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Effects of Flavonoids and Antioxidants of Blackberry on Skin Whitening and Erythema

RIZWANA KAUSAR[1,*], NAVEED AKHTAR[1], HAJI MUHAMMAD SHOAIB KHAN[1] and MARIA HASSAN KIANI[2]

[1]Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Bahawalpur, Pakistan
[2]Hamdard Institute of Pharmaceutical Sciences, Islamabad, Pakistan

*Corresponding author: Tel: +92 3451062472; E-mail: rizvi_awan@yahoo.com; swaira.awan@yahoo.com

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The aim of this study is to evaluate the effects of flavonoids and antioxidants of blackberry fruit extract incorporated into newly developed emulgel on skin melanin and erythema. Blackberry extract was entrapped in o/w emulsion and then incorporated into gel to formulate emulgel. Base (without extract) and the formulation (containing 4 % blackberry extract) were applied to the cheeks of thirteen healthy female human volunteers for 12 weeks. The base showed insignificant ($P \geq 0.05$) while formulation showed significant ($P \leq 0.05$) decrease in the skin melanin and erythema content. It can conclude that the formulation can be used as skin whitening treatment without causing any skin irritation.

Keywords: Melanin, Blackberry, Erythema, Flavonoids, Antioxidants.

INTRODUCTION

Melanin, found in skin is a biological pigment produced by melanocytes in the stratum basal of epidermis. Melanin is produced by the conversion of amino acid tyrosine to dopaquinone which is catalyzed by tyrosinase enzyme. Hyperpigmentation is the most common skin disorder. Accumulation and over production of melanin causes facial disorders such as melasma, age spots and freckles. It has been revealed that most of the cosmetic products inhibit tyrosinase enzyme activity leading to skin whitening [1]. Traditional whitening agents such as kojic acid, hydroquinone and arbutin are effective depigmenting agents. However, these agents have some safety problems with long term use. Botanical extracts give an idea to formulate new cosmetic products to overcome hyperpigmentation [2]. It has been studied from previous literature that the natural agents such as flavonoids and natural antioxidant block melanogenesis. Flavonoids such as kaempferol, quercetin and anthocyanin are polyphenolic natural antioxidants present in fruits and vegetables. The most common flavonol, quercetin (Fig. 1) was formerly isolated as the common tyrosinase inhibitor [3]. When emulsions and gels are used in combined form the dosage forms are referred as emulgels. Actually, the presence of a gelling agent converts a classical emulsion into an emulgel. Emulgels for dermatological use have several favourable properties such as being greaseless, thixotropic, easily removable, easily spreadable, non-staining, emollient, water soluble, longer shelf life, bio-friendly, pleasing appearance and transparent [4].

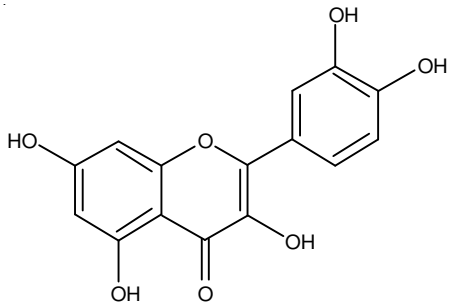

Fig. 1. Chemical structure of quercetin

Additional values can be given to emulgels by incorporating plant or fruit extracts with specific cosmeceutical effects.

Blackberry, belonging to the family *Rosaceae* is a fruit has many traditional and pharmacological uses. Blackberry fruit and leaves are traditionally used as anti-inflammatory, antiseptic, antidiabetic, anticancer, antimicrobial and antioxidant agents [5]. Blackberry is enriched in quercetin, ellagitannins, anthocyanins and gallic acid [6]. Blackberry has been used as an antioxidant, anti-inflammatory and for the treatment of skin diseases like eczema and psoriasis [6]. This study indicates blackberry actives as effective topical treatments for skin erythema and hyperpigmentation.

EXPERIMENTAL

Distilled water and methanolic extract of blackberry fruit were prepared in the laboratory of Department of Pharmacy,

The Islamia University of Bahawalpur, Pakistan. Propylene glycol, triethnolamine, liquid paraffin and methanol were obtained from Merck (Germany), Span 20, Tween 20, methyl paraben and carbopol 940 were purchased from Sigma, USA.

Electrical balance (Precisa BJ-210, Switzerland), Rotary evaporator (Eyela, Co. Ltd. Japan), refrigerator (Dawlance, Pakistan), pH-meter (WTW pH-197i, Germany), Mexameter® MPA 5 (Courage + Khazaka, Germany), visioscan® VC98 (Courage + Khazaka, Germany), homogenizer (Euro-Star, IKA D 230, Germany) and UV spectrophotometer-16 (Shimadzo Japan).

Blackberry fruit was purchased from The Metro Cash & Carry Store Islamabad, Pakistan. Identification of the fruit was made at the Botany Department of University of Sargodha, Pakistan. The specimen (Voucher# 6027) was deposited in the Laboratory of Biosystematics & Medicinal Plants, Department of Botany, University of Sargodha, Pakistan, for future research reference.

Determination of flavonoids: An efficient method of Zu *et al.* [7] with slight modification was applied for the evaluation of flavonoids in blackberry fruit. The studied sample was ultrasonified at high frequency. Ethanol (70 %) was used as extraction solvent at 30 °C for period of 1 h. Water-methanol-acetonitrile with 1 % acetic acid was used as mobile phase. Diode array detection was performed at wavelength of 368 nm for the detection of flavonoids. Flavonoids were determined with successful recoveries about 83.93 mg QE/g (milligram of quercetin equivalent/gram).

Determination of free radical scavenging activity of blackberry: The antioxidant activity of blackberry was determined using DPPH (1,1-diphenil-2-picrylhydrazyl) assay [8]. Equal concentrations of the 0.5 µL extract DPPH (6×10^{-3}) in absolute ethanol and maintained for 20 min at room temperature. The absorption of the mixture was measured spectrophotometrically at 517 nm wavelength. Ascorbic acid was used as standard. The antioxidant activity of blackberry was calculated as % inhibition according to the following formula:

$$\text{Inhibition } (\%) = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

The antioxidant activity of blackberry was 82 % in comparison to the standard. It may be attributed to the presence of natural antioxidants in blackberry fruit such as flavonoids, vitamin C, β-carotenes and tocopherols.

Preparation of emulgel: The oil phase (liquid paraffin and Span 80) was heated up to 75 ± 1 °C, aqueous phase (Tween 20, propylene glycol, methyl paraben and distilled water) heated to the same temperature. 4 % Blackberry fruit extract was added to the oil phase and then oil phase was added drop by drop to aqueous phase with continuous stirring to prepare o/w emulsion. Carbopol 940 (2 g) was dispersed in water with q.s. (quantity sufficient) to 100 mL. After complete dispersion, triethnolamine was added drop wise and after each addition pH was checked until it reached within the range of skin pH (5-6.5). Finally, prepared o/w emulsion was added gradually in prepared gel under continuous stirring at 2000 rpm by homogenizer for 15 min. Then, the homogenizer speed was reduced to 1000 rpm for a period of 5 min and then the speed further was decreased to 500 rpm for 5 min for proper homogenization.

Study protocol for emulgel evaluation on skin: A single-blinded study was designed in the month of February to April. Thirteen healthy female human volunteers who signed consent form, with ages between 25 and 30 years were selected. Prior to the dermatological tests, a dermatologist examined the volunteers for any skin disorder especially on cheeks. All the skin parameters were measured at 21 ± 1 °C and 40 ± 2 % relative humidity conditions [9]. The investigational study was carried out on the cheeks of volunteers on the first day; patch test was carried out on the forearms of each individual to determine any possible irritation to the emulgels. Each volunteer was provided with two formulations. One formulation was the base and the other formulation was active formulation containing 4 % blackberry fruit extract. Each formulation was marked with "left" or "right" indicating the application of base and active formulation respectively. Each volunteer was instructed to apply 5mg of both base as well as active formulation twice a day for the period of 12 weeks. Each volunteer was instructed to come at 2nd week, 4th, 8th, 10th and 12th week of investigational period for the skin tests.

Patch test: To evaluate skin compatibility, patch tests were performed on both forearms of each volunteer on the first day of dermatological testing. A 5 cm × 4 cm region on the forearm was marked. The patch for the right forearm was saturated with 1 g of base while the patch for the left forearm was saturated with 1 g of active formulation. The marked regions were covered with surgical dressing after application of base and active formulation. The patches were removed after 48 h and the skin of forearms was washed with physiological saline [10]. Skin observed for any irritation/redness using a 4 point scale from 0-3. Where 0 indicates absence of erythema, 1 stands for mild erythema, 2 for moderate while 3 for severe erythema as shown in Table-1.

Panel test: Each volunteer was given a questionnaire to assess the sensory values of the formulations. This performa was asked to be completed at the end of study by each volunteer. This questionnaire comprised of seven parameters to be assessed and every parameter assigned 11 values from -5 to +5 representing very bad to very good, respectively. The seven parameters were 1, spreadibility; 2, ease of application; 3, sense just after application; 4, sense in long term; 5, shine on skin; 6, sense of softness; 7, irritation or redness. From the average

TABLE-1
SCORE GIVEN BY VOLUNTEERS TO BASE AND ACTIVE FORMULATION ON THE BASIS OF IRRITATION/ITCHING*

Score		0	1	2	3
Number of Volunteers	Base	9	2	2	0
	Formulation	12	1	0	0

*No severe erythema occurred in any of volunteer, mild erythema occurred in 2 and 1 volunteers, moderate erythema occurred in 2 and 0 volunteers, whereas no erythema occurred at all in 9 and 12 volunteers for both base and active formulation, respectively.

reply of volunteers it was found that base and active formulation caused no irritation on the skin, as these were assigned 0.00 score for irritation by all the volunteers. Softness of the skin and shine on the skin were more for active formulation than base. With the help of paired sample t-test it was found that there was an insignificant difference between the average points of sensitivity for active formulation and base. From patch test it was observed that there was no great variation between the base and active formulation regarding the sensory point of view.

Ethical approval: This investigational study was approved by the Pharmacy Research Ethics Committee (PREC) for *in vivo* studies (Ref. No. 66-2015/PREC), The Islamia University of Bahawalpur, Pakistan and was directed according to the international guidelines of Helsinki Declaration.

Mathematical and statistical analysis: The percentage changes with respect to zero hour value of volunteers for different parameters, measured at 2nd, 4th, 6th, 8th, 10th or 12th week were calculated using following formula;

$$\text{Change}\ (\%) = \left(\frac{(A-B)}{B}\right)\times 100$$

where, A = individual value on any parameter at 2nd, 4th, 8th, 10th and 12th week. B = zero hour value of that parameter.

The measured values observed for different parameters (skin melanin and erythema) were evaluated using SPSS 20.0 on a computer. Paired samples t-test was used for variation between the two formulations; two-way ANOVA was used for variation between different time intervals at 5 % level of significance.

RESULTS AND DISCUSSION

Skin melanin: The percentage change in the skin melanin contents after the application of active formulation respective its base have been shown in Fig. 2.

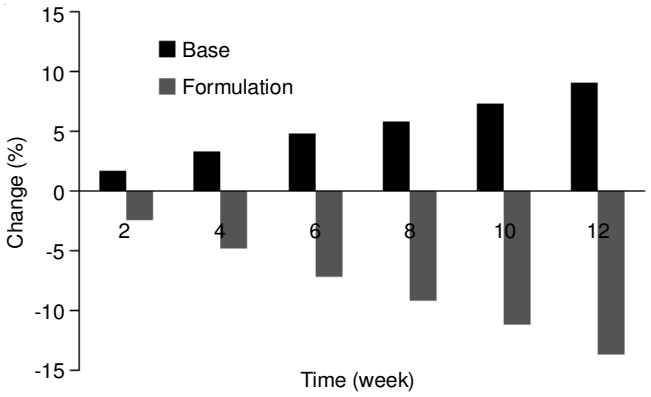

Fig. 2. Percentage of change in skin melanin content after the application of active formulation and respective base

Skin melanin: In this investigational study, the effects of active formulation *versus* its base on melanin contents were evaluated. The percentage change in skin melanin was measured for 12 weeks at different time intervals in each volunteer. After application of base it was observed that there was gradual increase in melanin throughout the study period. While in case of formulation there was continuous decline in the skin melanin throughout 12 weeks of study time (Fig. 2). By applying ANOVA test it was found that base showed insignificant ($P \geq 0.05$) while formulation showed significant ($P \leq 0.05$) effects with respect to time. With the help of paired sample t-test, significant differences were found between the melanin effects of base and formulation from the 3rd week till the end of investigational study.

It has been revealed from previous literature that flavonoids are important tyrosinase inhibitors. The tyrosinase inhibitory activity of the flavonoids may be linked to chelating the active center of tyrosinase enzyme leading to decreased melanin production [11]. The decline in skin melanin might be due to the flavonoids such as quercetin, anthocyanins and kaempferol and good free radical scavenging activity of blackberry fruit.

Skin erythema: The percentage change in the skin erythema contents after the application of active formulation respective its base have been shown in Fig. 3. In this study, the effects of active formulation with respect to base on skin erythema were assessed. The percentage change in skin erythema was measured for 12 weeks at different time intervals in each individual. After application of base it was observed that there was irregular increase in erythema throughout the study period. While in case of formulation there was gradual decrease in the skin melanin throughout 12 weeks of study time (Fig. 3). By applying ANOVA test at 5 % level of significance it was observed that base showed insignificant ($P \geq 0.05$) while formulation showed significant ($P \leq 0.05$) effects with respect to time. With the help of paired sample t-test, significant differences were found between the erythema effects of base and formulation from the 2nd week till the end of study period.

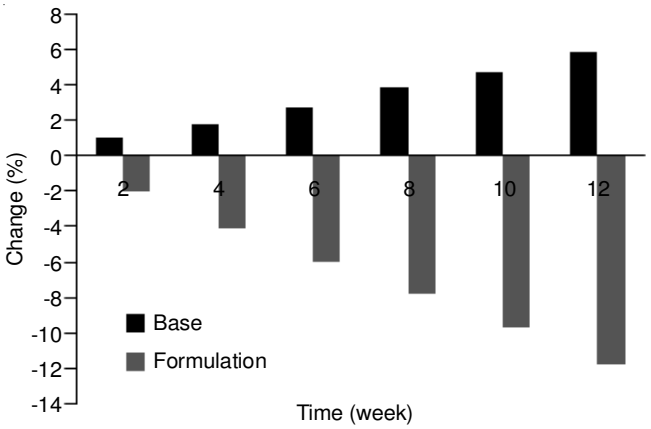

Fig. 3. Percentage of change in skin erythema content after the application of active formulation and respective base

Damage of skin proteins, cellular lipids and DNA by free radical agents are considered to be the major cause of erythema. β-Carotene and tocopherols are very effective free radical scavengers [12]. Blackberry is enriched in β-carotene and tocopherols. More decrease in erythema content after application of formulation can be due to the anti-inflammatory properties exhibited by blackberries [13].

Conclusion

From the results, it is concluded that a stable topical emulgel containing blackberry fruit extract produced a pronounced

decline in skin melanin content indicating that the formulation has skin whitening properties. The formulation was found to decline skin erythema significantly which shows that formulation has anti-inflammatory effects. A targeted study needs to be performed in future in patients with melasma, inflammation, eczema and freckles so that actual potential of this fruit against these skin disorders can be explored.

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