Extract from *Ceratonia siliqua* exhibits depigmentation properties

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Abstract

Skin hyper-pigmentation is a condition initiated by the overproduction of melanin existing in the melanocytes. Melanin pigment is responsible for the colour of skin in humans. It is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of the key enzyme tyrosinase. In continuation with our efforts to identify tyrosinase inhibitors from plants sources, the methanol extract from leaf, bark and fruit of *Ceratonia siliqua* were screened for tyrosinase inhibition and diphenolase activity. The bark extract exhibited significant inhibition on mushroom tyrosinase using *L*-tyrosine as a substrate and showed diphenolase activity. The extract further significantly inhibited tyrosinase mRNA levels in B16-F10 mouse melanocytes. Bioassay-guided fractionation led to the isolation of six compounds. Compounds (-)-epicatechin-3-*O*-gallate, 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose and Gallocatechin-3-O-gallate showed tyrosinase inhibitions with the IC_{50} values of 27.52, 83.30 and 28.30 µg/mL, respectively. These compounds also exhibited *L*-DOPA activities with IC_{50} values of >200, 150 and 200 µg/mL, respectively. A clinical study was conducted using 20 volunteers in a patch testing trial for irritancy potential and skin depigmentation. The clinical results showed the sample to be non-irritant with irritancy potential of -34.21 and depigmentation trial showed an improvement in the even skin tone of UV induced pigmentation at 3% after 28 days of application.

Keywords: *Ceratonia siliqua*; tyrosinase inhibition; DPPH activity; irritancy potential, UV-induced pigmentation

INTRODUCTION

Skin is an important component of body which protects the internal environment from the external one and adds to our beauty too. Beauty is a quality that gives pleasure to the senses, which is desired by many humans (Lall and Kishore, 2014). Skin hyperpigmentation is a condition initiated by the overproduction of melanin existing in the melanocytes. Melanin is a pigment that is responsible for the colour of skin in humans. There are two types of melanin pigments that can be produced by the melanocyte cells, eumelanin which is black or brown and pheomelanin which is red or yellow. The colour of human skin is determined by the type and distribution of melanin pigment. It is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase, the key enzyme in melanin biosynthesis (Mapunya *et al.*, 2011). The role of melanin is to protect the skin against UV light damage by absorbing UV sunlight and removing the reactive oxygen species (ROS). Over-activity of tyrosinase, the key enzyme in melanin biosynthesis, leads to the overproduction of melanin. There are several noteworthy tyrosinase inhibitors obtained from natural sources reported in literature which are used for depigmentation or for the disorder or hyperpigmentation of the skin. There is a variety of plant species that are used traditionally for the treatment of skin problems (De Wet *et al.*, 2013; Mabona *et al.*, 2013). South African plants have been found to play a major role in the treatment of skin hyperpigmentation and these species have significantly contributed in South Africa, towards the health care for skin (Lall and Kishore, 2014).

Ceratonia siliqua L., commonly known as the 'Carob tree', is an evergreen, highly drought resistant tree grown in many mild and semiarid parts of the world (Morton, 1987). It has a long history of use, both as food and as pharmaceutical. Medicinally, it is used to treat several conditions, including the treatment of diarrhoea, heartburn, obesity, vomiting, pregnancy conditions and high cholesterol (Custodio *et al.*, 2011). The seed gum is employed

in the manufacture of cosmetics, pharmaceutical products, detergents and insecticides. Many secondary metabolites have been previously isolated from this plant. These metabolites protect the organism from excessive production of free radicals and reactive oxygen species (Eldahshan, 2011). The use of seed gum from the plant in cosmetic formulation and reports of isolated flavonoids prompted us to select *Ceratonia siliqua* L. for the present study. Flavonoids are phenolic compounds that have been reported as good anti-tyrosinase agents (Chang, 2009; Mapunya *et al*., 2011; Rauniya *et al*., 2014). It has been explored many times before for its important bioactivity for diabetes and diarrhoea but not for tyrosinase inhibition. During our evaluation of the efficacy of South African flora for depigmentation activity, it was found that the leaf and bark showed high anti-tyrosinase activities. We proceeded further for the identification of active principles. In the present study, in an effort to develop a safe and effective melanin formation inhibitor from plant sources, the influence of crude extract and isolated compounds with arbutin and kojic acid as reference compounds, on tyrosinase activity and melanin synthesis in B16F10 melanoma cells, as well as their regulation on the irritancy potential and UV-induced hyperpigmentation of human skin were investigated.

MATERIALS AND METHODS

General experimental procedure.

L-Tyrosine, *L*-DOPA, tyrosinase, arbutin and kojic acid were obtained from Sigma-Aldrich (Kempton Park, South Africa). Cell culture reagents and equipment were purchased from Highveld Biological (Sandringham, South Africa), LASEC (Randburg, South Africa) and The Scientific Group (Midrand, South Africa). The B16-F10 mouse melanocyte cell line was obtained from Highveld Biological (Sandringham, SA). Column chromatography: silica gel 60 (70-230 mesh, Sigma-Aldrich). Sephadex LH-20 (Sigma-Aldrich). NMR spectra were

recorded on a Varian Oxford AV-200 MHz spectrometer, using reference line as a standard. IR spectra were recorded on a Nexus 670 FT-IR instrument from KBr pellets. All the chemicals were purchased from Sigma-Aldrich and Merck SA Pty Ltd.

Cell culture.

Mouse melanocytes, B16-F10 cell line (Highveld biological, South Africa) were cultured in a complete Minimum Essential Medium (MEM) containing 10% foetal bovine serum (FBS), 1.5g/L $NaHCO_3$, 2mM L-glutamine, 50µg/ml gentamicin, and 0.25µg/ml fungizone at 37°C in 5% CO2 humidified conditions. The cells were passaged in a ratio of 1:4 every third day.

Extraction and isolation.

The leaves, bark and fruits of *Ceratonia siliqua* were collected from the botanical garden of the University of Pretoria in July 2005 and identified at South H.G.W.J. Schweickerdt Herbarium (PRU) of the University. A voucher specimen (95502.1-2.3) was deposited at H.G.W.J Schweickerdt Herbarium, Department of Plant Science, University of Pretoria, South Africa. The air-dried and powdered leaves, bark and fruits were soaked in ethanol with stirring for 3 hours at 50 °C. The filtrates were collected and concentrated under reduced pressure by a rotavapor at 40 °C, with an equal volume of each respective extract. Bioassay of different parts of plant showed that the bark, leaves and fruits extracts demonstrated inhibition of monophenolase activity (Table 1). Due to the avoidance of harvesting bark for conservation purpose and due to the presence of higher tannins content in bark extract, the second best extract, *i.e.* leaf-extract was selected for further isolation.

Dried alcoholic extract of the leaves (95 g) was re-dissolved in 80% ethanol (ethanol: distilled water; 80:20) and partitioned with *n*-hexane, ethyl acetate and *n*-butanol. The organic layers were evaporated to dryness at 40 °C to give 11 g, 32.5 g and 19 g of *n*-hexane,

Table 1. Anti-tyrosinase activity and inhibition of melanin production by the extract and isolated compounds

Sample	Monophenolase IC_{50} (µg/ml)	Diphenolase IC_{50} (µg/ml)	Melanin inhibition (µg/ml)	% Reduction in melanin content	% Cell viability
Leaf extract	112.1±0.7	>400	1.563	25	100
Bark extract	40	>400	a	a	a
Fruit extract	150	400	a	a	a
Compound 1	27.52	>200	a	a	a
Compound 2	83.30	150	1.563	8	60
Compound 3	>200	200	1.563	8	100
Compound 4	>200	200	1.563	10	70
Compound 5	200	>200	a	a	a
Compound 6	28.30	200	a	a	a
Arbutin	149	>200	608.7	7	100
Kojic acid	1.138±0.05	50.51±1.7	1.563	45	100

[a]Not tested due to unavailability and sustainability

ethyl acetate and *n*-butanol fractions, respectively. Ethyl acetate fraction (32.5 g) was subjected to Sephadex column LH-20 (7X50 cm) using mixture of H_2O:Ethanol of decreasing polarity (0% to 100% Ethanol). Similar fractions were combined together according to TLC profile to give 10 main fractions (1-10). Fraction 4 (1.0 g) was chromatographed on Sephadex column using ethanol as eluent, the subfractions 9-11 were combined together (0.325 g) and chromatographed twice on Sephadex column (1.5X40 cm) to yield Myricetin-3-*O*-α-L-rhamnoside (**4**) (22 mg, 0.0022%, of dry plant material). Subfraction 12 of fraction 4 was

purified on preparative paper chromatography using 15% acetic acid to yield Myricetin-3-*O*-glucoside (**5**) (9 mg, 0.0009%, of dry plant material). Fraction 5 (2.216 g) was chromatographed on Sephadex column (3.0X40 cm) using 100% ethanol as eluent. Subfractions 5 and 6 were combined (0.071 g) and were further purified on preparative paper chromatography using 15% acetic acid to yield Quercetin-3-*O*-α-L-rhamnoside (**3**) (35 mg, 0.0035%, of dry plant material). Subfractions10 of fraction 5 was purified on preparative paper chromatography using 15% acetic acid to yield Gallocatechin-3-O-gallate (**6**) (17 mg, 0.0017%, of dry plant material). Fraction 9 (0.030 g) was purified on Sephadex column using ethanol as eluent. Subfractions (13-19) were seen to contain one main spot which were further re-purified on HPLC column (Phenomenex, Luna 5µ C18 (2), size 250X 10.00 mm) using methanol:H_2O (98:2) as mobile phase to yield (-)-epicatechin-3-*O*-gallate (**1**) (15 mg, 0.0015%, of dry plant material). Fraction 10 (0.802 g) was purified on preparative-paper chromatography using 15% acetic acid to yield 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose (**2**) (33.0 mg, 0.003%, of dry plant material).

Tyrosinase enzyme assay.

This assay was performed using the method described by Curto *et al.*, 1999 and Nerya *et al.*, 2003. Extracts were dissolved in DMSO (dimethyl sulphoxide) to a final concentration of 20 mg/ml. This extract stock solution was then diluted to 600 µg/ml in 50 mM potassium phosphate buffer (pH 6.5). Kojic acid and arbutin were used as control drugs (Lee *et al.*, 1997; Kim *et al.*, 2006). In the wells of a 96-well plate, each extract dilution was combined with tyrosinase (333 Units/ml in phosphate buffer) in triplicate. After incubation at room temperature for 5 minutes, the substrate (2 mM *L*-tyrosine or 12 mM *L*-DOPA) was added to each well. Final concentrations of the extract samples and positive controls ranged from 1.5 to 400 µg/ml while pure compounds were tested at the final concentrations ranging from 1.5

to 200 μg/ml. Incubation commenced for 30 minutes at room temperature. Optical densities of the wells were then determined at 492 nm with the BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa). All the experiments were repeated thrice. The extracts with significant anti-tyrosinase activities, were further investigated for their effect on melanin production by melanocyte cells.

Melanocyte cell culture and melanin inhibition assay.

The mouse melanocyte cell line, 'B16-F10', was cultured in complete basal medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM *L*-glutamine, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml fungizone at 37 °C with 5% CO_2 in a humidified atmosphere. Cells were sub-cultured in a ratio of 1:3 on every third or fourth day. For *in vitro* experiments, B16F10 cells, were re-suspended in complete DMEM medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM *L*-glutamine, 10 μg/ml penicillin, 10 μg/ml reptomycin, and 0.25 μg/ml fungizone.

Determination of extract toxicity and melanin content in melanocytes.

On the first day, B16F10 cells in complete DMEM medium were dispensed into the wells of a 96-well plate (10^4 cells per well) and 24-well plate (10^5 cells per well). After an overnight incubation at 37 °C in 5% CO_2 and a humidified atmosphere, extract samples were added to the cells to final concentrations of 1.5, 3.1, 6.2, 12.5, 25, 50 and 100 μg/ml. Kojic acid was used as a control drug (Nerya *et al.*, 2003). Incubation at 37 °C in 5% CO_2 and a humidified atmosphere followed for 3 days. The toxicity of the extracts on the B16F10 cells was assayed using XTT cytotoxicity assay (Zheng *et al.*, 2001). Fifty microlitres of XTT reagent (1 mg/ml XTT with 0.383 mg/ml PMS) was added to the wells and incubation commenced for 1-4 hrs.

The optical densities of the wells were then measured at 450 nm (690 nm reference wavelength). By referring to the control (medium with DMSO), cell survival was assessed. The effect of the extracts on melanin synthesis was determined by washing the cells in the 24-well plate with PBS, and lysing with 200 µl of sterile distilled water. Optical densities were determined at 405 nm. The effect on melanin production was determined by referring to the control sample (medium with DMSO).

Cell treatments and RNA extraction.

Approximately $1x10^6$ mouse melanocyte cells (B16-F10) were counted using a hemocytometer and treated with 100µg/ml of *Ceratonia siliqua* extract. Untreated cells were used as a negative control and a DMSO control was added which consisted of cells treated only with DMSO. This was done in order to take into account the effect that DMSO has on cells since it was used for dissolving of plant extracts. Either kojic acid or lipoic acid were used as positive controls to treat mouse melanocytes. Each cell treatment was performed in triplicate. The treatments were incubated for 72 hours at 37ºC in 5% CO_2.

After incubation, for both Semi quantitative RT-PCR and RT-qPCR, total RNA from treated cells was extracted using the Qiagen RNeasy Plant mini kit (Qiagen, Hilden, Germany) and was subjected to an on column RNase free DNase (Qiagen, Hilden, Germany) digestion to remove any contaminating genomic DNA. A NanoDrop™ 2000 spectrophotometer (Thermo scientific, Minnesota, USA) was used to determine the OD260/280 and OD260/230 ratios of each sample and denaturing formaldehyde agarose gel electrophoresis was performed to evaluate the quality of RNA. Extracted RNA (500ng) was used to prepare cDNA for each sample using the cDNA synthesis kit (Thermo scientific, South Africa).

PCR Primers.

Specific oligonucleotide primer pairs to be used for RT-PCR were purchased from Inqaba Biotechnical Industries (South Africa). As internal housekeeping control, mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was also amplified using mouse *gapdh* (M32599) primers. Tyrosinase primers were designed to the mouse tyrosinase gene (P11344). The sequences of the primers used for reverse transcription polymerase chain reaction (RT-PCR) were as follows: Tyrosinase forward primer: 5´ CGAGCCTGTGCCTCCTCTAA 3´, Tyrosinase reverse primer: 5´ CCAGGACTCACGGTCATCCA 3´, GAPDH forward primer: 5´ CCAATGTGTCCGTCGTGGAT 3´ and GAPDH reverse primer: 5´ GCTGTTGAAGTCGCAGGAGA 3´.

For the RT-qPCR study, gene specific primers were designed to mouse tyrosinase (P11344), mouse β- actin (NC_000071) and mouse carnatine palmitoyltransferase (NC_000070) genes (one target and two reference genes respectively) using Primer3 software (version 0.4.0) applying optimum parameters for RT-qPCR. The primers were further analysed in CLC bio Main workbench (version 6.6.5) software to assess their alignments to target genes. Primer sequences for each gene were as follows: Tyrosinase forward primer: 5' CCAGTGCCTTGTATATGC 3', Tyrosinase reverse primer: 5' CCTTGAACCGCTAGAGAA 3', β-Actin forward primer: 5' AAATCGTGCGTGACATCAAA 3', β-Actin reverse primer: 5' TCTCCAGGGAGGAAGAGGAT 3', Carnitine palmitoyltransferase 2 (Cpt2) forward primer: 5' CCTGCCAGAAGTGACACAGA 3', Cpt2 reverse primer: 5' ATCCAGGGGATATGCATTGA 3'.

Semi quantitative RT-PCR analysis of tyrosinase mRNA.

Two micrograms of total DNaseI-treated and column-purified RNA extracted from mouse cells treated with leaf extract and compound Quercetin-3-*O*-α-L-rhamnoside (**3**) at a

concentration of 25 µg/ml, and control mouse cells was reverse transcribed into first strand cDNA using ImpromII Reverse Transcriptase (Promega, Madison, WI) according to manufacturer's instructions. For semi quantitative PCR, each PCR amplification of tyrosinase or *gapdh* genes contained the following components: 1 µl of cDNA, 1 X buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer and 1 unit of Taq polymerase in a total reaction volume of 20 µl. DNA amplification was performed using a GeneAmp PCR System 2400 (Perkin-Elmer, California, USA). The cycling conditions were 95 °C for 5 min, 35 cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 6 min. Products were removed after 23, 25, 28, 30 and 32 cycles and run on a 2% (w/v) agarose gel alongside a standard. The gel was photographed under UV light and the amount of DNA represented in each DNA band was calculated using the VersaDocTM 4000 (Bio-Rad Laboratories, Inc. Life Science Research Group, California, USA). The data was entered in Microsoft Excel and the expression of the tyrosinase gene was standardised to the expression of the internal control gene, *gapdh*.

Real Time-quantitative PCR (RT-qPCR).

Real time-quantitative PCR was conducted on the Biorad CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Johannesburg, South Africa) using SYBR® Green Master Mix (Life technologies, Johannesburg , South Africa). The experiment consisted of three biological replicates and three technical replicates for each sample. Concisely, every reaction comprised of 5µl SYBR® Green Master Mix, 0.2 µl of 10 µM forward primer, 0.2 µl of 10 µM of reverse primer, 3.6 µl water and 1 µl cDNA template. The PCR was cycled as follows; 95°C for 3min, 40 cycles of 95°C for 10sec, 59°C for 30sec, 95°C for 10sec. A melting curve was incorporated at the end of the cycle, in which samples were heated from 65°C to 95°C with increments of 5 sec. Melting curves and amplification plots with Crossing

Point (CP) values were generated automatically by the CFX Manager™ software. Biogazelle qBasePlus 2.0 software (BioGazelle, Zwijnaarde, Belgium, Vandesompele *et al*., 2002) was used generate standard curves for all three genes, to assess the stability of the two reference genes across all samples, and to normalise tyrosinase gene expression relative to the two housekeeping genes.

DPPH antioxidant assay.

This assay was performed using the method described by Toit *et al.*, (2001) with slight modifications. Crude extract and pure compounds were prepared at the final concentrations of 7.8-1000 µg/ml for extract and 1.9-250 µg/ml for pure compounds for 1,2-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, South Africa) antioxidant assays. For each sample, dilution series (8 dilutions) was prepared in a 96-well ELISA plate by adding distilled water (100 µl) as a dilution medium. All the samples were prepared in triplicate. Later 90 µl (90 µM) of methanolic DPPH was added to each well. Vitamin C was used as control and its stock solution was prepared using boiling water. The plates were covered with aluminium foil and incubated at room temperature for 1 h before spectrophotometry. The radical scavenging capacities of the samples were determined by using a BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa) to measure the disappearance of DPPH at 550 nm.

The radical scavenging activity was measured in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) (Toit *et al.*, 2001). The EC_{50} value for each sample was determined graphically by plotting the absorbance of DPPH as a function of the sample concentration in µg/ml for the standard and samples. The EC_{50} is the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50%. The

results are expressed as the mg vitamin C equivalents/g dry weight and are calculated as follows:

EC_{50} Vit C mg/ml/EC_{50} sample (g/ml) = × mg vitamin C equivalents/ g dry weight

Zero mg/ml was taken as 100%.

Statistical analysis.

The final results are expressed as the mean (standard deviation, ±SE.S). The group means were compared using the ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple Range Test. Values determined were significant when *p* was less than 0.01 ($p<0.01$).

Clinical study.

Crude extract samples (Ethanol extract of leaves, S_1(3%) and ethanol extract of leaves + existing depigmenting agent; Niacinamide, S_2(3%) each in 1:1 ratio) were submitted for *in vivo* clinical study to the "FUTURE COSMETICS" 287 Sinovich Street, Grootfontein Country Estate, South Africa (Reg. No.: 2001/055088/23). Sample was trialled for skin lightening on identified spot and UV induced pigmentation. A standard aqueous cream was used in the study as a placebo.

The study was to determine the skin lightness/brightness efficacy on identified pigmentation spots for a test product compared to a placebo on the face of human subjects after twenty-eight (28 days) of consecutive use. Twenty (20) subjects between the ages of 20 and 68 were recruited. Nineteen (19) subjects complied with the rules and specifications of the study and all results recorded were used in calculations. The study was carried out at an ambient temperature of 22 ºC ± 4 ºC and a relative humidity of 40 %RH ± 15 %RH. The procedure of testing was explained to them verbally and a form of consent and medical

history was signed by each subject. All subjects were instructed to rest for 20 minutes before any testing was conducted. The designated left or right face was cleansed with a standard eye make-up remover and allowed to air dry for three minutes. The temperature and relative humidity were recorded during the time of the study. Subjects were restricted from using any topical products or any medication not approved by the study sponsor for the duration of the study. Subjects returned to the testing facility on days 14, 28 respectively.

RESULTS AND DISCUSSION

Inhibition of tyrosinase by crude extract.

The exploration of traditionally used plants to treat skin disorders is a good source for the discovery of novel active compounds. Although there are numerous natural products from plant origin with potent inhibitory effects on tyrosinase, their use in the pigmentation disorder and cosmetics industry has been demonstrated. The main purpose of the present study was to investigate the potential of plant crude leaf, bark, fruit extract and isolated compounds from the active one for hyper-pigmentation therapy and whitening application. Ethanol extracts of the leaves (**L**), bark (**B**) and fruits (**F**) of *C. siliqua* showed significant inhibition ($p<0.01$) of tyrosinase with 87%, 86% and 97% at 200 µg/ml when *L*-tyrosine was used as substrate (IC_{50} values of 112±0.7 µg/ml, 40 µg/ml and 150 µg/ml for L, B and C, respectively). Also a significant ($p<0.01$) inhibition of diphenolase activity (43% and 23%) was observed by the L at 400 and 200 µg/ml, respectively (Table 1). They also reduced the melanin content of mouse melanocytes without being significantly toxic at that concentration. The tyrosinase inhibitory activity of this plant has been reported for the first time.

Cytotoxicity and inhibition of melanin production by crude extract.

The cytotoxicity of the extract has been tested in comparison with kojic acid. The cultured cells were treated with various concentrations and the cell viability was assessed using the MTT assay. Leaf extract exhibited 45% reduction in melanin content at 12.5 µg/ml in melanocytes without being significantly ($p<0.01$) toxic to the cells. Kojic acid (positive control) showed no significant toxicity to B16F10 cells at the highest concentration tested and exhibited 60% reduction in melanin content at 3.1 µg/ml.

Identification of isolated compounds.

The ethanol extract of dried and powdered leaves of *C. siliqua* subjected to a series of chromatographic purification on silica gel and saphadex LH-20 resulted in isolation of six known compounds. Structural assessment of these compounds was characterized by Mass, ^{1}H and ^{13}C NMR spectroscopic data. Assignment of signals was facilitated by COSY, HSQC and HMBC experiments. The compounds obtained in this study, (-)-epicatechin-3-*O*-gallate (**1**) (Eldahshan, 2011), 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose (**2**) (Xiang *et al.*, 2011), Quercetin-3-*O*-α-L-rhamnoside (**3**) (Kim *et al.*, 2004), Myricetin-3-*O*-α-L-rhamnoside (**4**) (Sharma *et al.*, 2013), Myricetin-3-*O*-glucoside (**5**) (Kazuma *et al.*, 2003) and Gallocatechin-3-O-gallate (**6**) (Anke *et al.*, 2008) were identified by comparison of their physical and spectroscopic data with literature reports.

Anti-tyrosinase activity of isolated compounds.

The isolated compounds from the ethanol extract were tested for anti-tyrosinase activities with reference to arbutin and kojic acid. These isolated compounds exhibited tyrosinase inhibitions with IC_{50} values of 27.52 µg/ml ((-)-epicatechin-3-*O*-gallate), 83.30 µg/ml (1,2,3,6-tetra-*O*-galloyl-ß-D-glucose), >200 µg/ml (Quercetin-3-*O*-α-L-rhamnoside), >200

µg/ml (Myricetin-3-*O*-α-L-rhamnoside), 200 µg/ml (Myricetin-3-*O*-glucoside) and 28.3 µg/ml (Gallocatechin-3-O-gallate) when *L*-tyrosine was used as substrate. The IC_{50} values of compounds ((-)-epicatechin-3-*O*-gallate), (1,2,3,6-tetra-*O*-galloyl-ß-D-glucose), (Quercetin-3-*O*-α-L-rhamnoside) and (Myricetin-3-*O*-α-L-rhamnoside) were >200 µg/ml, 150 µg/ml, 200 µg/ml and 200 µg/ml, respectively, for diphenolase (*L*-DOPA) activity. Arbutin and kojic acid exhibited the IC_{50} values of 149 µg/ml and 1.1 µg/ml of *L*-tyrosine activity, respectively. The IC_{50} values of >200 and 50.5 µg/ml were found when *L*-DOPA was used as substrate for arbutin and isoliquiritigenin, respectively. Compounds (-)-epicatechin-3-*O*-gallate exhibited the best inhibition of monophenolase activity followed by Gallocatechin-3-O-gallate, 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose, Quercetin-3-*O*-α-L-rhamnoside, Myricetin-3-*O*-α-L-rhamnoside and Myricetin-3-*O*-glucoside. Compounds (-)-epicatechin-3-*O*-gallate, 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose and Gallocatechin-3-O-gallate exhibited the IC_{50} values less than arbutin.

The melanin inhibition of compound Quercetin-3-*O*-α-L-rhamnoside in B16F10 cells was found to be comparable to that of positive control arbutin; and this compound exhibited better anti-melanogenesis activity in comparison to positive control arbutin. It has been reported earlier that the compound (-)-epicatechin-3-*O*-gallate isolated from green tea exhibited more than 60% tyrosinase inhibition at 40.0 µM. The anti-tyrosinase activity of compound Myricetin-3-*O*-α-L-rhamnoside isolated from the leaves of *Cercis chinensis* and compound 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose isolated from the bark of *Paeonia moutan* have been reported before. The tyrosinase inhibitory activity of compound Quercetin-3-*O*-α-L-rhamnoside has not previously been reported.

Cytotoxicity and melanin inhibition by isolated compounds.

Compound 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose exhibited 30% reduction in melanin content at 6.250 µg/ml but the cell viability was 50% while compound Quercetin-3-*O*-α-L-rhamnoside showed 28% inhibition of melanin production at 6.250 µg/ml without being toxic to B16F10 melanoma cells (cell viability = 100%). 10% reduction in melanin production was observed at 1.563 µg/ml by compound Myricetin-3-*O*-α-L-rhamnoside (cell viability=70%) whereas positive control, arbutin exhibited 7% inhibition at 608.7 µg/ml while the cells were viable (Table 1). Kojic acid showed no significant toxicity to B16F10 cells at the highest concentration tested and exhibited similar reduction in melanin content; 60% at 3.1 and 25 µg/ml.

Expression of the tyrosinase gene in *C. siliqua*-treated mouse melanocyte cells.

It was considered beneficial to determine whether inhibition of the mushroom tyrosinase and overall melanin production inhibition in mouse melanocytes by CS leaf extracts was related to the inhibition of gene activity at the transcriptional level. Therefore, the semi-quantitative PCR was applied to determine the degree of expression of tyrosinase mRNA following treatment of mouse melanocytes (B16F10) with ethanol leaf extract, compound Quercetin-3-*O*-α-L-rhamnoside treated cells and kojic acid. In comparison to untreated cells, kojic acid, ethanol extract and compound Quercetin-3-*O*-α-L-rhamnoside exhibited 5.1%, 46.4% and 61.5% inhibition, respectively, of the expression of mouse tyrosinase gene (Figure 1b). The gene for GAPDH served as the housekeeping gene. At the transcription level, ethanol extract and compound Quercetin-3-*O*-α-L-rhamnoside (25 µg/ml) exhibited slight tyrosinase inhibitory activity compared to control (untreated cells) (Fig. 1b).

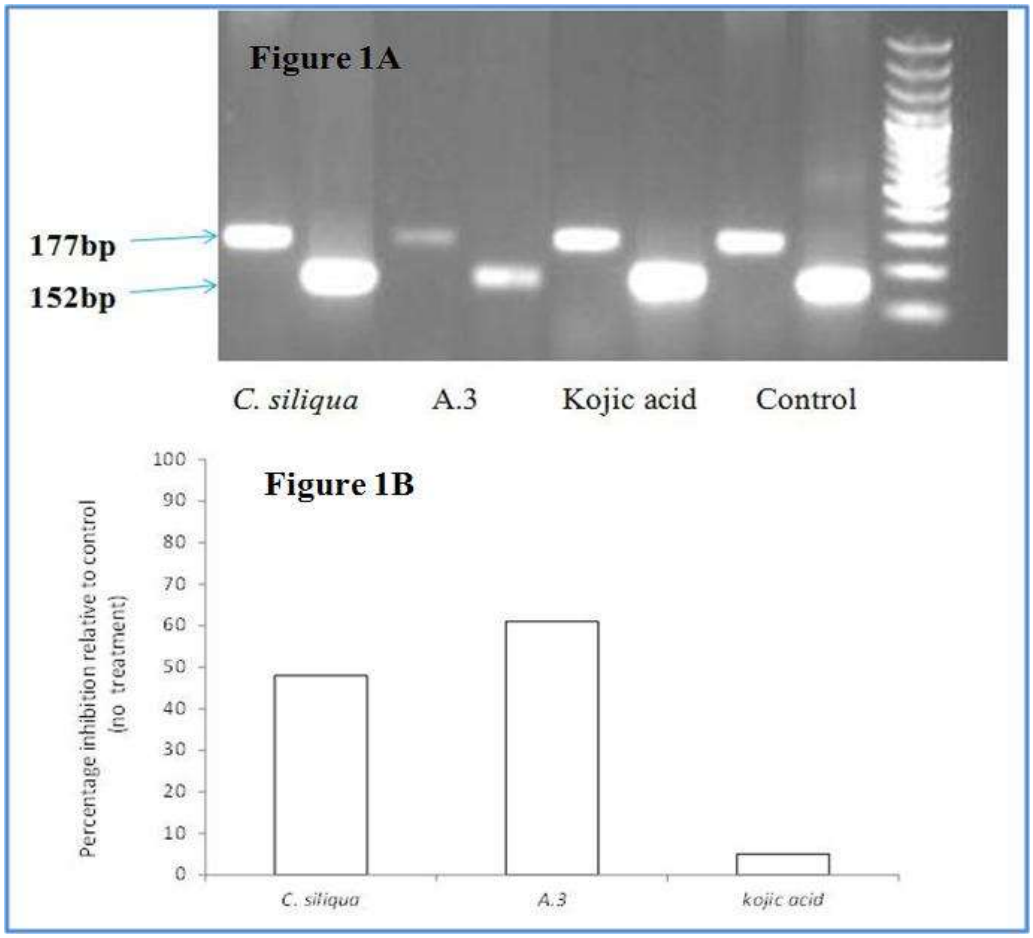

Figure 1. Reduction of tyrosinase mRNA level by *Ceratonia siliqua* (ethanolic extract of the leaves) extract and quercetin-3-*O*-α-L-rhamnoside **(A.3)**. The extract, **(A.3)** and kojic acid were used at the same concentration (25µg/ml). (A) Agarose gel electrophoresis image of tyrosinase (177bp) and GAPDH (152bp) in samples treated with *C. siliqua* and the controls. (B) Percentage inhibition of tyrosinase gene expression in samples treated with *C. siliqua* extract, quercetin-3-*O*-α-L-rhamnoside **(A.3)** and kojic acid compared to untreated control samples (based on quantification of gene expression in A).

In order to statistically quantify the level of tyrosinase gene expression inhibition in mouse melanocyte cells by CS leaf extracts, we performed RT-qPCR on RNA isolated from CS, DMSO and lipoic acid treated mouse melanocyte cells. In this instance, lipoic acid was used as a positive control as it is known to decrease pigmentation by reducing the activity of the microphthalmia-associated transcription factor, which regulates the transcription of the tyrosinase gene (Lin et al, 2002). B-Actin (a common housekeeping control) (Huggett et al., 2005) and

carnitine palmitoyltransferase 2, which showed constant expression levels when analysed with the Genevestigator V3 database (Hruz et al., 2008), both served as the housekeeping genes . Levels of mRNA encoding tyrosinase were found to be down-regulated by CS as compared to the untreated control values; expression was decreased by 75% at the concentration of 100ug/ml (Figure 2). This evidence supports results obtained by Momtaz (2007) who showed that constituents from *Ceratonia siliqua* extract have melanin inhibitory effects. The constituents are therefore responsible for down-regulating the gene expression of tyrosinase as is the case with lucidone from the fruits of *Lindera erythrocarpa* (Kumar et al., 2010) and hirsein from the leaves of *Thymelaea hirsute* (Miyamae et al., 2009). Thus results from this study suggest that a *C. siliqua* leaf extract acts as a potential melanin production inhibitor, targeting both tyrosinase gene expression and tyrosinase enzyme function, and therefore can be used in de-pigmentation products.

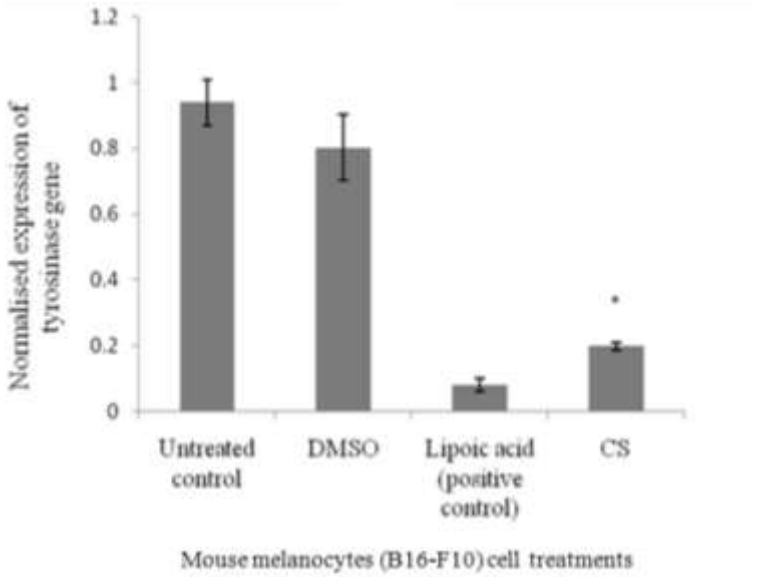

Figure 2. Expression of TYR mRNA in CS-treated and control B16 melanocyte cells. Expression of the tyrosinase gene in all samples was normalized to the expression levels of both of β-Actin and *cpt2* mRNAs. Treatments were performed in triplicate and the mean expression of samples was calculated relative to those of untreated controls. The 100µg/ml CS (*Ceratonia seliqua*) extract significantly inhibited tyrosinase gene expression (*P*-value < 0.05) when compared to the untreated control and positive control (lipoic acid), which was tested at 20µg/ml**.**

Antioxidant activity of crude extract and isolated compounds.

The ethanolic extract of the leaves showed the antioxidant activity of 19.33 µg/ml. The compound (-)-epicatechin-3-*O*-gallate exhibited the highest DPPH scavenging activity with EC_{50} value of 2.26 µg/ml followed by Gallocatechin-3-O-gallate (EC_{50} = 4.5 µg/ml), Myricetin-3-*O*-glucoside (EC_{50} = 7.2 µg/ml), 1,2,3,6-tetra-*O*-galloyl-ß-D-glucose (EC_{50} = 42.55 µg/ml) and Quercetin-3-*O*-α-L-rhamnoside (EC_{50} = 76.59µg/ml). Vitamin C was used as standard control, which showed an EC_{50} value of 3.339 µg/ml (Table 2). Compound Myricetin-3-*O*-α-L-rhamnoside was not tested due to the unavailability of enough quantity needed for this experiment. Melanin is the root cause of blackening of the skin. Its formation beneath the skin proceeds through free radical mechanism. UV-radiations facilitate this chain reaction, and it could be disrupted by selective use of antioxidants, potent enough to poison this reaction. Therefore, crude extract can be considered as either anti-tyrosinase or antioxidant agent. Based on our results, all the compounds showed strong antioxidant activities. The DPPH-scavenging activity of methanol extract was 2 times better than the

Table 2. Antioxidant activity of leaf extract of *Ceratonia siliqua* (EC_{50} values) and isolated constituents (Vitamin C, EC_{50} = 3.339 µg/ml)

Samples	EC_{50} (µg/ml)	mg vitamin C equivalents/g dry weight (EC_{50} value)
Leaf extract	19.33	172.7
Compound 1	2.26	1473.8
Compound 2	42.55	78.47
Compound 3	76.59	43.59
Compound 4	a	a
Compound 5	7.2	462.5
Compound 6	4.5	740.1

[a]Not tested

antioxidant activity of quercetin. Our results differ from the one obtained by other scientists.

Compound Quercetin-3-*O*-α-L-rhamnoside isolated from the methanolic extract of *Sanicula graveolens* exhibited 29% and 25% DPPH discolouration at 100 µg/ml and 10 µg/ml, respectively (Viturro *et al.*, 1999). Yan *et al.*, (2002) demonstrated that compound Quercetin-3-*O*-α-L-rhamnoside isolated before from the methanolic extract of Cranberry fruit (*Vaccinium macrocarpon*) showed DPPH radical scavenging activities with EC_{50} values of 12 µg/ml. The difference in results could be due to different methods used.

Clinical study.

Two samples (Ethanolic leaf extract and ethanolic leaf extract + existing depigmenting agent; Niacinamide) were found to have significant anti-tyrosinase activities. The toxicity and melanin inhibition of the samples were determined on B16F10 mouse melanocytes. Ethanol extract inhibited 45% of melanin production while a cell viability of over 60% was maintained. Ethanol extract with Niacinamide maintained a cell viability of over 90% at 45% of melanin inhibition. These samples were then submitted for clinical trials as they complied with the aforementioned investigations. The results achieved were measured by means of a Chromameter. The information needed to reach the aim of the current study is based on values that represent the smoothness characteristics of human subjects and can thus be classified as interval data, since the quantitative data can be represented in a quantitative manner. A distinction is made between probability and non-probability sampling. In this study use was made of a non-probability sampling method.

The researcher only included respondents who were 20 - 68 years of age. A sample of convenience was used in this study. A total number of nineteen (19) respondents were sampled. The data was captured onto excel and converted to extended excel statistical tests in order to do the analysis. Since the sample was relatively small and consisted of four groups of

twenty (20) respondents, use was made of a parametric test, the (unequal or equal variances) t-test for comparison of two sets of data. The t-test is used to determine whether a given treatment had a significant effect on a population. Statistically significant differences between variables are indicated by a significance value p. If the value of p is equal to or less than 0.05, it gives an indication that there is a statistically significant difference, at the 5% level of significance. The irritancy potential of the samples was determined using 20 volunteers in a patch testing trial. Both samples were found to be non-irritants with irritancy potentials of -34.21 and -14.56, respectively. In a depigmentation clinical trial, ethanol leaf extract was found to improve even skin tone of UV induced pigmentation at 3% after 28 days of application. S_2 was also found to lighten the pigmentation after 28 days at 3%. Ethanol extract was combined with niacinamide for the same trial at the same concentration in which it was found that pigmentation was lightened after only 14 days of application. Ethanol extract was combined with niacinamide and submitted to an additional skin lightening clinical trial on existing pigmentation in volunteers. The pigmentation was found to improve after 14 days when applied to skin, twice daily.

CONCLUSIONS

The present study validated the plant *Ceratonia siliqua* for anti-tyrosinase activity and melanin inhibition in B16F10 melanoma cells, as well as the UV activated pigmentation in human. The extracts from the leaves, bark and fruits of *C. siliqua* showed significant inhibition of the monophenolase activity of mushroom tyrosinase. They also proved to reduce the melanin content of mouse melanocytes without being significantly toxic at that concentration. The results provide both *in vitro* and *in vivo* evidences for the depigmenting effect of ethanol extract. These findings on isolated compounds and crude extracts warrant further clinical investigation for skin pigmentation disorder therapy and cosmetic industry.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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