

Whitening efficacy of plant extracts including *Hippophae rhamnoides* and *Cassia fistula* extracts on the skin of Asian patients with melasma

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Abstract

Introduction: Melasma/hyperpigmentation and solar damage of the skin remains a difficult problem to treat. Various types of whitening agents are used to treat hyperpigmentation. A change has been observed recently to use plant extracts as skin whitening agents.

Aim: To compare the effectiveness of emulsion formulations containing plant extracts that include catechins/polyphenols and placebo without plant extracts, on patients with melasma.

Material and methods: Two groups of 25 patients each (aged 21–35 years), who reported to the outpatient department of BV Hospital and Personal clinic of a dermatologist, were included in the study. Volunteers applied the formulations with plant extracts and placebo to one side of the cheek. Prior to the study, signed consent was obtained from each patient. The tyrosinase inhibitory activity of the extracts and formulations was tested *in vitro*. The pigment density of patients was evaluated biometrologically using Mexameter® and subjectively using a visual survey before and after treatment of 12 weeks. The approval of the Institutional Ethics Committee of Faculty of Pharmacy, the Islamia University of Bahawalpur was obtained before the study. One-way ANOVA and Kruskal-Wallis tests were used in the statistical analysis.

Results: A significant decrease in the level of melanin was determined in all 50 patients who used a plant extract containing catechin ($p \leq 0.05$). The difference between pre- and post-treatment levels of melanin was statistically significant ($p = 0.05$). Formulations prepared with plant extracts containing catechin were found effective on melasma, compared to the placebo.

Conclusions: Formulations containing plant extracts that are not yet being used widespread commercially on melasma could be an effective alternative treatment of melasma.

Key words: mexameter, melasma, *Hippophae rhamnoides*, *Cassia fistula*.

Introduction

Melasma is a general disorder of hyperpigmentation and usually involves the face and neck. Hyperpigmentation is particularly common in darker complected patients and is often hard to treat [1]. Skin-lightening agents are used to treat hyperpigmentation. Researchers have discovered and conducted various *in vivo* and *in vitro* studies on skin-lightening agents. Arbutin, hydroquinone and several plant extracts are tried to achieve the skin-lightening effect

and are being used in cosmetics. Skin-lightening agents inhibit the conversion of tyrosine to melanin by the enzyme tyrosinase, thus tyrosinase is the rate-limiting enzyme in melanin synthesis. Tyrosinase is a copper-containing enzyme that catalyzes melanin biosynthesis in three steps: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), oxidation of DOPA to DOPA quinone and oxidation of 5,6-dihydroxyindole to indolequinone. Tyrosinase inhibitors play a vital role as skin-lightening agents [2].

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Many plant extracts have a good inhibitory effect on melanin formation and may be a good choice for cosmetic purposes of whitening facial skin and defense against skin darkening. In addition, they have comparatively less side effects [3]. In cosmetic preparations, many plant extracts such as *Morus alba*, *Acacia bark*, *Glycyrrhiza glabra* and *Green tea* have been used as whitening agents [4–7].

Cassia fistula L, also known as the Golden Shower, is widely used in various countries as an ornamental tree for its beautiful bunches of yellow flowers. Recognized by the British Pharmacopoeia [8], *C. fistula*, a member of the Leguminosae family, is extensively used for its medicinal properties. In the Indian literature, this plant has also been described to be useful in skin diseases [9, 10]. *Cassia fistula* is a medicinal plant acknowledged to be rich in phenolics, containing catechin, epicatechin, Kaempferol, ellagic acid (EA) and Rhein [11].

The medicinal value of *Hippophae rhamnoides* was found more than 1000 years ago, but it has been used for making medicines and healthy products by using modern science and technology only for the last 30 years [12]. Various catechin like (+)-gallocatechin and (–)-epigallocatechin have been isolated from *H. rhamnoides* berries [13].

Aim

The aim of this study was to investigate the effects of formulations containing phenolic-rich (catechin) extract of *C. fistula* and *H. rhamnoides* *in vitro* and *in vivo*.

Material and methods

Plant materials and preparation of plant extracts

Cassia fistula pods were collected from the Islamia University of Bahawalpur in August. *Hippophae rhamnoides* berries were purchased from Pak Sea Buckthorn International Skardu, Pakistan in May. The identification of the plant materials was performed by a taxonomist, Prof. Dr. Muhammad Arshad at the Cholistan Institute of Desert Studies (CIDS), the Islamia University of Bahawalpur and voucher specimens were preserved (voucher # OB-LF-4-11-21 and HR-FT-03-11-23) at the herbarium of the Faculty of Pharmacy, Islamia University of Bahawalpur.

Plant materials were air-dried and then powdered. Each powdered plant was extracted with 70% methanol solution, and after filtration, the filtrates were evaporated under vacuum. These extracts were used for further studies of tyrosinase inhibition and emulsion formulations.

Drugs and chemicals

Methanol, catechin and gallic acid were obtained from Sigma (St Louis, MO, USA). Paraffin oil, Stearic acid, Tween80, Span20, Bees wax and Cetomacrogol were taken from Merck (Germany).

In-vitro tyrosinase inhibitory activity of plant extracts

Tyrosinase inhibitory activity is usually determined using a spectrophotometer. The procedure is briefly described here: a reaction mixture of 100 μ l, 40 μ l 100 mM phosphate buffer, pH 6.8, 20 μ l mushroom tyrosinase enzyme (6 units) and 10 μ l 0.5 mM test compound mixed in 96-well plate. The mixture was pre-incubated at 37°C for 5 min. Thirty μ l of 10 mM L-dopamine (substrate) was added. Contents were mixed and incubated for 10 min more. Absorbance was taken at λ 490 nm using Synergy HT BioTek 96-well plate reader. The percent enzyme inhibition was calculated as: %Inhibition = 100 – (Abs of test sample/Abs of control \times 100).

We determined the effect of the test compound on tyrosinase inhibition by IC₅₀ using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc., Amherst, USA).

Preparation of formulations

For investigation of the tyrosinase inhibitory effect of formulations, an oil-in-water (O/W) type emulsion (F1/F2) containing plant extracts and a placebo (F3) without plant extracts were prepared. The composition of the formulations is given in Table 1. The formulations were found stable for pH, centrifugation, electrical conductivity, temperature stability tests at 8 \pm 0.1°C, 25 \pm 0.1°C, 40 \pm 0.1°C and 40 \pm 0.1°C with 75% relative humidity, physical nature i.e. color, creaming and liquefaction and rheological characteristics.

In-vitro tyrosinase inhibitory activity of formulations

Ozer *et al.* method with a slight modification was applied for *in-vitro* tyrosinase inhibitory activity of formulations [14]. Approximately 1.5 gm of F1, F2 and F3 was weighed accurately into a centrifuge tube. Ten ml of methanol (50%) was added and stirred in an ultrasonic bath for 30 min. The suspension was centrifuged at 5000 rpm for 10 min. It was further diluted at a ratio of 1 : 10 with 50% methanol and tested by the dopachrome method for determination of tyrosinase inhibitory effect.

Table 1. Composition of formulations (% w/w)

| Composition | F1/F2 | F3 |
|-----------------|-------|-----|
| Liquid paraffin | 24 | 24 |
| Stearic acid | 5 | 5 |
| Span 20 | 2 | 2 |
| Bees wax | 7 | 7 |
| Cetomacrogol | 5 | 5 |
| Tween 80 | 6 | 6 |
| Plant extract | 5 | Nil |
| Preservative | 1 | 1 |
| D/W | 45 | 50 |

Subjects

Two groups of 25 healthy subjects each (aged 21–35) were enrolled in this study after their informed consent was obtained. All volunteers completed the study successfully. None of them had any pathological condition on their cheeks. They were sufficiently informed about the use of products. Moreover, they were told not to use any other skin products, especially whitening agents, throughout the 12-week duration of the study.

In-vivo study design

A single blinded, placebo-controlled study was conducted to assess the effects of formulations on the improvement of the skin melanin (anti-melasma effects). This study was conducted in October-December and an expert investigator ensured the proper handling of biometrological measurements, allowing for obtaining uniform results. Tests were carried out on both the right and left cheeks of volunteers. Measurements were carried out at baseline and on week 1, 2, 3, 4, 6, 8, 10 and 12.

Approximately 500 mg of formulations were applied to the cheeks twice daily (mornings and evenings) over a period of 12 weeks. Prior to all measurements, volunteers were asked for at least 15 min wait in order to allow for complete skin adjustment to the room temperature.

Ethical considerations

The institutional ethics committee of the Faculty of Pharmacy, the Islamia University of Bahawalpur approved the protocol of this study (No. 942/Acad). Before enrollment and inclusion of a volunteer in this study, signed informed consent was obtained from each volunteer.

Instrumental measurements

Biometrologically, a Mexameter® (Courage & Khazaka, Germany) with noninvasive probes was used in this study for assessing the skin melanin. Mexameter® is a narrow-band reflectance spectrophotometer, designed to measure the intensity of skin melanin. It has mainly been used for cosmetic research but recently it has also been used to assess hyperpigmentation of skin (melasma).

Table 2. Tyrosinase inhibition activity of *H. rhamnoides* and *C. fistula* extracts

| Plant extracts | Tyrosinase inhibition | |
|----------------------|----------------------------------------------|-----------------------|
| | Average inhibition percentage (%) at 0.05 mg | IC ₅₀ [µg] |
| <i>H. rhamnoides</i> | 72.57 ± 0.34 ^a | 45.9 |
| <i>C. fistula</i> | 77.64 ± 0.62 ^b | 39.2 |
| Control | Kojic acid | 6.00 ± 0.01 |

All values were based on three different samples. All assays were determined in triplicates per run and data presented as mean ± SD. For column of each sample, values followed by the same letter are not significantly different at $p < 0.05$

Visual survey

After 12 weeks of treatment, a visual survey was carried out to indicate 3 parameters: change in photograph, change in size and change in pigmentation. Photographs of only those volunteers, who allowed openly for reproduction, were taken.

Mathematical analysis

The percentage changes in melanin after every week for each volunteer were calculated by using the following formula: %Change = $[(Tx - T0)/T0] \times 100$, where: T0 – baseline values, Tx – value obtained every week.

Statistical analysis

The measured values were analyzed using GraphPad Prism version 5 statistical software. A paired sample *t*-test for variation between the active formulation and placebo was performed. Standard Parametric one-way ANOVA with Dunnett Multiple Comparisons Post Test was used to observe the correlation between baseline values and the values at different weeks. Values of *p* of less than 0.005 were considered statistically significant.

Patch Test (Burchard Test)

Prior to sampling, a patch test was performed to find any possible sensitivity of formulations on forearms of each volunteer. A specified area was marked on the forearms. Placebo was applied to the right forearm while the active formulation was applied to the left forearm. The areas were covered with surgical dressing (Patches) saturated with formulations. After 48 h, the patches were removed and the forearms were washed with saline. Scores were noted for the presence of itching/irritation using a 4-point scale ranging from 0 to 3; where 3 stands for severe response, 2 for moderate, 1 for mild and 0 stands for no response. Each volunteer was requested to note the degree of itching/irritation and allocate a score from the same scale.

Results

In-vitro tyrosinase inhibitory activity of plant extracts and formulations

Table 2 summarizes the results of anti-tyrosinase inhibition activity of plant extracts, where inhibition is expressed as IC₅₀ values. Results indicate that *H. rhamnoides* had slight poor antityrosinase activity (72% inhibition at 0.05 mg/ml concentration) as compared to *C. fistula* extracts (77% inhibition at 0.05 mg/ml concentration). Evaluation of the formulations on tyrosinase inhibition confirmed that the active formulations (F1 and F2) containing plant extracts at 5% concentration have the inhibitor activity. Placebo without any plant extract was used as a control sample, and no inhibitory effect was observed. Tyrosinase inhibitory effects of formulations are given in Table 3.

Patch test

There was no severe response of itching/irritation in any volunteer, mild response for formulation F1 and F2 occurred in 4 volunteers each, moderate erythema occurred in 2 and 4 volunteers, whereas no itching/irritation occurred in 19 and 17 volunteers, respectively, as shown in Table 4.

In-vivo tyrosinase inhibitory activity of formulations (mexametric evaluation)

The percent changes for the skin melanin following the 12 weeks' treatment with F1 and F3 along with statistical analysis are shown in Figure 1. The percent changes for the skin melanin following the 12 weeks' treatment with F2 and F3 along with statistical analysis are shown in Figure 2.

In the case of F1 and F3, it was noted that F3 has shown a mean initial change in melanin (2.52%) after the 1st week and the effect remained on the increase after the 12th week up to 5.8% compared to the baseline melanin values of the 25 volunteers.

In contrast to the F3 treatment effects, an excellent reduction in the skin melanin was noticed after treatment with F1, i.e. -3.5% mean initial reduction in melanin after the 1st week of treatment, -7.0% reductions after 1 month, and -16.35% reductions in melanin at the end of the 12th week compared to baseline values. When one-way analysis of vari-

Table 3. Tyrosinase inhibitory effect of formulations (n = 3)

| Formulation | Tyrosinase inhibitory effect [%] |
|-------------|----------------------------------|
| F1 | 58.6 ±0.425 |
| F2 | 64.0 ±0.107 |
| F3 | 0 |

F1 – Formulation containing *H. rhamnoides* extract, F2 – formulation containing *C. fistula* extract, F3 – placebo

ance, ANOVA (Kruskal-Wallis test) was applied, there was a high significant ($p < 0.0001$) difference between F1 and F3. When Dunn's multiple comparison tests were applied, there were insignificant ($p > 0.05$) differences between various time intervals.

F2 produced a noticeable reduction in melanin with an initial mean reduction of -7.07% after the 1st week and the effect was maintained until the 12th week up to -13.0%, compared to the baseline skin melanin values of the 25 volunteers. When one-way analysis of variance, ANOVA (Kruskal-Wallis test) was applied, there was a significant ($p < 0.0001$) difference between F2 and F3. When Dunn's multiple comparison tests were applied, there were insignificant ($p > 0.05$) differences between various time intervals except the 2nd and 4th week and the 1st and 12th week, where significant results were obtained.

Table 4. Score allocated by volunteers to formulations on the basis of itching/irritation

| Formulation type | Score | | | |
|------------------|--------------------------------------------------------|---|---|---|
| | 0 | 1 | 2 | 3 |
| | <i>No. of volunteers indicating itching/irritation</i> | | | |
| F1 | 19 | 4 | 2 | 0 |
| F2 | 17 | 4 | 4 | 0 |

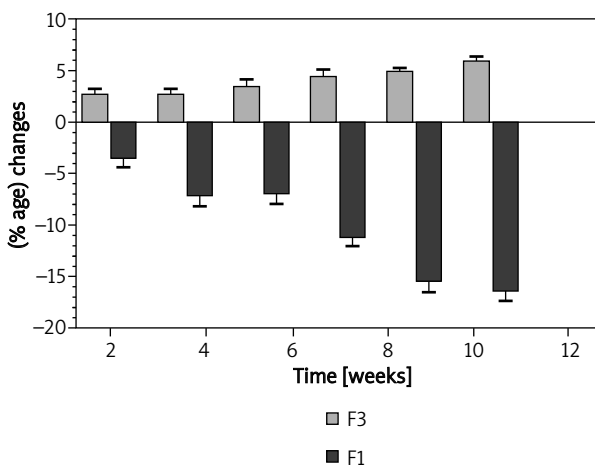


Fig. 1. Percentage of changes produced in skin melanin after the application of F3 (placebo) and F1 (*H. rhamnoides*)

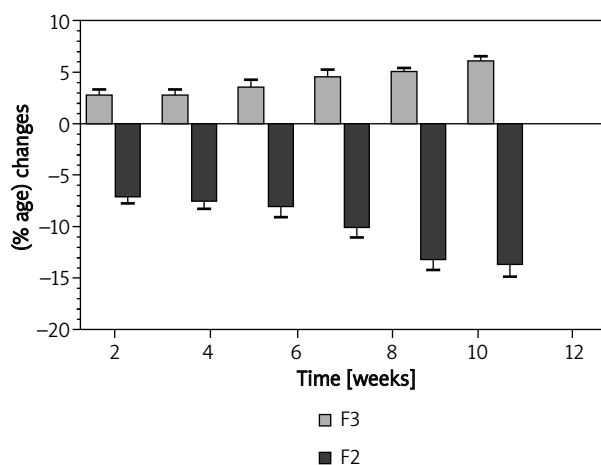


Fig. 2. Percentage of changes produced in skin melanin after the application of F3 (placebo) and F2 (*C. fistula*)

Table 5. Visual survey of formulations

| Parameter | Condition | F1 (n = 25) | F2 (n = 25) | F3 (n = 25) | Value of p |
|------------------------|--------------|-------------|-------------|-------------|------------|
| Change in photograph | Improved | 14 | 19 | 0 | 0.08 |
| | Not improved | 10 | 4 | 44 | |
| | Deteriorated | 1 | 2 | 6 | |
| Change in size | Improved | 18 | 20 | 0 | 0.057 |
| | Not improved | 5 | 4 | 40 | |
| | Deteriorated | 2 | 1 | 10 | |
| Change in pigmentation | Improved | 18 | 19 | 0 | 0.055 |
| | Not improved | 6 | 5 | 36 | |
| | Deteriorated | 1 | 1 | 14 | |
| Net result | Improved | 21 | 22 | 0 | 0.061 |
| | Not improved | 3 | 2 | 42 | |
| | Deteriorated | 2 | 1 | 8 | |

Visual survey

Subjectively using a visual survey, the number of volunteers who responded to various parameters is shown in Table 5 for F1, F2, F3. In the paired sample *t*-test, an insignificant difference between the three conditions was observed, which showed that there was no variation between the active formulations.

Discussion

Hyperpigmentation is one of the most common skin disorders. An increase in melanin synthesis or an irregular dis-

tribution of melanin results in hyperpigmentation or spots, especially on the face. Melasma is an example of pigmentation observed mainly in females, though in Asia it is commonly seen in males as well. It is more common in South Asian countries due to skin type IV to V (darker complexion) and increased UV radiation. Treating melasma is challenging and the main treating agent is hydroquinone. The major problem with high concentration (above 4%) of hydroquinone is contact dermatitis. Though various other treatment options are also available, all are very costly with delayed results [15].

Various types of whitening agents are used to treat hyperpigmentation. These agents include arbutin, hydro-

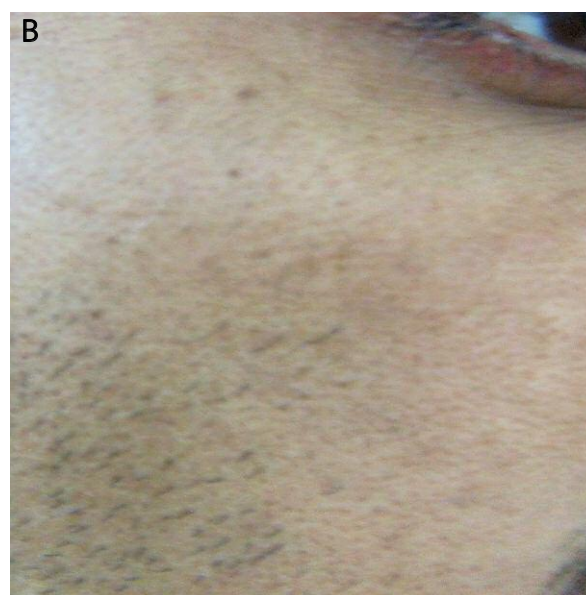


Fig. 3. A – Baseline photo of a patient treated with F1. **B** – Clinical improvement seen after 12 weeks of therapy with F1



Fig. 4. A, B – Baseline photos of patients treated with F2. **C, D** – Clinical improvement seen after 12 weeks of therapy with F2

quinones, azelaic acid and kojic acid. Though these agents are effective, they have side effects. A change has been observed recently to use plant extracts as skin-whitening agents in different types of formulations [16].

It has been reported by several researchers that plants rich in alkaloids, flavonoids, unsaturated fatty acids (linoleic acid, oleic acid) and mineral have anti-tyrosinase activity [17, 18]. Similarly, catechins, including (+)-gallic acid, (-)-epicatechin, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) are studied for anti-tyrosinase activity [19].

Both plants, *H. rhamnoides* and *C. fistula*, are reported to contain catechins, gallic acid, flavonoids, quercetin, organic acids (linoleic acid, oleic acid). The reduction in skin

melanin (anti-melasma effects) can be credited to these constituents present in F1 and F2.

Conclusions

The good agreement between the results of *in-vitro/in-vivo* evaluations and those of visual surveys showed that the plant extracts demonstrate efficacy in comparison to placebo in whitening the skin as well as melasma on the face of patients. The plant extracts, which demonstrated a significant tyrosinase inhibitory activity, were described for the first time to possess this biological property. In Pakistan, where sun exposure is severe, the pos-

sibility of developing melasma is higher. Instead of expensive and risky treatments, there is a demand for natural remedies which are cheaper and effective. *H. rhamnoides* and *C. fistula*, therefore, offer a possible solution to the problem.

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