ethanolic saffron extract (20, 40 and 80 mg/kg) has significant hypoglycaemic effect by increasing the number of  $\beta$ -cells in pancreas and insulin plasma level and reaction with free radicals in alloxan induced diabetic rat (Mohajeri et al., 2009). Another study also demonstrated that saffron extract has hypoglycemic effects on healthy male rats. It extract could increase their insulin secretion from pancreatic  $\beta$ -cells (Arasteh et al., 2010). An *in-vitro* model study indicated that saffron strongly enhanced glucose uptake and the phosphorylation of AMP-activated protein kinase (AMPK)/acetyl-CoA carboxylase (ACC) and mitogen-activated protein kinases (MAPKs); AMPK plays a major role in the effects of saffron on glucose uptake and insulin sensitivity in skeletal muscle cells (Kang et al., 2012). Oral administration of saffron extract at the three different doses 200, 400 and 600 mg/kg caused significant increase in serum insulin level in all treated diabetic rats, while significantly reduced blood glucose levels. In addition, saffron extract (600 mg/kg) improved hypertrophy and hyperplasia of  $\beta$ -cells of islets of Langerhans associated

with pyknosis of their nuclei in alloxan induced diabetic rats (Elgazar et al., 2013).

Administration of crocin significantly reduced the blood glucose level in diabetic animals (Rajaei et al., 2013; Shirali et al., 2013; Tamaddonfard et al., 2013). In experimental model of DM also observed that safranal (0.25, 0.5 and 0.75 mg/kg/day for 4 weeks) administration led to a significant decrease in glucose, MDA and NO content accompanied by a significant increase in plasma GSH content and CAT and SOD activities. This finding indicated that safranal exerts anti-hyperglycemic and hypoglycemic properties by modulation of oxidative stress in STZ diabetic rats (Samarghandian et al., 2013). Also, kianbakht et al. (2011) confirmed antihyperglycemic activity of saffron, crocin and safranal in alloxan diabetic rats. The findings of one study indicated that saffron and its main ingredients (crocin, crocetin and safranal) may have anti-hyperglycemic and blood insulin level elevating effects without hepatic and renal toxicities in the alloxan-diabetic rats (kianbakht et al., 2011). Based on these results, the protective effect of saffron extract on pancreas of diabetic rats might be attributed directly to scavenging activity due to its major constituents including crocin, crocetin and safranal. These compound as natural antioxidant may be very important in mitigating impaired insulin secretion and action in insulin resistance and prevent diabetes complications

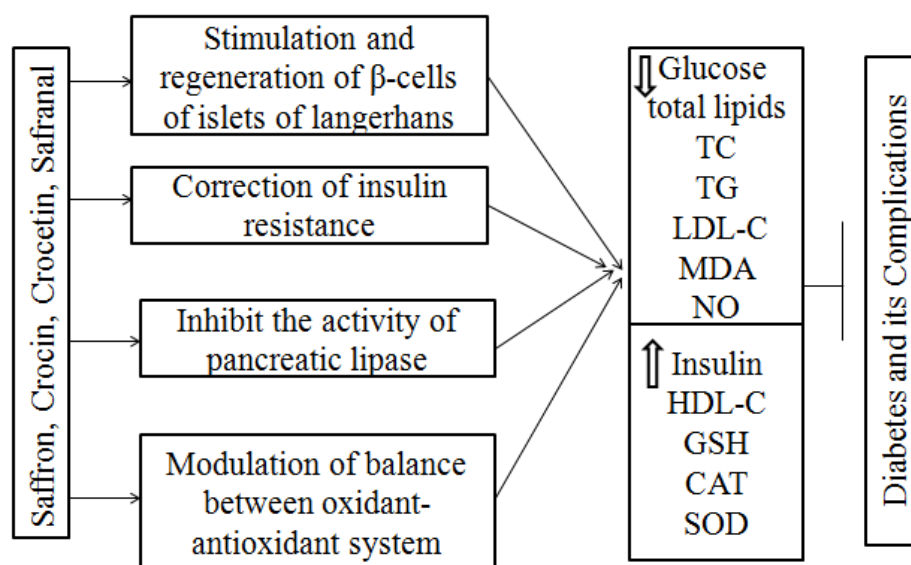
### **Dislipidemia control and arthrosclerosis management**

Dyslipidaemia describes a group of conditions in which there are abnormal levels of lipid and lipoprotein in the blood (Bhalodia et al., 2010). The level of serum lipid fractions is often increased in DM and such an elevation plays a major role in coronary artery disease. This abnormal high level of serum lipid fractions is mainly due to the decrease in the action of lipolytic hormones in the adipocyte tissue. Under normal circumstances, insulin activates the enzyme lipoprotein lipase, which hydrolysis triglycerides. However, insulin deficiency or insulin resistant in diabetic patients leads to hypertriglyceridemia and hypercholestermia by inactivating lipoprotein lipase (Sharma et al., 2003).

Diabetes mellitus usually includes lipid abnormalities such as elevated circulating levels of TG, TC, LDL-C and usually accompanied by decreased circulating levels of HDL particles (Sharma et al., 2003). During diabetes, persistent hyperglycemia causes increased production of free radicals, especially reactive oxygen species (ROS). Lipids when react with free radicals, they undergo peroxidation to form lipid peroxides. The increase in the level of ROS in diabetes could be due to their increased production and/or decreased destruction by nonenzymic and enzymic catalase (CAT), reduced glutathione (GSH),

and superoxide dismutase (SOD) antioxidants (Logani and Davis, 1979). These findings may constitute the predominant mechanism in STZ and alloxan induced complications of hyperglycemia. Several mechanisms for the hypolipidemic effects of saffron extract and its constituents have been suggested: (1) modulatory effects on the oxidant-antioxidant system (Xiang et al., 2006); (2) inhibitory effect on pancreatic lipase. It may act by reducing the absorption of fat and cholesterol through inhibiting pancreatic lipase activity (Sheng et al., 2006). In several studies, treatment animals with different concentrations of saffron showed improved lipid profile (Elgazar et al., 2013). Regarding the hypolipidemic effects of saffron, Xu et al. (2005) reported that in experimental hyperlipemic rats with 2 months feeding of heavy cholesterol, crocin decreased largely the content of cholesterol, triglyceride and density lipoprotein in blood and increased the content of high-density lipoprotein. In agree with this result, Sheng et al. (2006, 2008) indicated that crocin has lipid lowering properties and selectively inhibits the activity of pancreatic lipase as a competitive inhibitor. Moreover, He et al. (2005) found that crocin has a potent hypotriglyceridemic and hypocholesterolemic activity in atherosclerotic quails. Shirali et al. (2013) also showed that crocin significantly decreased the levels of triglyceride, total cholesterol, and low-density lipoprotein and increased the high-density lipoprotein in the diabetic rats by improving insulin resistance in the diabetic rats. Other investigations indicated that crocetin has increased insulin sensitivity and ameliorated abnormalities related to insulin resistance such as impaired glucose tolerance, hyperinsulinemia, dyslipidemia and hypertension due to high-fructose diet, high fat diet and dexamethasone injection in rats (Xi et al., 2005, 2007a; Shang et al., 2008). Crocetin attenuated the palmitate-induced insulin insensitivity in the rat adipocytes (Xi et al., 2007b).

The antioxidant effects of crocetin may, at least in part, explain the ability of this compound to attenuate insulin insensitivity. In addition, Samarghandian et al. (2013), indicated that safranal inhibits elevation of the serum lipid level by controlling oxidative and nitrosative systems. Lipids change to form lipid peroxides when it reacts with free radicals. Lipid peroxides decompose to form numerous products including malondialdehyde (Raghuvanshi et al., 2007). The toxicity of oxygen, or of its radical derivatives, is often accompanied by the peroxidation of lipids. Lipid peroxidation as induced by low-level exposures to nitrogen dioxide appears to proceed either by hydrogen atom abstraction or by nitrogen dioxide addition to the olefin. These abnormalities may be further exacerbated by the increased oxidizing environment which enhances the formation of oxidized LDLs (ox-LDLs), glycated LDL and oxysterols (formed from the oxidation of cholesterol). It has been suggested that these oxidized lipid products can bind to specific receptor proteins or activate inflammatory proteins which generate ROS



**Figure 2.** The molecular targets of diabetes and its complications modulated by saffron and its main ingredients.

(Johansen et al., 2005). The import of ox-LDLs in the vascular wall is an important mechanism by which ROS and oxidative stress induce atherosclerosis. Crocetin could also prevent progression atherosclerosis by inhibition adhesion of leukocytes to the bovine aortic endothelial cells (BEC) induced by advanced glycation end products (AGEs) and AGEs-induced BEC apoptosis possibly through its antioxidant activity and thus it has been suggested that crocetin may prevent diabetes-associated vascular complications (Xiang et al., 2006a, 2006b). Increasing evidence suggests that oxidative stress and changes in nitric oxide formation or action plays major roles in the onset of atherosclerosis. Samarghandian et al. (2013) also indicated that safranin might prevent the occurrence of atherosclerosis by reduction of serum NO content. Therefore, saffron and its main ingredients are beneficial for curing of atherosclerosis by controlling lipid profile through correction of insulin resistance, oxidant-antioxidant system and inhibition pancreatic lipase. Table 1 shows some studies that evaluate the effect of saffron, crocin, crocetin and safranin on glucose and lipid profile in diabetic model and proposed mechanisms. Figure 2 indicates the molecular targets of diabetes and its complications modulated by saffron, crocin, crocetin and safranin.

## CONCLUSION

This review highlights the effects of saffron and its main ingredients on various parameters of diabetes including unveiling potential biochemical pathways involved. Saffron

extracts influence the content of free radicals and antioxidants in treated animals, suggesting that the levels of free radicals and antioxidants are associated with the diabetic state. Saffron and its main ingredients, in addition to reducing blood glucose level in diabetic rats, lead to an increase of GSH, CAT, GST and SOD whose activities used to be decreased by diabetic conditions.

Scientists worldwide are more attracted to show that consumption of saffron positively correlates with a lower risk of diabetes and its complications, and they also studied the attribution of the large number of phytochemicals in saffron. Among these phytochemicals, crocin, crocetin, and safranin are considered the most medicinally bioactive and the most frequently examined in many *in vitro* and *in vivo* studies. Different hypotheses for the mode of antidiabetic action of saffron and its ingredients have been suggested and in detail discussed in this review. Several studies suggested that hypoglycemic and hypolipidemic effects of saffron and its main components seem to exert mechanisms including stimulation of glucose uptake by peripheral tissues inhibition of intestinal glucose, absorption inhibition of insulinase activity in both liver and kidney, inhibition of endogenous glucose production, inhibition of renal glucose reabsorption or correction of insulin resistance stimulation of  $\beta$ -cells of islets of Langerhans to release more insulin regeneration of  $\beta$ -cells islets of Langerhans.

Recently, many studies have shown that saffron and its main ingredients ameliorated the diabetogenicity of STZ and alloxan in animals by reacting with free radicals formed from STZ and alloxan during its interaction with the  $\beta$ -cell, or prevent radical formation.

**Table 1.** Effect of saffron, crocin, crocetin and safranal on glucose and lipid profile in diabetic model and proposed mechanisms.

Saffron and its ingredients	Cell lines/Animal models	Effect	Mechanism of action	References
Saffron extract	Alloxan diabetic rat	Serum glucose, TG and TC Serum insulin	Increased the number of $\beta$ -cells in pancreas, modulation of balance between oxidant-antioxidant system	Mohajeri et al. (2009), Kianbakht et al. (2011), Elgazar et al. (2013)
	Skeletal muscle cell	Glucose uptake in muscle cells	Phosphorylation of AMPK muscle cells, regeneration of $\beta$ -cells of islets of Langerhans	Kang et al. (2012)
Crocine	Alloxan and STZ diabetic rat	Serum glucose, MDA, TG, TC and LDL-C GSH and HDL-C	Modulation of balance between oxidant-antioxidant system, correction insulin resistance, inhibition of the activity of pancreatic lipase	He et al. (2005), Xu et al. (2005), Sheng et al. (2006), Kian bakht et al. (2011), Rajaei et al. (2013), Shirali et al. (2013), Tamaddonfard et al. (2013)
Crocetin	Fructose-fed rat, high-fat diet rat dexamethasone- exposed rat	Serum glucose, TG and TC Serum insulin	Stimulation and regeneration of $\beta$ -cells of islets of Langerhans, correction insulin resistance, modulation of balance between oxidant-antioxidant system	Xi et al. (2005, 2007a, b), Sheng et al. (2008)
	Bovine aortic endothelial cell	Inhibition adhesion of leukocytes to the bovine aortic endothelial cells and prevent progression atherosclerosis	Modulation of balance between oxidant-antioxidant system	Xiang et al. (2006a, b)
Safranal	Alloxan and STZ diabetic rat	Serum glucose, total lipids, TC, TG, LDL-C, MDA and NO Serum insulin, HDL, GSH, CAT and SOD	Modulation of balance between oxidant-antioxidant system	Kianbakht et al. (2011), Samarghandian et al. (2013)

To date, the exact mechanism of antidiabetic effect of saffron is not clear. However, the most medicinally bioactivity of saffron belongs to carotenoids. Carotenoids exhibit biological activities as antioxidants, and act as a membrane-associated high-efficiency free radical scavenger. Several studies pointed out the use of some of them, such as crocin, crocetin and safranal, in diabetic management. These compounds are lipid-soluble and might act as free-radical scavengers, and prevent protein, lipid and carbohydrate oxidation.

In conclusion, this review proposes that the antidiabetic activity of saffron and its compounds is more closely related to the antioxidant reinforcement, rather than to other possible mechanisms. However, present findings have not yet been verified by clinical trials in humans and

in-depth studies need to define the efficacy of saffron in diabetic management. In addition, the possible long-term toxic effects of saffron extract and protective effects of different doses also need to be determined.

#### Conflict of interest

The author(s) confirm that this article content has no conflicts of interest.

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