In vitro effects of the methanolic leaf extract of *Otholobium fruticans* in murine B16 melanoma cells: implications for the treatment of skin hyperpigmentation.

Abstract

Hyperpigmentation is a cosmetically important skin disorder which commonly affects the face and neck regions and impacts negatively on the self-esteem of affected persons. Most of the current treatment agents for hyperpigmentation are cosmetic additives and prescription medications which generally act to suppress melanogenesis. However, many of these products are known to have limited effectiveness, deleterious side effects, and induce adverse reactions especially after prolonged use, hence safe and efficacious treatments are required. Herbal formulations are a putative alternative, considering their use for generations in traditional medicine for treating many diseases, including skin-related conditions. In this study, the methanolic leaf extract of *Otholobium fruticans*, a gardening and ornamental plant common to the South African Cape provinces, was evaluated for its possible anti-melanogenic effects based on evidence from its traditional use. The 50 µg/mL extract concentration was found to be non-toxic to murine B16 melanoma cells, to significantly reduce tyrosinase activity, increase intracellular reactive oxygen species (iROS) levels and down-regulate some melanogenesis-related genes (TYR, TRP-1, TRP-2, MITF and MC1R), except the upregulated β-catenin gene. These findings tend to suggest that the depigmentation potential of the methanolic extract of *O. fruticans* could be mediated through an interplay of mechanisms that inhibit tyrosinase activity, the cAMP-dependent pathway and increased iROS levels. Further studies involving the chemical isolation, characterization and testing of the activities of the constituent compounds in *O. fruticans* are recommended to fully understand the basis for the current traditional uses of *Otholobium* plants for the treatment of skin conditions.

Key words: *Otholobium fruticans*; tyrosinase; melanin; hyperpigmentation; cosmeceuticals; plant extract.

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1. Introduction

Many plants belonging to the *Fabaceae* family have been used for generations in folk medicine for the treatment of different human ailments in many parts of the world [1]. The *Otholobium* genus belongs to this plant family, with some species distributed mainly in the South-Eastern and Eastern parts of Africa, extending into the Mediterranean climate areas of South Africa (the Great Cape Flora region). Plants in the *Fabaceae* family have been reported to have tyrosinase and melanin synthesis inhibition potential in a previous study with the methanolic extract and bioactive compounds from the flowers of *Vicia faba* L (broad bean) [2]. There are now more plants classified under the *Otholobium* genus following the recent re-classification of many *Psoralea* species due to available evidence of similarities in their chemical constituents and biological action [3].

Unlike the *Psoralea* species that have been extensively studied and shown to contain many melanogenesis-inhibiting phytomedicines like psoralen [4,5] the health benefits of *Otholobium* plants have yet to be fully studied. *Otholobium fruticans* is a semi-shrub that grows up to 40 cm tall and is branches can spread as wide as 0.5 to 1 m in all directions, from the centre of the bush. Its tri-foliate leaves have smooth superior surfaces and hairy under surfaces, with are pea-like, triplet flowers. *O. fruticans* is known to spill over walls and terraces and is used in landscaping in fynbos gardens because of its showy sprays of blue and purple colour. This species only occurs on the steep slopes of the mountains in the Cape Peninsula of South Africa, at 180–600 m altitude [61].

The present study therefore aims to evaluate the melanin inhibition potential and mechanisms of action of *Otholobium fruticans* in B16 melanoma cells as compounds in most medicinal plants are generally considered to be relatively safe and efficacious for use.

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2. Materials and Methods

2.1.Plant extraction

The *O. fruticans* plant material was harvested from the Cape Flats Nature Reserve in Western Cape, South Africa during November 2013 and was identified by a plant systematist and curator of the University of the Western Cape Herbarium, Mr Frans Weitz, and a voucher specimen (#BC/27-4) was deposited in the UWC Herbarium. The leaves of *O. fruticans* were allowed to dry at room temperature and 100 g of the dried material was blended with

methanol, thoroughly mixed and filtered after 24 h using a Buchner vacuum filter. The extraction process was repeated twice and the filtrates were combined and evaporated under reduced pressure at 45°C using a rotary evaporator. The final methanol extract was kept under cold conditions (4°C) until use.

2.2.MTT Cytotoxicity Assay

The 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is a colorimetric assay used to determine cell viability based on the ability of succinate dehydrogenase enzymes in the mitochondria of live cells to metabolize yellow MTT to insoluble purple colour formazan product which can be measured spectrophotometrically between 500 and 600 nm wavelengths.[46,4] Briefly, cells were grown at 6.0 x 10^4 cells/mL in 12-well culture plates and treated with plant extracts at various concentrations for 72 h. Thereafter, 10 µL of a 5 mg/mL MTT solution in PBS was added to each well and incubated for 3 hours at 37 ˚C in a 5 % CO_2 condition. Spent medium was removed and 100 µL of DMSO was added and the plates were shaken for 5 sec to solubilize the formazan. The absorbance was measured at 570 nm using the microplate reader (POLARstar Omega BMG LABTECH, Germany).

2.3.Determination of Cellular Melanin Content

The experiment was performed according to previously described protocols [6] with slight modifications. Briefly, a stock density of 6.0 x 10^4 cells/mL of B16 melanoma cells was prepared and 500 µL of this solution was dispensed into each well of the 12-well plates and incubated for 24 h to allow cells to adhere to the plate. After incubation, the cells were treated for 72 h with serially diluted concentrations of the extract in 500 µL of DMEM and kojic acid was used as a positive control. After treatment, the medium in each well was dispensed into appropriately labelled Eppendorf tubes and spun at 4 000 rpm for 3 min at 25°C and the supernatant was removed and the tubes rinsed with 200µL of PBS and then lysed with 400 µl of a 1M NaOH solution. The cell suspension was transferred to appropriately labelled Eppendorff tubes and solubilized on heating blocks (Eppendorf Thermomixer Comfort, Merck Chemicals Ltd, South Africa) at 60˚C with mixing at 300 rpm for 60 min. At the completion of solubilisation, 200 µL of the cell suspension was transferred into appropriate 96-well plates and absorbance was read at 405 nm with a microplate reader to determine intracellular melanin content. The lysing solution made up of 400 µL of the 1M NaOH solution which passed through the same processes as the samples, was used as blank.

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2.4.Intracellular Tyrosinase Activity assay

Tyrosinase activity was determined by measuring the rate of L-DOPA oxidation as previously described by Kim *et al.* with some modifications [49]. Cells were treated with the plant extract dilutions as described previously, washed with PBS and lysed in 200 µL of 50 mM Sodium phosphate buffer (pH 6.5) containing 1% Triton X-100 and 0.1 mM Phenylmethylsulfonyl fluoride (PMSF). Lysates were then frozen at -80 ^{o}C for 30 min, thawed in a water bath at 37 ^{o}C for 2 min and then mixed. Cellular extracts were clarified by centrifugation at 12, 000 rpm for 30 min at 4 ^{o}C and the protein content was determined using the Nanodrop ND-1000 spectrometer (ThermoScientific, South Africa). This was followed by addition of 120 µL of a 0.067 M PBS (pH 6.8) solution and 20 µL of the cellular extracts and incubation at 37 ^{o}C for 15 min. Thereafter, 40 µL of a 2.5 mM L-DOPA solution was dissolved in a 0.067 M PBS (pH 6.8) solution in a 96-well plate and immediately monitored for the formation of dopachrome (□ = 3700 $M^{-1}cm^{-1}$) by measuring the linear increase in absorbance at 475 nm for 60 min.

$$\text{Tyrosinase activity} = \frac{K}{10^{-6} \in \times V \times 2.5 \times 0.1}$$

Where K = Slope of the curve

V = Test volume of cell extract, and

$\in$ = Dopachrome extinction coefficient

2.5.Dihydroxyphenylalanine (DOPA) Staining Assay

The DOPA-staining assay was performed as previously reported by Pintus *et al.* with slight modifications[50]. Briefly, cells were treated with extracts for 72 h as described in the previous section, harvested with lysis buffer and the supernatant was collected for analysis of the protein content using the Nanodrop ND-1000 spectrometer. Protein extracts (5µg) were mixed with 10 mM Tris-HCl buffer, pH 7.0, containing 1% SDS, 25% glycerol, 1% Bromophenol blue without mercaptoethanol or heating and resolved by 8% SDS-polyacrylamide gel electrophoresis at initially 100V until gel front enters the resolving gel; then at 200V until the end of the run. After running the gel, it was rinsed in 0.1 M phosphate buffer (pH 6.8) and equilibrated for 15 min in the same buffer. The gel was then transferred to a staining solution containing 0.1 M phosphate buffer (pH 6.8) with 5 mM L-DOPA incubated in the dark for 4 h at 37 ^{o}C. Tyrosinase activity was visualized in the gel as dark melanin-containing bands.

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2.6.Evaluation of intracellular Reactive Oxygen Species (ROS)

Evaluation of iROS was done as described by Koptyra *et al*. with slight modifications [51]. Briefly, cells were cultured in a 12-well plate at a density of 6.0 x 10^4/mL and treated with 100 µg of *O. fruticans* extract for 72 h and 300 µg of kojic acid for 48 hours. After treatment, cells were washed with PBS and stained with 7.5 µM of CM-H_2 DCFDA (Invitrogen, USA) prepared in PBS from a DMSO stock solution and incubated for 30 min at 37°C in a humidified CO_2 incubator. The cells were then washed twice with ice-cold PBS and 10,000 events analyzed on a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen, San Diego, CA, USA) fitted with a 488 nm argon laser.

2.7.RNA extractions and cDNA synthesis

Confluent murine B16 melanoma cells were treated with the *O. fruticans* extract in T25 flasks and with kojic acid (as positive control); some cells were untreated and used as the negative control. Total RNA was extracted from the cells using the Qiagen RNeasy minikit (Qiagen, Germany). The additional on-column DNAse digestion was done using the RNase-free DNase purification kit (Qiagen, Germany), which was included to eliminate genomic DNA contamination. The quality of the RNA samples was assessed using the Nanodrop ND-1000 spectrometer (ThermoScientific) by obtaining the OD260/280 and OD260/230 ratios. Further evaluation of the RNA quality was done using a Seakem® LE Agarose (Lonza Rockland, USA) gel electrophoresis for all the RNA samples and the extracted RNA was then used to prepare cDNA with the cDNA synthesis kit (ThermoScientific, South Africa).

2.8.PCR Primers

Gene-specific primers used for qPCR are shown in Table II. The tyrosinase gene (TYR), tyrosinase-related protein-1 gene (TYR-1), tyrosinase-related protein-2 gene (TYR-2), microophthalmia associated transcription factor (MITF) gene [51], melanocortin-1-receptor gene (MC1R) [52], and β-Catenin gene [53] were previously reported sequences. Each primer of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was designed to be 20 bp long using the NCBI Primer-BLAST algorithm, accessible at https://www.ncbi.nlm.nih.gov/tools/primer-blast/. The oligonucleotide sequences were sent to Inqaba biotech http://www.inqababiotec.co.za/ for synthesis and the primers were delivered as a lyophilized pellet. A 10 µM working stock solution was prepared by re-

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suspending the pellet in 1X TE buffer (10 mM Tris, pH 7.5 to 8.0, 1 mM EDTA). The primers were finally stored at -20 °C.

2.9.Analysis of gene expression profiles of the genes using qPCR

Expression profiles of the genes were analysed via qPCR in the control, extract-treated and kojic acid-treated B16 melanoma cells and the housekeeping gene, GAPDH was used as the calibrator. All reactions were performed on the LightCycler® 480 System (Roche diagnostics) instrument.

2.10. Morphological evaluation of cells

B16 melanoma cells were cultured in DMEM in 12-well plates at 6.0 x 10^4 cells/mL and treated after 24-hour incubation at 37°C in a humidified incubator after attachment to the bottom of the plates. Cell morphology was studied using the Zeiss light microscope (Carl Zeiss, Germany).

2.11. Statistical Analysis

Data are expressed as means ± SEM. The values were analyzed using the One-Way ANOVA, followed by Tukey's multiple comparism test on the GraphPad Prism version 6.05 software for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. Results

3.1.Cytotoxicity and melanin-inhibition effects of kojic acid

Kojic acid is widely used as a positive control in experimental studies of melanogenesis due to its inhibitory effects on melanin synthesis[6)] and is an ingredient in many products used for the treatment of hyperpigmentation. Thus, there need to determine the safe concentration of this product that can inhibit melanin synthesis in the cell model used in this study since the effects of the *O. fruticans* extract will have to be compared with it for benchmarking purposes.

Figure IA shows the cytotoxicity screening of kojic acid at increasing concentrations over 48 h and 72 h durations respectively, using the MTT assay. The results show that after 48 h treatment, the 300µg/ml concentration of kojic acid only resulted in a 12.3% reduction in cell viability relative to the control, albeit not statistically significant. (Figs. 1A). On the other

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hand, treatment for 72 h resulted in a statistically significant reduction in cell viability even at lower concentrations (Fig. IB), hence only exposure to kojic acid for 48 h was non-toxic (Figs. 1A; 2A) and considered appropriate for this study, since this compound is used a control for the plant extract.

Following the results in Figure 1, the non-toxic 300 µg/ml concentration at 48 h exposure was selected for melanin synthesis experiments and found to result in a 17.6% reduction (Fig. IIB), albeit not statistically significant when compared with the control. The 6% dimethylsulphoxide (DMSO) solution used as the positive control, produced significant reduction in melanin synthesis.

3.2. Cytotoxic and melanin inhibition effects of *O. fruticans* extract on B16 melanoma cells

The cytotoxic effects of the methanolic leaf extract of *O. fruticans* were evaluated using the MTT assay, following a 72-h exposure of murine B16 melanoma cells to the extract. Testing was done with serial dilution concentrations of the methanolic extract (12.5, 25 and 50 µg/mL) respectively, while 6% dimethylsulphoxide (DMSO) was used as the positive control. The respective cell viability values recorded at these dilution concentrations of the extract were 99.8%, 91.9% and 79.2%, indicating a dose-dependent decrease which was however, not statistically significant when compared with the untreated control. The concentrations of the kojic acid (100µg/mL) and the positive control (6% DMSO) used, however showed significant decrease in cell viability (Fig. IIIA).

For intracellular melanin synthesis, treatment with the 12.5, 25 and 50 µg/mL extract concentrations produced 120.9%, 115.6% and 61.1% values respectively, relative to the untreated cells, indicating a dose-dependent decrease in intracellular melanin which was only statistically significant at the 50µg/mL extract concentration (38.9% reduction; $p<0.05$) as well as at the 100µg/mL kojic acid concentration (66.0% reduction; $p<0.001$), compared to the untreated control (Fig. IIIB). Thus, the 50µg/mL concentration was considered to be a safe, non-toxic concentration of this plant extract and was the concentration of choice for subsequent experiments. As shown in Fig. IIIA, most of the cells died following DMSO treatment, hence melanin synthesis by the DMSO-treated cells was absent in Fig. IIIB.

3.3. Cytotoxic effects of the *O. fruticans* extract on normal keratinocytes

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It is known that melanocytes interact with keratinocytes at the basal layer of the skin, at the ratio of one melanocyte to 30-40 keratinocytes, to form epidermal melanin units.[7)] Therefore, any substances that affect the normal functions of melanocytes could affect proximal keratinocytes. In this study, the effects of all treatments were evaluated on the HaCaT cell line, derived from spontaneously transformed, aneuploid immortal adult human skin keratinocytes. The 50µg/mL concentration of the methanolic leaf extract of *O. fruticans* was considered safe to normal HaCaT cells, as no significant difference was observed in cytotoxicity when compared with the untreated control after 72 h exposure (Fig. IV). However, treatment with the positive control (6% DMSO) resulted in significant cell death. Thus, the 50µg/mL of *O. fruticans* extract could be considered to be safe for both the melanocytes and neighbouring keratinocytes, following treatment for 72 hours (Fig 2).

3.4.Effects of the *O. fruticans* extract on murine B16 melanoma cell morphology

Murine B16 melanoma cells were treated with the 50µg/mL of the methanolic leaf extract of *O. fruticans* for 72 h and 6% DMSO was used as positive control. As shown in Fig. V, the *O. fruticans*-treated cells were smaller in size (Fig. VB) while cells treated with 6% DMSO (Fig. VC) appeared more round-shaped, a deviation from the typical dendritic, oval or fusiform shape of mature melanocytes as seen in the untreated control cells (Fig. VA). However, detailed morphological features of the melanocytes could not be observed at the magnification used in this study.

3.5.Tyrosinase enzyme activity assay

In melanogenesis, L-tyrosine is first converted into L-dihydroxyphenylalanine (L-DOPA) by tyrosinase, the rate limiting enzyme of melanin synthesis. [59] In this study, the intracellular tyrosinase enzyme activity in B16 melanoma cells was determined by measuring the rate of L-DOPA oxidation by tyrosinase in cell lysates. Briefly, a mixture of L-DOPA in phosphate-buffered saline (PBS) solution was added to separate samples of cell lysates, and PBS served as control. The results showed that the usually clear L-DOPA solution was oxidized to a pink-coloured dopachrome when active tyrosinase enzyme was added to the L-DOPA-PBS mixture, and the absorbance was monitored every minute, for 60 mins.

An enzyme-substrate reaction kinetics analysis was done, using the Michaelis-Menten plot (Fig. VI) and the results showed that tyrosinase enzyme activity in the lysates from control cells (blue hyperbolic plot), was 512.15 ± 17 µmol/min (Table I), while no enzyme activity

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was recorded for the lysates from B16 cells treated with *O. fruticans* for 72 h, represented by the horizontal pink line in the Michaelis-Menten plot. Thus, inhibition of tyrosinase enzyme activity was more in the cell lysates treated with *O. fruticans* compared with the untreated control. No tyrosinase activity was recorded from the lysates of cells treated with kojic acid (red plot), the mixture of PBS and L-DOPA (yellow plot) as well as with PBS only (green plots), indicating tyrosinase enzyme inhibition (Fig. VI).

Thus, both the 50µg/ml concentration of *O. fruticans* extract and 300 µg/mL kojic acid concentration, showed potent tyrosinase inhibition effects in B16 melanoma cells.

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3.6.L-Dihydroxyphenylalanine (L-DOPA) staining for intracellular tyrosinase activity

The effect of the methanolic leaf extract of *O. fruticans* on intracellular tyrosinase activity was further confirmed by zymography, a technique used to assess the enzymatic activity either *in situ* or by separation electrophoresis. The B16 melanoma cells were treated with 50 µg/mL of the extract for 72 h and with 300 µg/mL of kojic acid for 48 h, respectively. Tyrosinase activity was detectable with a visible band in the untreated control cells while no visible bands were seen in the *O. fruticans* and kojic acid-treated cell lysates (Fig. VIIA & B). These findings are in agreement with the results from the L-DOPA oxidation assay, further confirming the possible anti-melanogenic effects of the extract.

3.7.Flow cytometry analysis of the effects of *O. fruticans* on intracellular ROS (iROS)

The treatment of B16 melanoma cells with the methanolic leaf extract of *O. fruticans* was seen to cause elevated iROS, using the cell-permeant dye chloromethyl-2’, 7’-dichlorofluorescinediacetate (CM-H2DCFDA) assay in the flow cytometer. The overlaid histograms in Figure VIII show the unstained control cells (black), the stained control cells (red) as well as the *O. fruticans* extract-treated cells (dark-green) and kojic acid-treated cells (light-green). The shift of the red and light-green histograms to the right relative to the black histogram indicates an increase in iROS caused by staining (Fig. VIIIA) and by staining and treatment with *O. fruticans* extract (Fig. VIIIB). However, treatment with kojic acid did not cause significant increase in iROS (Fig. VIIIC).

3.8.Effects of the *O. fruticans* extract on the expression of melanogenesis-related genes

To determine the mechanism by which *O. fruticans* inhibited melanin synthesis, the expression of six different genes involved in various melanogenesis pathways was evaluated

using the real-time quantitative polymerase chain reaction (RT-qPCR) test. Experiments were done in triplicates and the relative expression of each gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a housekeeping gene. Figure IXA shows that 5 of the 6 melanogenesis-related genes were down-regulated viz: TYR (-1.557 ± 1.826), TRP-1 (-2.987 ± 3.237), TRP-2 (-1.740 ± 1.265), MITF (-2.943 ± 3.099), and MC1R (-0.540 ± 4.837) while the β-Catenin gene was up-regulated (5.093 ± 4.837).

Kojic acid is used as positive control in melanin inhibition experiments due to its known inhibitory effects on melanin synthesis.[7] Unlike in cells treated with *O. fruticans*, only four genes were upregulated, namely, TYR (3.810 ± 3.593), TRP-1 (3.077 ± 2.835), MC1R (4.567 ± 4.494) and β-Catenin (0.817 ± 1.404) following Kojic acid treatment while TRP-2 (-3.847 ± 3.284) and MITF (-2.307 ± 2.48) were down-regulated (Fig. IX B).

4. Discussion

Medicinal plants have been used as traditional treatments for many human diseases for thousands of years and in many parts of the world, especially in most rural areas of developing countries where plants remain the primary source of medicine [12]. Natural products are an abundant source of biologically active compounds, many of which have provided the basis for the development of new lead compounds for the pharmaceutical industry [55]. However, most developing countries have huge medicinal and aromatic plant resources that remain untapped.

Cosmeceuticals are increasingly popular alternatives to standard depigmenting agents [13] possibly because of their affordability and ready availability in most retail outlets [14]. Some cosmeceutical products formulated with plant-derived phytochemicals have been shown to have varying cellular actions for various skin pigmentation-related diseases [14,15]. To the best of our knowledge, there is currently no evidence-based information on the biological effects of *O. fruticans* on skin pigmentation, hence the present study was done to evaluate the melanin-synthesis inhibition potential as well as other mechanism(s) of action of the methanolic leaf extract of this plant.

The anti-melanogenic effects of the *O. fruticans* extract were evaluated using murine B16 melanoma cells which are widely used for this purpose, possibly because their relative ease for *in vitro* culture and their biochemical similarity with normal human melanocytes [16]. Kojic acid, which has known inhibitory effects on melanin synthesis, was used as the positive

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control [l7]. The results showed that *O. fruticans* inhibited melanin synthesis as much as Kojic acid but without the side effects seen with treatment with kojic acid, especially at high concentrations [60]. An effective therapeutic treatment formulation for skin depigmentation must have no toxic effects on both the target cells and the surrounding tissues. One melanocyte is known to interact with 30-40 keratinocytes to form an epidermal melanin unit, with several such units present at the basal layer of the skin [5].

The 50 µg/mL concentration of the *O. fruticans* that caused a reduction in melanin synthesis was not toxic to the normal, non-cancerous HaCaT keratinocytes (Fig IV) used in this study, with supporting evidence from the cell morphology images (Fig. V) which showed no significant changes in the extract-treated cells compared with control cells.

The Fabaceae family is one of the most studied group of plants [17,18] possibly because of its potent bioactive effects due to their chemical flavonoid, alkaloid, coumarin and other constituents [17] *O. fruticans* is a member of this family of plants known to have melanogenesis-inhibiting effects. Reports on the effects of *O. fruticans* are not available in literature but studies on other members of the Fabaceae family are well documented. In one clinical trial, 2.5% g of the extract of *Glycyrrhiza glabra* (Liquorice), a popular medicinal plant of the Fabaceae family was prepared in a cream and applied topically for four weeks by 100 females, for the treatment of melasma, a skin condition characterized by brown or blue-gray patches or freckle-like spots on the cheeks, forehead, nose and chin [19]. The results showed significant improvement of melasma symptoms when compared to the placebo group, with no side effects reported. In another study, 1.0 µg/mL of glabridin, a bioactive compound derived from Liquorice, was found to inhibit tyrosinase activity in B16 murine melanoma cells without affecting DNA synthesis [20]. Yet another study on bakuchiol, a bioactive compound isolated from *Otholobium pubescens*, showed hypoglycaemic effects in mice [21] as well as anti- ageing effects through retinol- like regulation of gene expression [22]. Since there is no documented information on the constituent phytochemical compounds in *O. fruticans*, studies in this area are recommended, to ascertain if it contains similar compounds as other plants in the Otholobium genus or the Fabaceae family.

To further elucidate the mechanism(s) by which *O. fruticans* reduced melanin synthesis in murine B16 melanoma cells, the effects of its crude extract on tyrosinase activity were evaluated. Tyrosinase (TYR) is a very important enzyme in melanogenesis [23] the primary enzyme that catalyzes the first two chemical reactions of the pathway, viz, the hydroxylation

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of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone [24]. In this study, the *O. fruticans* extract was found to inhibit tyrosinase enzyme activity as did kojic acid (Fig 6), and this was further confirmed with the results from zymography (Fig 7), indicating the effectiveness of this plant extract and its promise as an ant-melanogenesis agent.

Many plant metabolites have been reported to modulate activities in the ageing or hyperpigmentation processes, especially those that involve the tyrosinase enzyme [25,26]. For pigment spot lightening, a number of topical agents like azelaic acid [27] kojic acid [28] retinoic acid (vitamin A) [29], ascorbic acid (vitamin C) [30] and arbutin [31] have been used. However, as a constituent of skin lightening formulations, kojic acid is known to cause skin irritation and such side effects as cytotoxicity, dermatitis, and skin cancer [32]. Thus, cosmeceuticals are increasingly becoming popular alternatives to standard depigmenting agents to overcome these adverse effects [33] and the results from this study tend to suggest that the methanolic extract of *O. fruticans* could be considered a better alternative to Kojic acid as an inhibitor of melanin synthesis.

The impact of *in vitro* or *in vivo* experimental interventions on cells can best be determined not only from the biochemical results obtained and morphological observations, but also from the corresponding effects at molecular levels. Gene expression analysis allows for the comparison of the levels of expression of one or more genes from different samples [56,57] and in this study the RT-qPCR technique was used for accurate determination of the effects of the different treatments on the expression of the melanogenesis-related genes TYR, TRP-1, TRP-2, MITF and MC1R in B16 melanoma cells.

Tyrosinase (TYR) is the rate-limiting enzyme that controls melanin synthesis or production through catalysing the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA) and subsequent prompt oxidation of DOPA to DOPA quinone [24] which then immediately becomes converted into the intermediate DOPAchrome. TRP-1 and TRP-2 together with TYR, are downstream enzymes in the melanogenesis pathways and the re-arrangement of DOPA to dihydroxyphenylindolecarboxylic acid (DHICA) is catalysed by TRP-2 [37].

The microphthalmia-associated transcription factor (MITF) has been documented to be the master-regulator of melanocyte differentiation, proliferation, pigmentation, and survival [38]. As a major transcription factor, it regulates TYR, TRP-1, and TRP-2 expression [9]. As

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such, a decrease in MITF expression leads to the down-regulation of differentiation markers and inhibition of melanogenesis [34]. In this study, MITF was 3-fold and 2-fold down-regulated by *O. fruticans* and kojic acid, respectively and possibly caused the down-regulation of the TYR, TRP-1 and TRP-2 genes. Interestingly, down-regulation of MITF did not lead to down-regulation of TYR and TRP-1 upon treatment with Kojic acid which suggests a possible difference in mechanism but in both cases the TRP-2 gene was down-regulated compared to the control.

The melanocortin 1 receptor (MC1R), also known as melanocyte-stimulating hormone (MSH) receptor or melanin-activating peptide receptor or melanotropin receptor, is a G protein–coupled receptor that regulates skin pigmentation processes, ultraviolet (UV) radiation responses, and melanoma risk [33]. The intracellular cAMP-mediated pathway is known to activate enzyme activity and regulate gene expression [10] in many biochemical processes including melanogenesis [39] The binding of α-melanocyte-stimulating hormone (α-MSH) to MC1R activates cAMP [10,40] and causes an increase in the expression of the MITF gene via activation of the cyclic adenosine 3′,5′-monophosphate (cAMP) response element binding protein (CREB) transcription factor [41] In this study, the MC1R gene was down-regulated by *O. fruticans* but up-regulated by kojic acid treatment, possibly suggesting that the cAMP-mediated pathway was not activated by treatment with *O. fruticans*.

Another important regulatory pathway for melanin synthesis is the Wnt/β-catenin pathway and when this signal pathway is not activated, β-catenin becomes phosphorylated by a complex of many proteins comprising casein kinase 1 (CK1), axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β), and then get degraded by ubiquitin proteasomes [42]. On the other hand, if the Wnt pathway is activated, interactions of Wnt 1, Wnt 3a, and Wnt 8 with frizzled receptors and low-density lipoprotein-related co-receptors 5 and 6 (LRP5/6) leads to the down-regulation of GSK3β [43]. In the absence of this regulator, cytoplasmic β-catenin gets translocated into the nucleus and binds to the MITF promoter, causing transcriptional activation of MITF [44,45,11].

In this study, the β-Catenin gene had a 5-fold up-regulation following the treatment with *O. fruticans* (Fig 9) while the MITF gene was suppressed, suggesting that the Wnt/β-catenin pathway was not activated despite the expression of the β-Catenin gene and the inactivation of the cAMP pathway was predominant. These results are in line with previous findings in

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which *Glabridin*, Liquorice-derived bioactive compound was found to inhibit tyrosinase activity at 1.0 μg/mL without affecting DNA synthesis in B16 melanoma cells [20]. Also, *Vachellia karroo*, a member of Fabaceae family, was reported to significantly down-regulate the expression of the tyrosinase gene [46] Kojic acid treatment produced different results; TYR, TYR-1 and MC1R were up-regulated while TYR-2 and MITF were suppressed while the β-Catenin gene was up-regulated albeit less than observed following treatment with the *O. fruticans* extract. Thus, the downregulation of MC1R, MITF and consequently, TYR, TRP-1, and TRP-2 seen in this study following treatment with the *O. fruticans* extract could be associated with the reduced melanin synthesis seen in murine B16 melanoma cells possibly mediated through the inhibition of the cAMP-mediated pathway and increased iROS.

Intracellular ROS are continuously being generated from many normal cellular events like aerobic respiration, and can cause damage to the body, hence the need to regulate the body's responses through the counteractive actions of many endogenous antioxidant proteins [33]. Depending on the levels produced, iROS could be useful to cell signalling and regulation or injurious to cell survival as by-products of metabolic processes [58]. Some studies have reported that natural depigmentation processes such as hair greying and vitiligo could be caused by iROS generation [34]. Also, some previous studies have reported that MITF, known to play an important role in melanocyte development by regulating the expression of tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1), is often phosphorylated and then degraded in response to iROS stimulation [35,36] consequently leading to depigmentation. In another study, a short pulse of hydrogen peroxide (H_2O_2) applied to B16 murine and human melanoma cells was found to induce a transient repression of melanogenesis via mechanisms that include MITF-mediated regulation of melanogenesis-related enzymes [34]. It is therefore possible that the *O. fruticans*-induced increase in iROS levels (Fig 8) and the concomitant reduction in melanin synthesis (Figs. 1B, 6, 7) seen in this study, are also MITF-mediated. This is supported by the findings from the gene expression experiments in this study that showed down-regulation of TYR, TRP-1, TRP-2/dopachrome tautomerase, MITF, and MC1R genes relative to the untreated control, with only the β-Catenin gene being up-regulated.

5. Conclusions

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The crude extract of *O. fruticans* was evaluated for its depigmenting potential and found to inhibit melanin synthesis as much as kojic acid. This study demonstrated that the inhibition of melanin synthesis by *O. fruticans* occurs through inhibition of cAMP-mediated pathway (Fig. X) and increased reactive oxygen species with no cytotoxicity to the non-cancerous HaCaT keratinocytes. Thus, this plant may possess great potential for use as a candidate anti-melanogenic agent or an ingredient of safe cosmeceutical products for the treatment of hyperpigmentation disorders, while enhancing skin tone. Further research aimed at elucidating the bioactive components of the *O. fruticans* extract is suggested.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table I: Inhibition of tyrosinase enzyme by *O. fruticans* extract

Inhibitor	Concentration (µg/mL)	Km (mM)	Vmax (µmol/min)	Slope K (Km/Vmax)	Enzyme Activity (µmol/min)
Control	0	2.36±0.36	0.2494±0.05	9.48±0.32	512±17
O. fruticans	50	-0.108	0.1065	0	0
Kojic acid	300	-0.0508	0.1065	0	0

Table II: Primer sequences for PCR amplification of cDNA

Primers	FWD 5'- 3'	REV 3'- 5'	Ref	Temp/°C
TYR	GTCGTACCCTGAAAATCCTAACT	CATCGCATAAAACCTGATGGC	(Kwak et al., 2011)	61
TYR-1	CTTTCTCCCTTCCTTACTGG	TCGTACTCTTCCAAGGATTCA	‘‘	61
TYR-2	TTATATCCTTCGAAACCAGGA	GGGAATGGATATTCCGTCTTA	‘‘	62
MITF	GTATGAACACGCACTCTCGA	GTAACGTATTTGCCATTTGC	‘‘	62
MC1R	TGACCTGATGGTAAGTGTCAGC	ATGAGCACGTCAATGAGGTT	(Chang et al., 2015)	61
β-Catenin	ATGGCTACTCAAGCTGAC	CAGCACTTTCAGCACTCTGC	(Jho et al., 2002)	62
GAPDH	ACCCACTCCTCCACCTTTG	CTCTTGTGCTCTTGCTGGG		61/62

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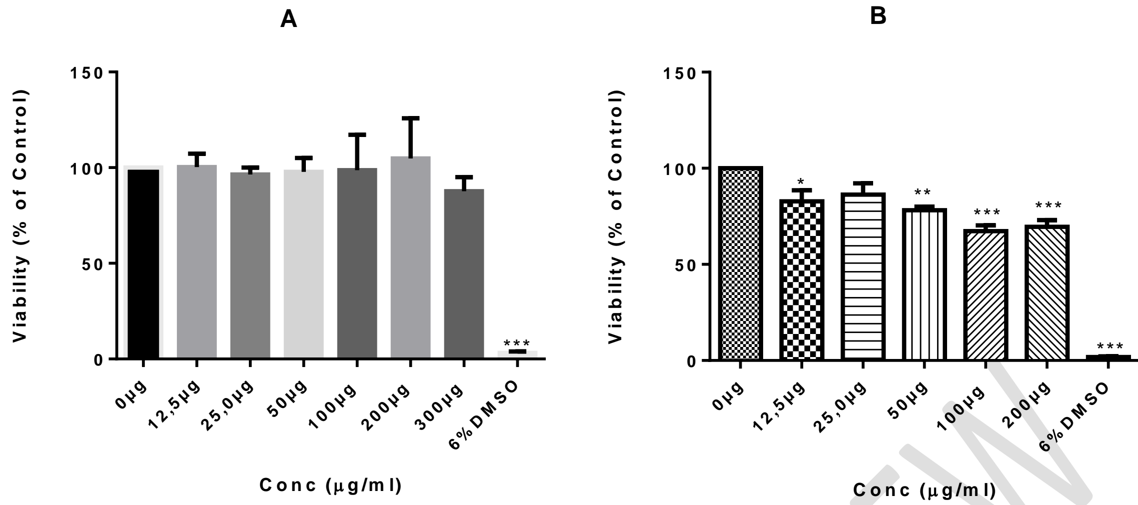

Figure I: Cell viability following treatment with kojic acid for 48 hours (A) and 72 hours (B).

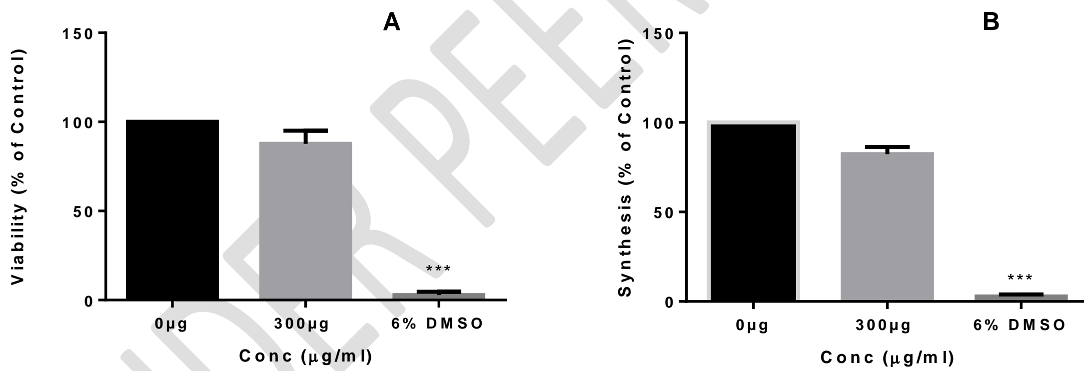

Figure II: Effects of Kojic acid (300 µg/mL) on the viability (**A**) and intracellular melanin concentration (**B**) at 48 hours in B16 melanoma cells.

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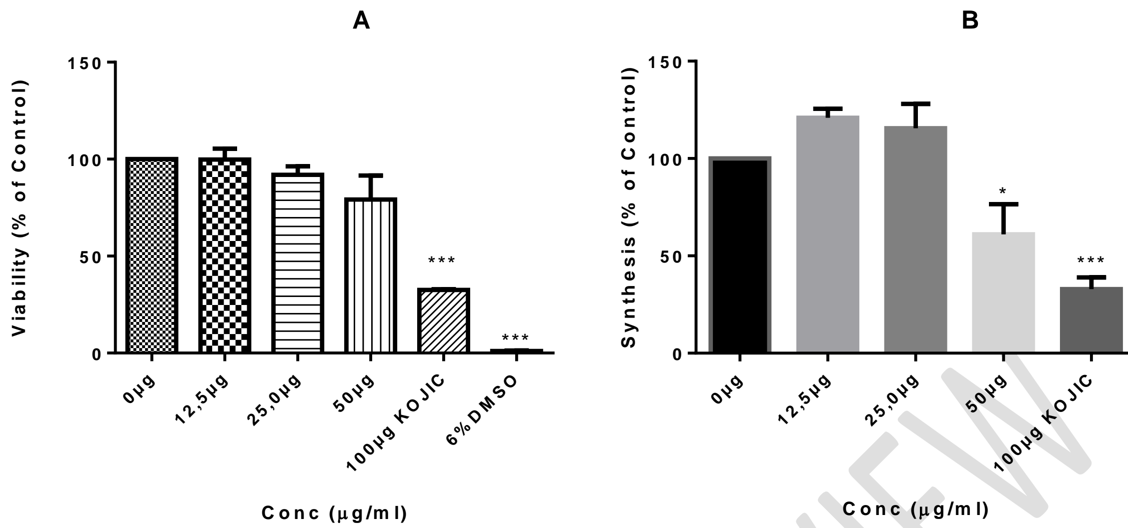

Figure III: Effects of the methanol extract of *O. fruticans* on the viability (**A**) and intracellular melanin percentage concentration; (**B**) of B16 melanoma cells following 72 h treatment.

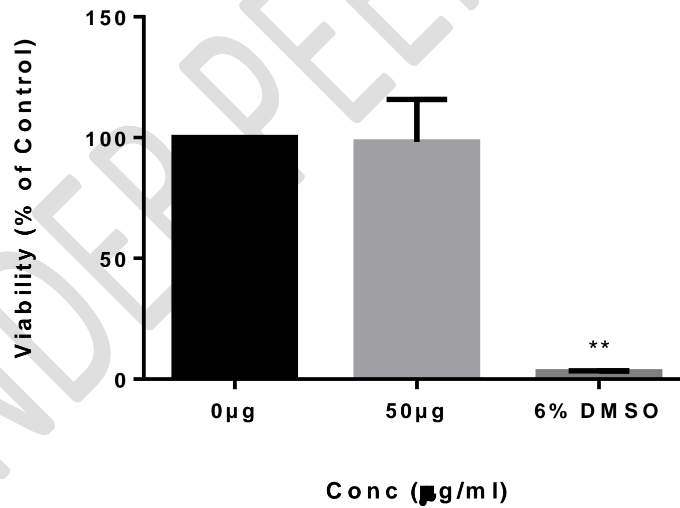

Figure IV: Effects of the 50 µg/mL concentration of *O. fruticans* on HaCaT cells following 72 h treatment.

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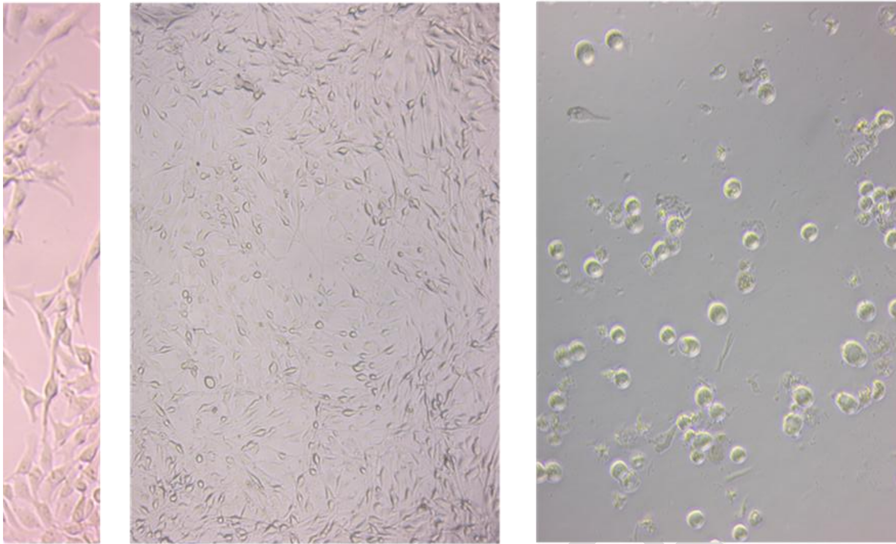

A **B** **C**

Figure V: Photomicrographs of B16 melanoma cells showing effect of *O. fruticans* extract treatment after 72 h. (Mag. x 200) **A**: Untreated control. **B**: 50µg/mL *O. fruticans*. **C**: 6% DMSO.

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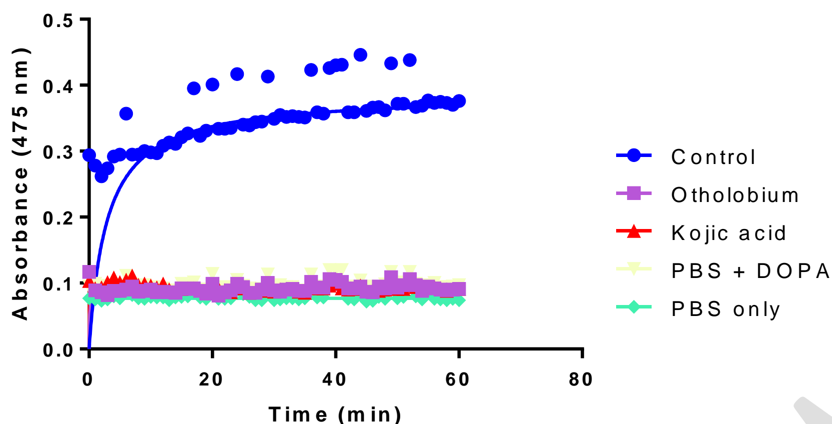

Figure VI: Michaelis-Menten plots for evaluating the inhibition of intracellular tyrosinase activity in B16 melanoma cells, by *O. fruticans* extract using L-DOPA as the substrate. Kojic acid was used as positive control. The Km and Vmax values were obtained from the graph using GaphPad Prism version 6 software.

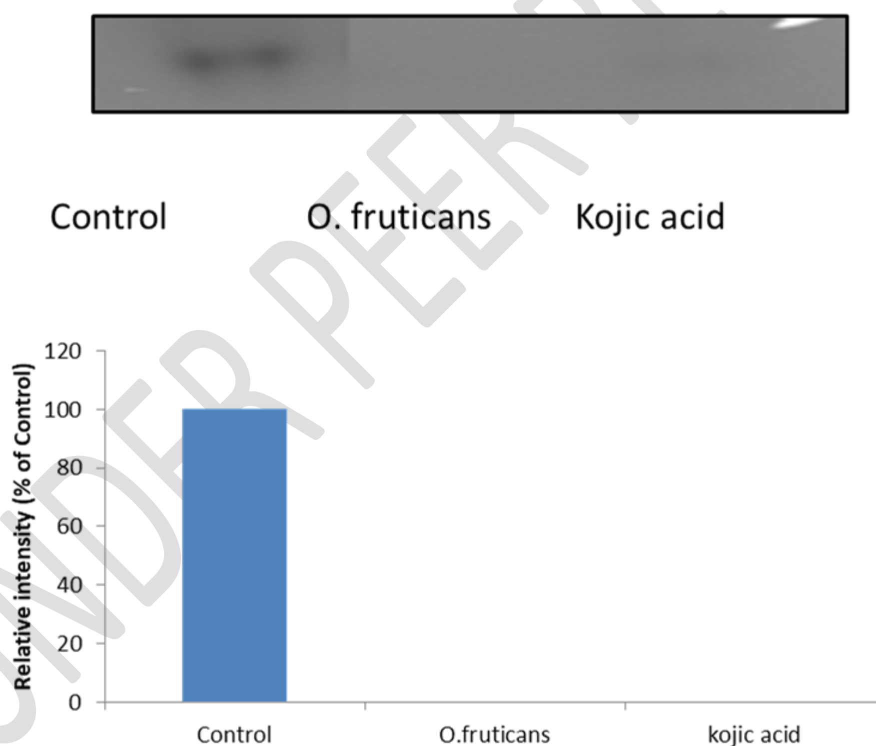

Figure VII: Effects of the methanol extract of *O. fruticans* leaves on B16 melanoma cells by L-DOPA staining. Tyrosinase activity was estimated by zymography (**A**) and the relative intensity of the bands was analysed with Image J software (**B**).

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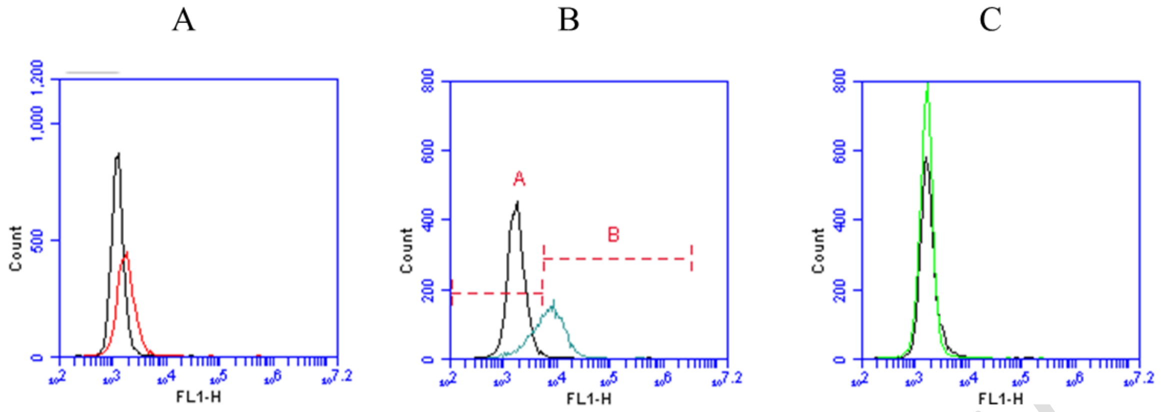

Figure VIII: Representative histograms of cells stained with CM-H2DCFDA dye and evaluated by flow cytometry. The black histogram in (A, B and C) represents the unstained control cells while the red histogram represents the stained control cells; the dark green histogram represents cells treated with *O. fruticans* while the light green histogram represents cells treated with kojic acid.

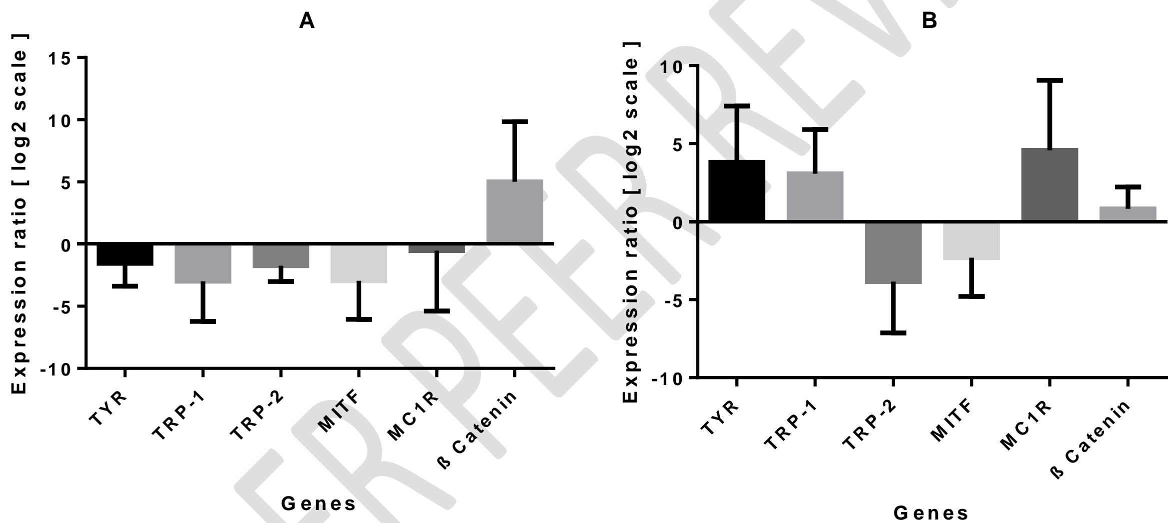

Figure IX: Relative expression ratio plot of genes for *O. fruticans* (A), and Kojic acid (B), compared with untreated control.

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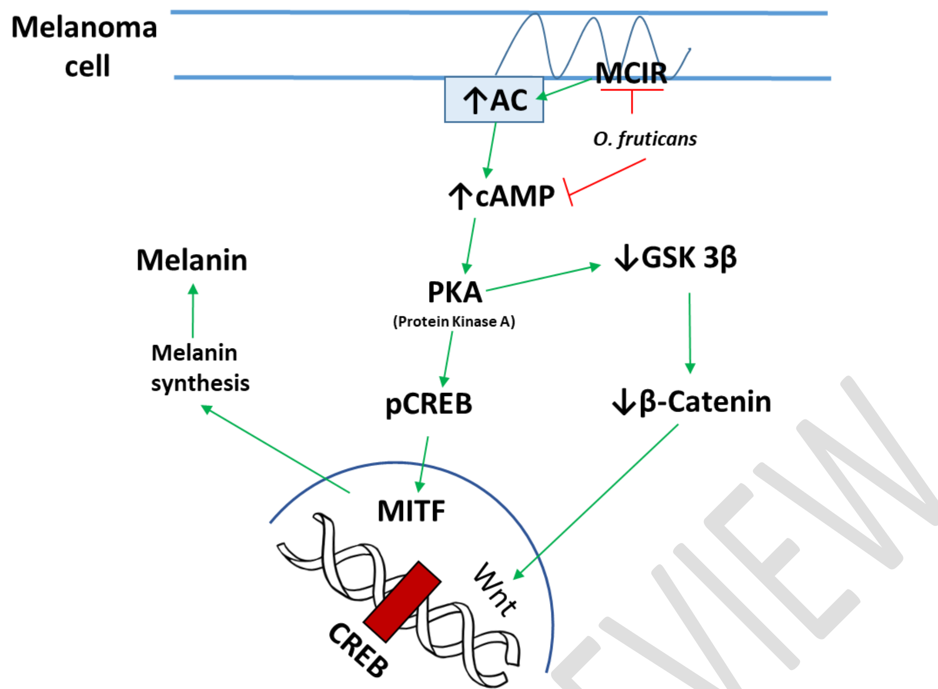

Figure X: Potential pathway by which *O. fruticans* regulates melanogenesis in melanoma cells

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