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ORIGINAL ARTICLE

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

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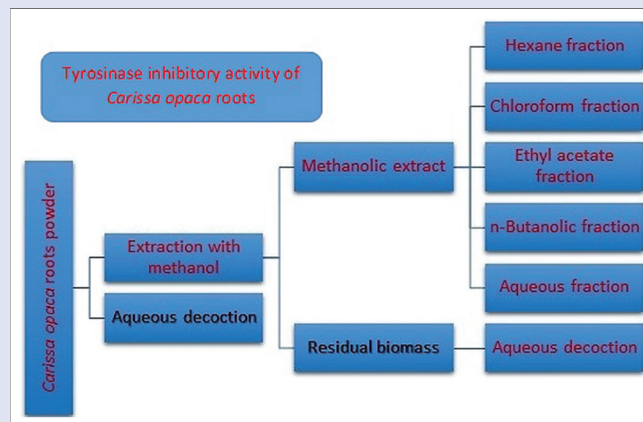
ABSTRACT

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 IC_{50} 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 IC_{50} of ME was 34.76 μg/mL while that of hexane, chloroform, ethyl acetate, *n*-butanolic, and aqueous fractions was 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-,(3 β)-, lupeol, lup-20(29)-en-3-one, lup-20(29)-en-3-ol, acetate,(3 β)-, 2(1*H*) 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-methylenecyclohexanone 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 a possible application of the plant in cosmetic as whitening agent subject to further pharmacological studies.

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.



Abbreviations used: AD: Aqueous decoction; ME: Methanolic extract; BAD: Biomass aqueous decoction; GC-MS: Gas chromatography-mass spectrometry.

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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-threating 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.[8] 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] Its 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] While the aerial parts of the plant have been quite extensively investigated,[11,12] limited work has been so far done on its roots. Our group has recently reported a number of studies on them.[13-16] 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, 2,4,6,7,8,8ahexahydro-3,8-dimethyl-4-(1-methylethylidene)-,(8S-cis), limonene, vanillin, lupeol, 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.[8]

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 Heän (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 wee 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. Dopaquinone 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_s and A_c 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-spectrums 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 IC_{50} (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 IC_{50} 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.[18] 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.[19-23]

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.[8] 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

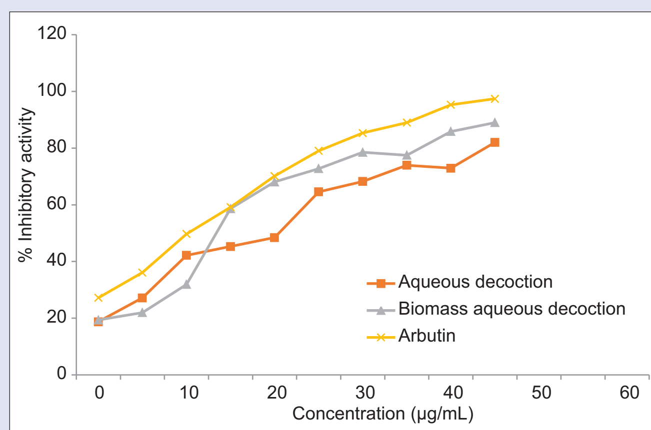

Figure 1: The percentage tyrosinase inhibitory activities of aqueous decoctions of *Carissa opaca* roots along with the standard arbutin as a function of concentration (*n* = 3)

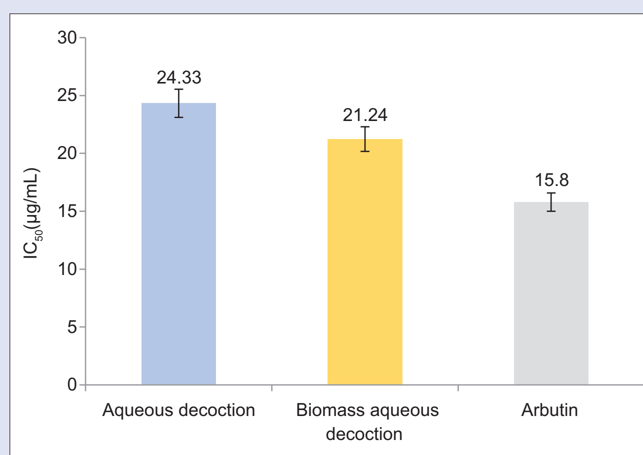

Figure 2: The IC_{50} of aqueous decoction and biomass-aqueous decoction of *Carissa opaca* roots along with arbutin against tyrosinase (*n* = 3)

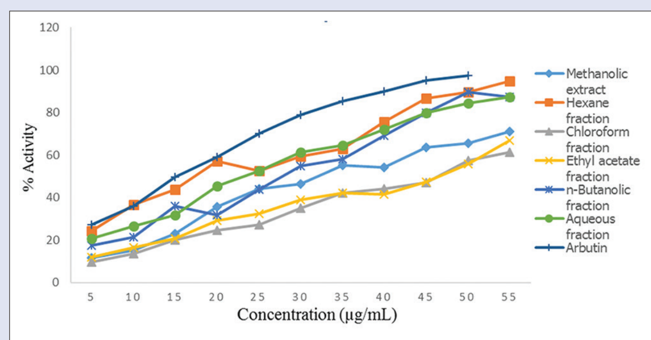

Figure 3: % Tyrosinase inhibitory activities of methanolic extract of *Carissa opaca* roots and its different fractions as a function of concentration (*n* = 3)

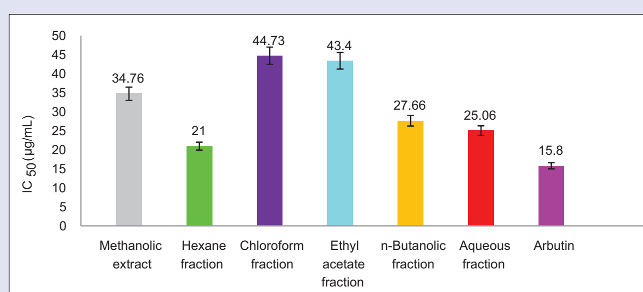

Figure 4: The IC_{50} of methanolic extract of *Carissa opaca* roots and its different fractions against tyrosinase along with the standard arbutin (*n* = 3)

Table 1: Phytochemicals found in aqueous decoctions, methanolic extract and fractions of *Carissa opaca* roots identified by gas chromatography-mass spectrometry

Retention time (min)	Compound name	Molecular mass	Percentage of total
	Methanolic extract		
19.472	Hexadecanoic acid, methyl ester	270	10.120
19.642	2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	218	1.630
19.854	Hexadecanoic acid	256	7.512
20.186	Hexadecanoic acid, 14-methyl-, methyl ester	284	1.370
21.111	9,12-octadecadienoic acid, methyl ester, (E, E)-	294	3.875
21.179	9-octadecadienoic acid (Z), methyl ester	296	13.559
21.545	Oleic acid	282	5.422
25.121	[1.1'-Bicyclopropyl]-2-octanoicacid, 2'-hexyl-, methyl ester	322	2.437
30.201	Campesterol	400	2.087
30.439	Stigmasterol	412	1.861
30.068	Gamma-Sitosterol	414	5.675
31.629	Alpha-Amyrin	426	5.106
31.816	Lup-20(29)-en-3-one	424	3.601
32.028	9, 19-cyclolanost-24-en-3-ol, (3 beta)-	426	1.870
32.206	Lupeol	426	33.873
	Hexane fraction		
9.388	Ethanone 1-(2-hydroxyphenyl)	136	18.142
18.376	Hexadecanoic acid, methyl ester	270	1.517
18.836	2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	218	17.931
20.058	9-octadecadienoic acid (Z), methyl ester	296	3.422
22.241	Oleic acid	282	1.469
30.286	Lupeol	426	5.368
	Chloroform		
19.480	Hexadecanoic acid, methyl ester	270	5.901
19.888	Hexadecanoic acid	256	10.736
21.188	9-octadecadienoic acid (Z), methyl ester	296	12.817
21.570	Lupeol	426	6.639
23.974	9,19-cyclolanostan-3-ol, 24-methylene-, (3 beta)-	440	6.036
25.486	Lup-20 (29)-en-3-ol, acetate, (3 beta)-	468	34.346
31.085	Gamma-Sitosterol	414	5.262
31.654	Alpha-Amyrin	426	7.945
31.832	Lup-20(29)-en-3-one	424	6.355
	Ethyl acetate fraction		
18.784	2(1*H*) naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-	218	46.519
20.058	11-Octadecenoic acid, methyl ester	296	17.693
	***n*-Butanolic fraction**		
18.555	14-methylpentadecanoic acid, methyl ester	270	13.095
18.937	2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	218	67.923
20.002	11-octadecenoic acid, methyl ester	296	9.280
	Aqueous decoction		
16.629	2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	218	64.328
	Aqueous decoction of biomass		
16.629	2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	218	35.863

was fractionated sequentially into solvents of increasing polarity to obtain hexane, chloroform, ethyl acetate, *n*-butanolic, 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* roots was comparable to that of arbutin, having IC_{50} values 24.33 (R^2 = 0.9853) and 15.80 μg/mL (R^2 = 0.9631), respectively. The BAD also showed excellent enzyme inhibitory potential with IC_{50} of 21.24 μg/mL (R^2 = 0.9016). The tyrosinase inhibitory activity of the ME and its fractions was also noteworthy. The IC_{50} value of ME was 34.76 μg/mL (R^2 = 0.9665) while that of hexane, chloroform, ethyl acetate, *n*-butanolic, 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-methylenecyclohexanone (molecular formula $C_{15}H_{22}O$; molecular mass 218) by GC-MS. The retention time was 16.629 min. This compound seems to be a major constituent of the roots of this plant as it was found in ME and its hexane and *n*-butanolic fractions as well. It was in good percentage [Table 1]. A number of triterpenoids and related compounds were identified in ME or its fractions, which included campesterol,

stigmasterol, gamma-sitosterol, alpha-amyrin, 9,19-cyclolanostan-3-ol, 24-methylene-,(3 β)-, lupeol, lup-20(29)-en-3-one, lup-20(29)-en-3-ol, acetate,(3 β)-, and 2(1*H*) naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-. Other compounds included fatty acids and their esters. Some of these compounds are being first time reported here from this plant.

These compounds may, at least, partly explain the antienzymatic property of the fractions. The compound 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone that appears to be one of the major constituents of *C. opaca* roots may have this property. Other compounds with like tyrosinase inhibitory activity may include sterols, lupeol, and fatty acid esters. The findings are in agreement with those of similar studies by other researchers.[24]

CONCLUSIONS

The roots of *C. opaca* displayed considerable tyrosinase inhibitory activity the IC_{50} 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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