Tyrosinase Inhibitory Activities of Carissa opaca Stapf ex Haines Roots Extracts and Their Phytochemical Analysis

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ABSTRACT
Objective: Carissa opaca is a medicinal plant with rich folkloric applications. The present research was conducted to explore the tyrosinase inhibitory potential of aqueous decoction (AD) and methanolic extract (ME) of roots of C. opaca and its fractions in various solvents and their phytochemical analysis.

Materials and Methods: AD of the dried powdered roots of C. opaca was prepared by boiling in water. ME was prepared by cold maceration. Its fractions were obtained in solvents of increasing polarity, i.e., hexane, chloroform, ethyl acetate, n-butanol, and water. The biomass left after extraction with methanol was boiled in water to get its decoction Biomass aqueous decoction (BAD). Tyrosinase inhibitory activities of the samples were studied according to a reported method. Chemical compounds in the samples were identified by gas chromatography-mass spectrometry (GC-MS). Results: The AD, BAD, and ME and its fractions displayed remarkable tyrosinase inhibitory activity. The IC₅₀ of AD was 23.33 µg/mL as compared to 15.80 µg/mL of the standard arbutin and that of BAD was 21.24 µg/mL. The IC₅₀ of ME was 34.76 µg/mL while that of hexane, chloroform, ethyl acetate, n-butanol, and aqueous fractions were 21.0, 44.73, 43.40, 27.66, and 25.06 µg/mL, respectively. The hexane fraction was thus most potent followed by aqueous fraction. By phytochemical analysis, campesterol, stigmasterol, gamma-sitosterol, alpha-amyrin, 9,19-cyclolanostan-3-ol, 24-methylene-,(3β-, lupeol, lup-20(29)-en-3-one, lup-20(29)-en-3-ol, acetate, (3β-, 21H-naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-, and 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methyleneacyclohexanone were identified in the extracts by GC-MS. Other compounds included fatty acids and their esters. Some of these compounds are being first time reported here from this plant. Conclusions: The roots extracts exhibited considerable tyrosinase inhibitory activities, alluding to their possible application to treat disorders related to overproduction of melanin.

Key words: Carissa opaca, melanin, tyrosinase, whitening agents

SUMMARY
The present study aimed to explore the tyrosinase inhibitory potential of aqueous decoction and methanolic extract of roots of Carissa opaca and its fractions in various solvents and their phytochemical constituents. GCMS analysis was conducted to identify the phytochemicals. The extracts and fractions of C. opaca roots showed remarkable anti-tyrosinase activities alluding to their possible application to treat disorders related to overproduction of melanin.

INTRODUCTION
Tyrosinase (EC 1.14.18.1), a copper-containing metalloenzyme, is an important enzyme involved in the production of the brown/black pigment in our skin called melanin.[1,2] The pigment per se is a blessing as it protects the skin from detrimental sun rays. Its over or uneven formation may, however, result in harmful or undesirable effects. The hyperpigmentation can be caused by various factors such as diseases, drugs, or prolonged exposure to sunlight.[3] The problem can be solved by the use of tyrosinase inhibitors, the substance that can inhibit the enzymatic action of tyrosinase. This is a valid strategy which is followed by a number of whitening agents such as arbutin, kojic acid, and hydroquinone.[4] The toxicity and efficacy issues of many of them necessitate efforts to discover safer and more effective remedies.[5] The tyrosinase inhibitors also have a role in agriculture to prevent enzymatic browning in fruits and vegetables, which is one of the major causes of food decay.[2] Excessive production of reactive oxygen species in the body can cause degenerative diseases including cancer, cardiovascular disorders, aging, and skin wrinkles.[6] Antioxidants not only provide defense against cancer-like life-threatening diseases but are also important in cosmetics and skin-care strategies. Plant extracts having compounds with antioxidant and tyrosinase inhibitory activities can slow down the aging and wrinkle formation and inhibit hyperpigmentation.[7]

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Carissa opaca (family Apocynaceae) is a plant well-known for its rich ethnomedicinal uses.[9] It is an evergreen, thorny shrub that grows wild in the Himalayan mountainous areas of Indo-Pakistan subcontinent.[9] The leaves of the plant are used as remedy for jaundice, hepatitis, fever, and asthma.[8] It's fruit consists of small berries which are edible and are considered to have aphrodisiac properties. Its roots are used to heal wounds and injuries.[10][11] While the aerial parts of the plant have been quite extensively investigated,[12][13] limited work has been so far done on its roots. Our group has recently published a number of studies on them.[14][15][16] They have been found to contain 2H-cyclopropanaphthalene-2-one, 7-hydroxy-6-methoxy-2H-1-benzopyran-2-one, 3-(4-methoxyphenyl)-2,6-dimethylbenzofuran, 5(1H)-azulenone, 2,4,6,7,8aaxehydro-3,8-dimethyl-4-(1-methylthylidene)-(8S-cis), limonene, vanillin, luteolin, rutin, quercetin, β-sitosterol, Vitamin-E, 2-hydroxyacetophenone, naphthalenone, 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)6-methylenecyclohexanone, and 2-benzenedicarboxylic acid mono (2-ethylhexyl) ester. The methanolic extract (ME) of the roots and its fractions have been shown to possess good antioxidant, antimicrobial, and xanthine oxidase and alpha-amylase inhibitory activities.[9]

The aim of the present study was to explore the roots C. opaca for tyrosinase inhibitory activities. Aqueous decoction (AD) and ME and its fractions were investigated in the study. To our knowledge, these studies are being reported here for the first time.

MATERIALS AND METHODS

Chemicals

Solvents were of high-pressure liquid chromatography grade. Dimethyl sulfoxide (DMSO) was purchased from Merck (Germany). Tyrosinase enzyme (mushrooms origin), tyrosine, and arbutin were purchased from Sigma-Aldrich (USA). Dipotassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Riedel-de Héan (Germany).

Collection of plant material

The roots of C. opaca Stapf ex Haines were collected from Abbottabad region (Pakistan). The plant was identified by Dr. Muhammad Ajaib of GC University (Lahore), where a specimen of the plant is kept in its herbarium under serial 2271.

Preparation of aqueous decoction

After separating them from aerial parts of the plant, the roots were washed with distilled water and were dried under shade for 3 weeks. The raw crushed and ground into a fine powder. The roots powder (50 g) was soaked in distilled water (500 mL) and boiled on a hot plate for 2 h. The decoction was filtered at room temperature by Whatman filter paper 41. The filtrate was a colloidal solution. It was centrifuged at 4000 rpm for 10 min at 10°C. The supernatant was collected from the centrifuge tubes as a clear solution. It was used for bioactivities. To determine the concentration of the supernatant, the following method was used. The supernatant (50 mL) was concentrated on a rotary evaporator to obtain as a gummy material, which was weighed and found to be 0.25 g. Therefore, the concentration of supernatant obtained was 5 mg/mL.

Preparation of methanolic extract and its fractions

The roots powder (2 Kg) was extracted into methanol (3 L) for 15 days for maximum extraction at room temperature. It was filtered and the filtrate was concentrated by rotary evaporator under reduced pressure to obtain ME (300 g). A weighed amount of ME (100 g) was suspended in distilled water (200 mL) and fractionated sequentially into the solvents of increasing polarity using separatory funnel (1000 mL), i.e., hexane, chloroform, ethyl acetate, and n-butanol. The fractions so obtained were concentrated by rotary evaporator and weighed to calculate yield.

Preparation of roots biomass-aqueous decoction

The biomass of the roots left behind after extraction of the ME was once again washed with methanol. It was dried in an oven at 40°C. The dried biomass (19.35 g) was soaked in distilled water (300 mL) and boiled for 2 h. It was then filtered at room temperature and the filtrate was collected which was in a colloidal form. Filtrate was centrifuged at 4000 rpm for 10 min at 10°C. The supernatant was collected from centrifuge tubes, which was a clear solution. To determine the concentration of the supernatant, 50 mL was concentrated on rotary evaporator to obtain as a gummy material, which was weighed and found to be 0.56 g. Therefore, the concentration of supernatant obtained was 11.2 mg/mL.

Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activities of ADs and ME of dried powdered roots of C. opaca and its fractions in various solvents were determined according to a reported method.[17] In the assay, tyrosine is converted to L-dihydroxyphenylalanine, which is then converted to dopaquinone. Both the reactions are catalyzed by tyrosinase. Dopaaquinone spontaneously changes to dopachrome, the formation of which is monitored spectrophotometrically at 475 nm.[18]

The plant samples were prepared in DMSO at concentrations ranging from 5 to 50 µg/mL. For 138 U/0.1 mL enzyme solution, 0.0025 g enzyme was dissolved in 10 mL potassium phosphate buffer (0.1 M, pH 6.8). The substrate tyrosine solution was prepared by dissolving 0.04529 g tyrosine in 100 mL buffer solution. The reaction mixture contained 1.8 mL phosphate buffer, 600 µL distilled water, 100 µL sample solution, and 100 µL (138 units) enzyme solution. The mixture was incubated at 25°C for 5 min. After this, 400 µL (6.3 mM) substrate solution was added and mixed. The absorbance of the clear mixture taken in a tube was recorded at 475 nm. The formation of dopachrome (orange color) indicates the activity of enzyme. DMSO was used as a blank while the same mixture without test materials was used as negative control. Arbutin dissolved in distilled water was used as a positive control. The percentage tyrosinase inhibitory activity of a sample was calculated using the following equation:

\[
\% \text{ activity} = \frac{A_c - A_s}{A_c} \times 100
\]

Where \(A_c\) and \(A_s\) are absorbance of a sample and the negative control, respectively. The \(IC_{50}\) values were calculated by the linear regression analysis.

Identification of phytochemicals

Phytochemicals present in ME of C. opaca roots and its fractions and ADs were identified through gas chromatography-mass spectrometry (GC-MS) analysis. The following protocol was used. The GC system (7890A Agilent Technologies, USA) consisted of a fused silica capillary column HP-5MS. The column dimensions were 30 m × 250 µm with 0.25 µm stationary film thickness. Helium gas was used as carrier gas with a flow rate of 1.0 mL/min. Agilent Technologies 5975C Inert Mass Selective Detector with triple axis detector was used for the identification of separated components. The sample was run with 240°C MS source temperature and a 4.00 min solvent delay time. The mass selected ranged from 30 to 600 amu using 71 eV relative voltage. The components were identified by comparing mass-spectra with NIST 05 library software (Gaithersburg, MD, US).
Statistical analysis
Activity of each sample was measured thrice and statistical mean ± standard deviation was calculated using Excel 2010 (Microsoft Corporation, USA); the same program was employed to calculate IC₅₀ (the concentration of a sample that gave 50% inhibition of the enzyme) of a sample.

RESULTS
Tyrosinase inhibitory activity of aqueous decoction
The percentage age tyrosinase inhibitory activities of AD of roots of C. opaca and decoction of its residual biomass were determined as a function of concentration and the results are displayed in Figure 1. The IC₅₀ values were calculated and are shown in Figure 2.

Tyrosinase inhibitory activity of methanolic extract and its fractions
The percentage age tyrosinase inhibitory activities of ME s of roots of C. opaca and its fractions were determined as a function of concentration. The results are displayed in Figures 3 and 4.

Phytochemical constituents
The GC-MS analysis of the samples proved useful and a number of important phytochemicals were identified in the samples of C. opaca roots [Table 1].

DISCUSSION
Melanin is a brown pigment in our skin that protects the skin from damaging sun radiations. The process of melanin biosynthesis is catalyzed by a copper-containing multifunctional enzyme called tyrosinase. While normal production of melanin is a blessing, age, prolonged exposure to sunlight, and diseases may cause uneven pigmentation or hyperpigmentation affecting the beauty or skin health of a person. It is, therefore, desirable to find out materials that can inhibit tyrosinase. Such substances have great cosmetic value. Synthetic chemicals may further deteriorate the cosmetic problem by damaging skin cells or causing other side effects. Plant-based inhibitors of the enzyme, therefore, hold promise to provide safe and affordable remedies for over or uneven production of melanin. Consequently, numerous studies have been carried out recently on herbal extracts and natural products, which demonstrate them as potential tyrosinase inhibitors.

C. opaca is well-known as a medicinal plant. In the recent years, many studies, in vitro and in vivo, have been conducted on the plant to explore various bioactivities. Studies on its roots, however, are limited. Moreover, their AD has not been investigated for any activity so far. ME of the roots has been studied for some activities recently, but the data are still limited.

In the present work, two schemes were used for extraction. In the first scheme, powder of the dried roots of C. opaca was boiled in distilled water to get their AD. In the second scheme, the powder was extracted exhaustively into methanol at room temperature to obtain ME. The ME
was fractionated sequentially into solvents of increasing polarity to obtain hexane, chloroform, ethyl acetate, n-butanol, and aqueous fractions. The residual biomass was then boiled in water to get AD of the biomass (BAD).

With the aim to discover natural tyrosinase inhibitors, the roots of *C. opaca* were evaluated, in the present work, for their possible inhibitory action on the enzyme. The AD exhibited considerable ability to inhibit the enzymatic reaction. The tyrosinase inhibitory activity of AD of *C. opaca* was comparable to that of arbutin, having IC$_{50}$ values of 16.629 (R$^2$ = 0.9853) and 15.80 (R$^2$ = 0.9665) while that of hexane, chloroform, ethyl acetate, n-butanol, and fractions was 21.0 (R$^2$ = 0.9427), 44.73 (R$^2$ = 0.9904), 43.40 (R$^2$ = 0.9687), 27.66 μg/mL (R$^2$ = 0.9764), and 25.06 (R$^2$ = 0.9853), respectively. The hexane fraction was thus most potent followed by the aqueous fraction.

The encouraging results prompted us to explore the phytochemical constituents of the samples to provide possible leads for cosmetic industry. The major compound identified in AD and decoction of biomass both was a sesquiterpenoid 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylene cyclohexanone (molecular formula C$_{22}$H$_{24}$O; molecular mass 343.40 (R$^2$ = 0.9687), 27.66 μg/mL (R$^2$ = 0.9764), and 25.06 (R$^2$ = 0.9853), respectively. The hexane fraction was thus most potent followed by the aqueous fraction.

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CONCLUSIONS

The roots of C. opaca displayed considerable tyrosinase inhibitory activity the IC₅₀ values of the samples tested were proximity to that of the positive control arbutin. A number of triterpenoids and fatty acid esters were identified in the samples. The compound 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone appears to be one of the major constituents of C. opaca roots. Subject to confirmation by further studies, the roots of the plant may potentially find application in cosmetic and food industry to control over-pigmentation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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