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Evaluation of tyrosinase inhibitory potential in flowers of *Cassia auriculata* L. for the development of natural skin whitening formulation

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ABSTRACT

Introduction: The inhibition of tyrosinase has been a long-term target in the field of cosmetics for skin whitening purposes. Since the number of reports on issues related to toxicity of currently employed synthetic formulations is alarmingly increasing, there is an increased interest for plant based natural skin whitening agents. Therefore, this study focuses on the evaluation of skin whitening effect of the hydromethanolic extract prepared from the flowers of *Cassia auriculata* L., a plant widely employed in traditional medicine in Sri Lanka to improve complexion.

Methods: The skin whitening effect of the extract was determined by in vitro tyrosinase inhibitory assay while the antioxidant activity was evaluated by DPPH assay. Thereafter a topical formulation was developed using this extract and the tyrosinase inhibitory potential was further investigated.

Results: *C. auriculata* extract has displayed a strong tyrosinase inhibition with an IC_{50} of 42.49 µg/mL, while the IC_{50} value of the positive control (ascorbic acid) was determined as 33.70 µg/mL. The formulation developed from this extract has also displayed a potent inhibition of the enzyme with an IC_{50} of 70.70 µg/mL which was extremely remarkable than the commercial preparation of kojic acid. In addition, *C. auriculata* extract has exhibited a strong antioxidant activity with an EC_{50} value of 19.99 µg/mL, suggesting a possible correlation between tyrosinase inhibition and antioxidant activity.

Conclusion: The preliminary findings reveal that *C. auriculata* has a high potential to be used as a natural skin whitening agent due to the inhibition of tyrosinase enzyme as well as the strong antioxidant activity.

1. Introduction

The natural aspiration of humans to look beautiful has increased the awareness about skin and its colour. Most of women in Asian countries wish to have a fair skin because it is regarded as a very desirable feature. However, the majority of the South-East Asian population has a darker, ethnic skin which is mainly caused due to daily exposure to strong sunlight [1]. Therefore, many cosmetic companies develop skin-whitening cosmetic preparations to satisfy the desire of keeping the skin white.

Melanin is the major pigment which is responsible for determining the color of the human skin and is synthesized in melanocytes in the basal layer of the epidermis, within membrane bound organelle, 'melanosome' [2]. This process is known as "melanogenesis" and it starts from hydroxylation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA) and subsequent oxidation of L-DOPA to Dopaquinone [1]. Tyrosinase (EC 1.14.18.1) is the key enzyme which catalyzes the initial step of mammalian melanogenesis and in skin health research and cosmetics, inhibition of tyrosinase has been a long-term target [3]. Despite the significant number of reports on tyrosinase inhibitors of natural and synthetic origin, only a few of them are marketed as skin-whitening agents due to various safety concerns [4]. Thus, as a safer alternative, there is an increased interest for plant based natural skin whitening ingredients and several plant species such as *Aloe vera, Carica papaya, Cinnamomum zeylanicum, Curcuma longa, Rosa alba, Syzygium aromaticum* [1,5–7] etc., have been already identified with anti-tyrosinase activity. Sri Lanka is a country with a rich history of ethno-pharmacological practices where a large number of plant species have been utilized in the traditional medicine to enhance the complexion and to treat various dermatological diseases [8]. However, there is a dearth

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of scientific information on skin whitening potential of Sri Lankan medicinal plants apart from the study conducted to evaluate the anti-tyrosinase activity in *Camellia sinensis* [9]. In order to fill this knowledge gap, the present study has focused on evaluation of skin whitening potential of *Cassia auriculata* that has been extensively utilized in Sri Lanka for enhancing complexion and as a dermatological therapeutic. The ethnopharmacological usage of this plant species could also be rationalized with these findings.

2. Materials and methods

2.1. Plant material

Flowers of *C. auriculata* (Fabaceae) were collected from local cultivations in Sabaragamuwa province of Sri Lanka in 2016. The plant was identified by the author (MTN) and the descriptions given in the books "A Revised Handbook to the Flora of Ceylon-M.D. Dassanayake & F.R. Fosberg" and "Medicinal Plants (indigenous and exotic) used in Ceylon-D.M.A. Jayaweera" were used to confirm its identity. Further, the plant material was sent to the National Herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka for authentication. A voucher specimen (MN_CA_1605) was deposited in the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka for future references.

2.2. Preparation of the crude extract

Since our preliminary investigations on aqueous and organic extracts of *C. auriculata* did not reveal a significant tyrosinase enzyme inhibitory potential, the experiment was designed to access the tyrosinase inhibitory activity of the hydromethanolic extract.

The plant materials were thoroughly washed with running water and thereafter, dried in shade (30 °C) for six days. Dried flowers were powdered using a domestic grinder (Singer, model KA-MIXEE, India). The powdered material (15 g) was extracted in 300 mL of 70% methanol-water. The extract was evaporated into dryness with a rotary evaporator (HS-2005V-N, South Korea).

2.3. Anti-tyrosinase assay for the hydromethanolic extract of C. auriculata

The plant extract was dissolved in 50 mM potassium phosphate buffer (pH 6.5) and tested for the tyrosinase inhibition at concentrations of 166.6, 83.3, 41.6, 20.8 and 10.4 µg/mL at the 96-microwell plate as described by Curto et al. and Nerya et al. with slight modifications [10,11]. In brief, the extract (70 µL) was mixed with tyrosinase (30 µL, 333 units/mL in phosphate buffer). The mixture was incubated at room temperature (37 °C) for 10 min. Then the substrate, L-tyrosine (110 µL, 2 mM) was added to each well. The reaction mixture was incubated again at room temperature (37 °C) for 30 min. The absorbance was measured at 492 nm (Thermo Scientific-Multiscan Go Microplate spectrometer). The percentage inhibition of tyrosinase activity was calculated as follows [12].

$$\text{Percentage Inhibition} = (A - B) / A \times 100$$

where, A = absorbance without the test sample (control), B = absorbance with the test sample.

As the positive control, ascorbic acid was used. The experiments were carried out in triplicate and IC_{50} value was calculated using Graph-Pad Prism version 6.01.

2.4. DPPH radical scavenging capability

The method described by Blois 1958 [13] was employed with slight modifications to determine the radical scavenging capacity of the above extract. In brief, the reaction mixture consisting of test extract/positive control (at concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 µg/mL) and the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution, was incubated in the dark for 30 min. Thereafter, the absorbance was measured at 517 nm (Thermo Scientific-Multiscan Go Microplate spectrometer). The percentage antioxidant activity (AA) was calculated using the following formula and the EC_{50} was determined using Graph-Pad Prism version 6.01.

$$AA\% = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100$$

Bulyted hydroxyanisole (BHA) and ascorbic acid were used as positive controls. All the measurements were carried out in triplicate.

2.5. Preparation of topical formulation and evaluation of tyrosinase inhibition

The hydromethanolic extract was incorporated into the aqueous cream base (cetostearyl alcohol, white soft paraffin wax, sodium lauryl sulphate as ingredients) at different ratios and the ideal ratio of the extract and the cream base was determined based on the spreadability, homogeneity and grittiness of the formulation.

Thereafter the tyrosinase inhibitory potential of the formulation was evaluated against the commercial preparation of kojic acid as the positive control following the same procedure mentioned in 2.3. Similarly, the aqueous cream base that consists of cetostearyl alcohol, white soft paraffin wax and sodium lauryl sulphate was also subjected to the above assay as the negative control of this study.

2.6. Statistical analysis

All the above experiments were peformed in triplicate and the values were given as mean ± SD.

3. Results

3.1. Tyrosinase inhibition by the hydromethanolic extract

The concentration-response studies have indicated an extremely potent inhibition of the enzyme by the hydromethanolic extract (Fig. 1) with an IC_{50} value of 42.49 µg/mL. This potency is comparable with the positive control, ascorbic acid (IC_{50} = 33.70 µg/mL).

3.2. DPPH radical scavenging capability

The radical scavenging potential was extremely high in the *C.*

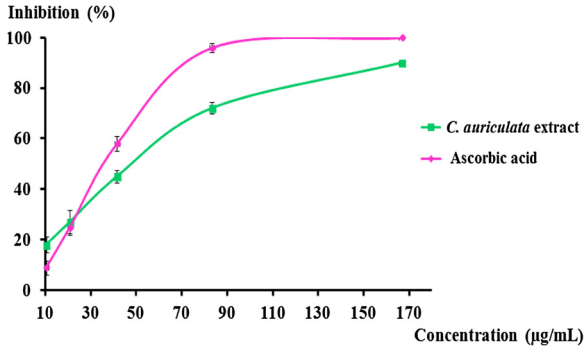

Fig. 1. Concentration- response curve for the crude extract of *C. auriculata* and ascorbic acid.
The crude extract of C. *auriculata* or ascorbic acid (70 µL) was mixed with tyrosinase (30 µL, 333 units/mL). After incubation of 10 min, 2 mM L-tyrosine (110 µL) was added and incubated further for 30 min. Thereafter the absorbance at 492 nm was measured and the percentage inhibition of tyrosinase activity was calculated. Data are means ± S.D, n = 3.

Fig. 2. Appearance of the formulation developed from *C. auriculata*.
The extract was incorporated into the aqueous cream base at different ratios to obtain a formulation with a good spreadability.

auriculata extract according to the DPPH assay. The EC_{50} value for this extract (19.99 µg/mL) was better than one of the two positive controls, BHA (EC_{50} = 23.12 µg/mL) and also comparable with the other positive control, ascorbic acid (EC_{50} = 18.03 µg/mL).

3.3. Preparation of topical formulation and evaluation of tyrosinase inhibition

The formulation appeared in chocolate brown color (Fig. 2) with a good spreadability.

The herbal formulation also exhibited a strong inhibition of the enzyme with an IC_{50} value of 70.70 µg/mL. Interestingly, the inhibition of the enzyme by the commercial preparation of kojic acid was very weak (Fig. 3) although it is widely popular as a skin whitening agent. Moreover, the negative control was not able to inhibit the enzyme even at 166 µg/mL, the highest concentration used in this assay.

4. Discussion

Cosmetics could be considered as one of the essential commodities in the modern society and widely used to alter the appearance and enhance beauty and attractiveness of a person. Even before the onset of the modern cosmetic industry, herbal preparations have been extensively utilized in the traditional systems of medicine in Sri Lanka to improve complexion and to treat various dermatological conditions. However, there has been hardly any scientific report on the skin whitening potential of Sri Lankan medicinal plants to rationalize the ethnopharmacological significance of these herbal preparations. Therefore, to the best of our knowledge, this is the first report to reveal the skin whitening potential of a popular traditional medicinal plant in Sri Lanka in the light of science, that aimed towards the development of a topical formulation. Thus the inhibition of tyrosinase was extensively evaluated as this enzyme is considered to be the key enzyme in the mammalian melanin biosynthesis.

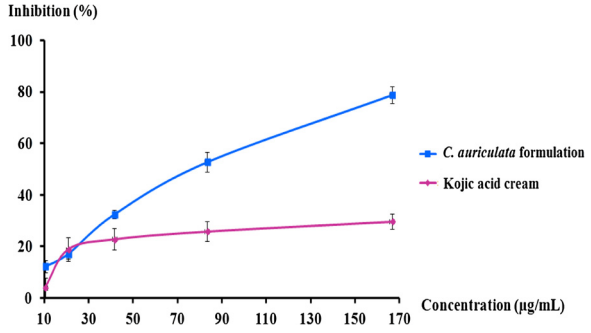

Fig. 3. Concentration-response curve for the formulation developed from the crude extract of *C. auriculata* and Kojic acid cream.
The formulation prepared from *C. auriculata* or commercial preparation of kojic acid (70 µL) was mixed with tyrosinase (30 µL, 333 units/mL). After incubation of 10 min, 2 mM L-tyrosine (110 µL) was added and incubated further for 30 min. Thereafter the absorbance at 492 nm was measured and the percentage inhibition of tyrosinase activity was calculated. Data are means ± S.D, n = 3.

There are different classes of tyrosinase inhibitors, for example, reducing agents, *O*- dopaquinone scavengers, specific tyrosinase inactivators and specific tyrosinase inhibitors etc. [14]. Ascorbic acid is well known as a skin whitening agent, although there are controversial views on the effect of ascorbic acid on the action of tyrosinase. Some authors suggested that specific structures of the enzyme in which copper is present were affected by ascorbic acid while others suggested that ascorbic acid is a reducing agent for the *O*-quinones produced by the enzymatic oxidation of substrates, showing no effect on the active site of the enzyme. However, most of the articles have shown that it is an effective agent which can momentarily retard the melanin biosynthesis pathway [15]. Therefore ascorbic acid was used as the positive control in this study and to compare the efficacy of the plant extract.

Our investigations revealed an extremely potent inhibition of the enzyme by the hydro methanolic extract prepared from the flowers of *C. auriculata* that supports the traditional claim as an enhancer of complexion. Moreover, the topical formulation prepared from this extract also displayed a strong inhibition of the enzyme, which is far better than the commercial preparation of kojic acid. Furthermore, the study indicated that the kojic acid preparation used in our experiments was not effective, despite its popularity within the local community as a skin whitening agent.

C. auriculata is highly reputed as an enhancer of the complexion in women and usually the dried flowers are boiled, brewed and subsequently taken as a drink [8]. Nevertheless, our preliminary investigations revealed that neither this aqueous extract nor the organic extracts prepared with hexane, dichloromethane, ethyl acetate and methanol could display a significant inhibition of tyrosinase enzyme. In contrast, the hydromethanolic extract has exhibited a potent anti-tyrosinase activity. This further supports the fact that, the extraction solvent has a vital role in the bioavailability of chemical compounds in an extract, hence, the bioactivity [16].

Several studies have reported the ability of polar substances of plant extracts to inhibit tyrosinase and it has been documented that the activities are partly contributory by antioxidant potentials of the extracts [17]. Therefore, the DPPH assay was employed to investigate the potential antioxidant activity of the plant extract where it has displayed EC_{50} = 19.99 µg/mL. Interestingly, this EC_{50} value was comparable with those of the two positive controls and it suggested that the anti-tyrosinase activity of this extract is due to its reducing capability. Most of the commonly used skin whiteners contain hazardous chemicals such as mercury, in the form of ammoniated mercury or mercuric chloride, as the active ingredients. As a result, the long term usage of such skin whitening products would lead to serious systemic effects such as irritability, muscle weakness, neuropathy and nephrotoxicity [3,18]. In this respect, the hydromethanolic extract prepared from the flowers of *C. auriculata* could be utilized as a safer alternative to the synthetic skin whiteners after being tested for the possible cytotoxicity in appropriate in vitro and in vivo models (zebrafish model etc.) /clinical trials [18]. Thus, the experiments are in progress to develop it as a commercial preparation.

5. Conclusions

The preliminary findings of this study reveals that Sri Lankan medicinal plant, *C. auriculata* has an extremely potent inhibitory action on tyrosinase, the key enzyme in melanin biosynthesis. Thus there's a

high potential for the development of herbal cosmetic from this extract.

Declarations of interest

None

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