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Anti-melanogenic effect of *Prunus davidiana* extract in melan-a melanocyte through regulation of OCA-2, TRP-1 and tyrosinase

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Abstract−*Prunus* spp. and locally available plants (used as folkloric medicine) were screened to find a novel and natural anti-melanogenic agent. Based on p-protein promoter reporter assay (PPRA) the candidate plants were screened in the quest for p-protein inhibitor. Expression profiling of key proteins revealed the molecular mechanism of the melanin inhibition as well as TEM analysis revealed melanosome structure. The screened plant extract through PPRA showed significant down regulation of p-protein, which led to melanin inhibition. Another key melanosomal protein like tyrosinase and TRP-1 was also found to be down-regulated. However, TRP-2 was not affected. TEM analysis of treated cells also revealed that the stage IV melanosomes were lowered in number compared to control. The present study shows the plants used in this study possess good anti-melanogenic properties. However, the *P. davidiana* has the highest anti-melanogenic property among screened plant extracts.

Keywords: Pink-eyed Dilution (p) Protein, Melanosome, TEM, *P. davidiana*, Anti-melanogenic Agent

INTRODUCTION

Melanin is a good devil, which is produced to combat against radiation-induced skin damage. However, overproduction causes skin tanning and results in a psychopathic complexity and other pathological symptoms, such as post inflammatory melanoderma, melasma, freckles, and solar lentigo. However, cosmetics provide an alternative, especially in hiding the phenotype but not a solution. Some fairness cosmetics which inhibit melanin synthesis have chemical constituents and mainly inhibit tyrosinase. Tyrosinase is a key enzyme in melanogenesis; however, this enzyme also plays a vital role in other physiological activities. Thus, a long-term use of such cosmetics causes various side effects, so a melanosome-specific target based natural anti-melanogenic agent is being required [1,2].

Among various proteins associated in melanogenesis a very few are melanosomal proteins, and targeting these proteins may provide more safe and effective cosmetics. However, these melanosomal proteins are also shared by other cells; therefore, it is important to target a melanosome-specific protein to develop a targeted cosmetic. Among various known melanosomal proteins the p-protein (product of OCA-2) could be a safe target to design a new cosmetic. In previous study in our lab [2,3] we successfully demonstrated that p-protein can be a better target. P-protein is an 833-amino acid long membrane protein, which consists of a 12 membrane spanning domain [4]. Mutation or deletion in this gene causes oculocutaneous albinism II (OCA2), one of the most common types of albinism [5]. Due to a lack of direct evidence based study, the role of p-protein in melanogenesis is still not clear. However, several reports have been presented on the role of p-protein in melanogenesis: sorting of tyrosinase to melanosomes [6], membrane transport [7] stabilization of melanosomal protein complex [5], regulation of melanosomal pH [8], arsenic sensitivity [5] and control of tyrosinase processing and cellular glutathione metabolism [5,9] and may involve in processing and trafficking of proteins to the melanosome. It has been hypothesized that the OCA2 gene encodes a transport or pore protein vital for melanosome function [6,7,10-12]. Based on previous studies [2], it was aimed to screen locally available various folkloric medicinal plants in a quest of p-protein inhibitor.

In this study, three plant-extracts from Rosaceae family were used; this family includes 95 to 100 genera with several species of economic importance, particularly edible fruit and ornamentals. These plants are also used for medicinal and nutraceutical property, especially in Chinese and Korean-Hanbang traditional medicines [13]. Among the screened plants, the *P. davidiana* (Rosaceae) has received more attention in Korea and is widely cultivated as an ornamental plant, especially in Korea, Japan, Taiwan and China. The stem of *P. davidiana* has been used as folkloric medicine to treat for neuritis, antidiabetic, anti-inflammatory and rheumatism in Korea [14,15]. No specific hazardous effect has been reported for this species; however, most members of the genus *Prunus* produce hydrogen cyanide. It is usually present in too small a quantity to do any harm; in small quantities, hydrogen cyanide has been shown to stimulate respiration and improve digestion, and is also claimed to be of benefit in the treatment of cancer. At a very high concentration, it can cause respiratory failure and even death; however, this is very rare from any natural source (http://www.pfaf.org/USER/Plant.aspx?LatinName=Prunus+davidiana).

Our aim was to screen selected plants based on the literature (use as traditional application and local availability), and further to

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characterize the mechanism of action. Because, the work is a part of comprehensive screening of folkloric medicinal plants in a quest of p-protein inhibitors. Therefore, a luciferase based promoter-reporter screening assay was performed and the respective cytotoxicity toward Melan-a cells was also evaluated. To understand the mechanism of action of the melanin inhibition, the RT-PCR for OCA2 expression, Western blot for MITF, TRP-1, TRP-2 and Tyrosinase was performed. To ensure the cellular physiology and melanosome structural integrity, TEM analysis was performed to elucidate the role of plant extracts in melanogenic pathways.

MATERIALS AND METHODS

1. Materials and Reagents

Roswell Park Memorial Institute-1640 (RPMI-1640), fetal bovine serum (FBS), trypsin EDTA (TE), phosphate buffered saline (PBS), Penicillin-Streptomycin, Lipofectamine LTX&PLUS reagent, PVDF membrane were purchased from Invitrogen Corp. (CA, U.S.A.). Sodium bicarbonate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-DOPA (3, 4-dihydroxy-1-phenylalanine), dimethyl sulfoxide (DMSO), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protein cell extraction kit, Phenylmethanesulfonylflouride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, USA). Goat polyclonal NCKX5, Tyrosninase, Actin antibodies as well as rabbit-antigoat and donkey antigoat were purchased from Santa Cruz Biotechnology Inc.,; Quaigen RNA extraction kit, Quaigen RT-PCR kit, QuantiTect SYBR® Green PCR Kit were purchased from, Quaigen. Primers were purchased from Bioneer (Daejeon, Korea). A luciferase assay kit (Secrete Pair™ Gaussia Luciferase Assay Kit) was purchased from GeneCopoeia (USA).

2. Cell Culture and Plant Extract

Melan-a immortal murine melanocytes were purchased from ATCC (American Type Culture Collection) and cultured in RPMI-1640 medium supplemented with 2 g/L of Sodium Bicarbonate, 6 g/L of HEPES, 10% (v/v) FBS, and 200 nM TPA at 37 °C in a humid atmosphere with 10% CO_2. The cells were passed every 4 days at the concentration of 1×10^6 cells per dish. Passage number of the cells that were used did not exceed passage number 40.

2-1. Reporter Plasmids

Expression vector pEZX-PG02 containing a reporter gene as Gaussia luciferase was purchased from GeneCopoeia. This plasmid confers resistance for the puromycin which was used as a selectable marker in the experiments. Product catalog number, MPRM12122-PG02, containing P protein promoter was used to establish a stable cell line. Negative control, which was replaced with p-protein promoter, NEG-PG02 was used to confirm normal expression of luciferase by the p-protein promoter.

2-2. Stable Expression of the Mouse P-protein Promoter Reporter Gene in Melan-a Cell

For stable transfection, 4×10^5 cells were seeded into 60 mm dish, incubated for 24 h, then transfected with 5 μg of luciferase reporter plasmid harboring the cloned p-Protein promoter sequence by using lipofectamine LTX&PLUS reagents as per manufacturer's protocol. The cells were cultured in Opti-MEM for transfection. After 24 h incubation, the medium was aspirated and fresh medium (RMPI) was added. After 24 h, 1.0 μg/mL of puromycin was added to the medium for selection of transformed cell. Transfected cells were subcultured when cells were 70-80% confluent. After four weeks of starting selection, the cell which was grown was analyzed for promoter assay by using the luciferase reporter gene.

3. Measurement of the Luciferase Activity and Cell Viability Assay

Melan-a cell transfected with pEZX-PG02 was seeded in 96 well plates at a density of 1.5×10^4 cells. At 24 h after seeding, the cells were treated with each sample for 48 h. The media in which cells grew were collected to measure luciferase activity, and cells on the bottom of plates were used to analyze cell viability. Luciferase activity was measured by using Secrete Pair™ Gaussia Luciferase Assay Kit (GeneCopoeia, USA). Luicferase activity was measured as relative light units (RLUs) emitted when 10 μL media were mixed with 100 μL coelenterazine solution. The luminescence was measured by a luminometer (TD-20e, TURNER BIOSYSTEMS, USA). Cell viability was assayed by using MTT reagent. The medium was changed with 100 μL of 0.5 mg/mL MTT solution and the cells were incubated for 3 h. MTT solution was removed and 200 μL of DMSO was added to dissolve the formazan produced in the cells. The absorbance of each well was then read at 540 nm [16].

4. Cell Viability Assay

The MTT based assay was used to determine the cell viability of melan-a cells. Melan-a cell was seeded in 96 well systems at the concentration of 0.5×10^4 cells/well respectively, for 24 h and then treated with the plant extract (0-100 μg/mL) in RPMI-1640 for 48 h. Then again the cells were treated and placed in 10% CO_2 for 24 h. Then medium was replaced with RPMI-1640 with 0.5 μg/mL (100 μL) MTT and kept in the 10%CO_2 incubator for 2-3 h. Further, media containing MTT were removed and 200 μL of 100% DMSO was added to the cells and cell viability was assessed by absorbance at 540 nM using an ELISA reader (Spectra Max 340 microplate reader) [17]. Untreated cells were used as a control group.

5. Measurement of Total Melanin Content

Melan-a cell (2.5×10^4 cells/well) and melan-p cells (2.5×10^4 cells/well) were seeded in 12 well plate systems and incubated for 24 h, and then treated with different concentration of methanol extract (25 μg/mL, 50 μg/mL and 100 μg/mL) of the plant extract. After 48 h cells were treated again and kept at 10% CO_2 incubator for next 24 h. Further, cells were washed with PBS and the cells were detached with trypsin/EDTA solution and harvested. The harvested cells were washed with PBS (2X) for 5 min at 5,000 rpm, and dissolved in 100 μL of extraction buffer (1 N NaOH, 10% DMSO). Afterwards, it was kept at 80 °C for 1 h and transferred to 96 well plate systems for reading under ELISA microplate reader (Spectra Max 340 microplate reader) at 405 nm [17]. Untreated cells were used as the control group for further analysis of results.

6. RT-PCR

Total RNA was isolated from the treated cells and control cells using the RNeasy mini kit (QIAGEN, USA) according to the manufacturer's protocol. Total RNA concentration and quality of the samples were checked using Nanodrop. Elimination of genomic DNA was performed at 42 °C for 2 min and then reverse transcription for the first strand cDNA synthesis was performed with 1 μg

of RNA in a 20 μL final volume at 42 °C for 15 min followed by heat inactivation of reverse transcriptase at 95 °C for 3 min by using the QuantiTect Reverse Transcription Kit (QIAGEN, USA). Real-time PCR was performed using Thermo Scientific PikoReal Real-Time PCR in a 20 μL of reaction mixture containing SYBR green master mix (QIAGEN, USA), 0.1 μg of template DNA, and 0.5 μM of gene-specific primer sets. PCR was carried out for 40 cycles of 30 s at 95 °C, and 30 s at 60 °C, with a final extension step of 5 min at 60 °C. The sequence of the primers (Bioneer, Daejeon, Korea) was: GAPDH, 5′-TGAACGGGAAGCTCACTGG-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (anti-sense); P protein, 5′-TGCGGTGGATAAAGATCACA-3′ (sense) and 5′-TTGGTCCTCCAGCCATTAAG-3′ (anti-sense). Data was analyzed by relative quantification.

7. Tyrosinase Activity Assay

To determine the cellular tyrosinase activity, melan-a cells were seeded in 100-mm dishes (6×10^5 cells/well) for 24 h and then treated with different concentration of methanol plant extract (25 μg/mL, 50 μg/mL and 100 μg/mL) for further 48 h. and again treated after 48 h, for further 24 h and then harvested as described in the previous section. The cells were lysed in lysis buffer (0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% Triton-100X). Further, cells were disrupted by sonication for 30 min at 4 °C and centrifuged at 13,000 rpm for 15 min at 4 °C. Protein quantification of the cell lysate was done by using a protein assay kit (Bio-Rad, USA). All the similar reaction mixtures consisting of 40 μg of protein, 40 μL of 5 mM L-DOPA and 0.1 M PBS (pH 6.8) were assayed on a 96-well plate at 37 °C. Absorbance reading was taken by ELISA reader (Spectra Max 340 microplate reader) at 475 nm and the percentage activation was calculated [3].

8. Electron Microscopy

After the treatment, the treated and control cells were harvested, and washed three times with 0.1 M phosphate buffer (pH. 7.2). These cells were primarily fixed by fixing solution containing 4% paraformaldehyde, 1% gluteraldehyde in 0.1 M Phosphate buffer for 4 h followed by secondary fixation with 1% osmium tertroxide at 4 °C for 30 min. Thereafter, dehydration was performed; using an increasing concentration of ethyl alcohol, i.e., 10 min each in 50, 70, 80, and 90%, while two times treatment in 95% for 15 min, and finally 1 h in 100% and each time it was centrifuged at 4,000 rpm for 5 min at 4 °C. After this step, the sample was treated with propylene oxide for 15 min. For the embedding of the sample, Embed 812 was used, which is a commercial quick-setting epoxy glue having Epoxy resin (mixture A, and mixture B mixed according to manufacturer with 1.4% DMP-30). Mixture A contained Embed 812 (EMS) and dodecyl succinic anhydride, while mixture B had Embed 812 (EMS) and nadic methyl anhydride. The resin (R) and hexylene glycol (HG) was combined in a 1 : 2 ratio, add to sample and again centrifuged as the previous manner after 15 min. and finally, replaced with 100% resin and place at room temperature for overnight. It was allowed to polymerize at 60 °C for three days. Then ultrathin sections of the sample were obtained by Ultramicrotome (MTX ULTRAMICROTOME). The specimen was treated with uranyl acetate and lead citrate for 30 and 15 min, respectively. This specimen was examined by transmission electron microscope (TEM). Cells from each lattice grid were observed and recorded.

9. Expression Profile of Tyrosinase, TRP-1, and TRP-2 and MITF by Western Blotting

To determine the effect of the methanol plant extract on the expression profile of melanosomal proteins like tyrosinase, TRP-1, TRP-2, MITF and possibly transport protein OCA^2 western blotting was performed. Melan-a cells (6×10^5 cells/well) were seeded on 100 mm dish and harvested as described in the previous section. Extraction buffer (40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40 (NP-40), 0.1 mM sodium orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride (PMSF)) containing and a protease inhibitor cocktail (Roche, Mannheim, Germany) was used to lyse the treated cells and control cells, and lysates were cleared by centrifugation at 13,000 rpm for 20 min at 4 °C. Afterwards, the supernatant was collected and quantification of proteins was done by using a protein assay kit (Pierce GST Protein Interaction Pull Down Kit, Thermo Scientific, USA). Albumin was taken as a standard for protein quantification. For western blotting, equal amount of protein was taken and denatured at 95 °C-100 °C and kept at room temperature for 5-10 min in loading dye (Novex, Invitrogen). Afterwards, subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to a PVDF membrane by using 1X transfer buffer. The membrane was further subjected to blocking solution (5.0 gm of nonfat dry milk in 100 ml of TBS) and incubated with antibodies against tyrosinase, TRP-1, TRP-2, MITF and OCA_2. The membranes were then transferred to anti-goat horseradish peroxidase-conjugated secondary antibody. Excess antibody was removed by three times washing with TBS-T buffer at the interval of 15 min. Immuno-reactive bands were detected by chemiluminescence using ECL western blotting detection reagents (Amersham). β-Actin was used as a loading control.

RESULTS

1. Screening of Potential Skin-whitening Compounds

To achieve the goal, melan-a cells were transfected with p-Protein promoter containing reporter plasmid. The transformed cells were selected by selectable marker puromycin. The independent clones which showed the highest activity were used for further study. The transfected cells were treated with #4 (Methyl 5-cyano-6-{[2-(4-methoxyphenyl)-2oxoethyl]sulfanyl}-2-methyl-4-(2-thienyl)-1,4-dihydro-3-pyridinecarboxylate), a known inhibitor for p-protein down-regulation reported by Morya et al. (2014). This compound inhibits melanogenesis by suppressing the expression of p-protein (Morya et al., 2014). The ten plant extracts were screened through the luciferase activity assay. This result revealed that the methanol extract of *P. davidiana* shows inhibition of luciferase activity by 26.1% without showing any cytotoxicity (Table 1).

2. In Vitro Screening of Anti-melanogenic Property

The hypopigmentation effects of the methanol extract of *P. davidiana* was analyzed after the treatment on melan-a cell. From the results, the methanol extract shows dose-dependent inhibition of the melanin content from 25 to 100 μg/mL treatment without affecting the cell viability of melan-a cell (Fig. 1). The concentration of methanol extract of *P. davidiana* more than 100 μg/mL was found

Table 1. Screening of putative p-protein inhibitor from luciferase assay

Concentration	% Luciferase activity	Cell viability
#4 2 μM	78.9±6.1	99.6±4.1
No. 1 Ginkgo biloba (Stem)	86.4±12.5	117.0±4.1
No. 2 Daphne genkwa (Stems, Roots)	81.9±13.3	101.0±4.0
No. 3 Acer mono (Leaf)	74.2±10.2	51.4±10.5
No. 4 Orostachys iwarenge (Whole plant)	116.7±12.4	91.2±1.3
No. 5 Acer mono (Stem-core)	88.9±17.3	104.4±4.3
No. 6 Quercus acutissima (Leaf)	110.7±12.4	83.0±4.3
No. 7 Zanthoxylum piperitum (Branch+Leaf)	79.7±7.1	118.0±5.3
No. 8 Pyrus ussuriensis (Leaf)	103.7±2.2	114.7±8.3
No. 9 Pyrus ussuriensis (Stem)	77.9±9.7	131.8±5.7
No. 10 Prunus davidiana (Stem-Bark)	73.9±3.5	116.4±10.7

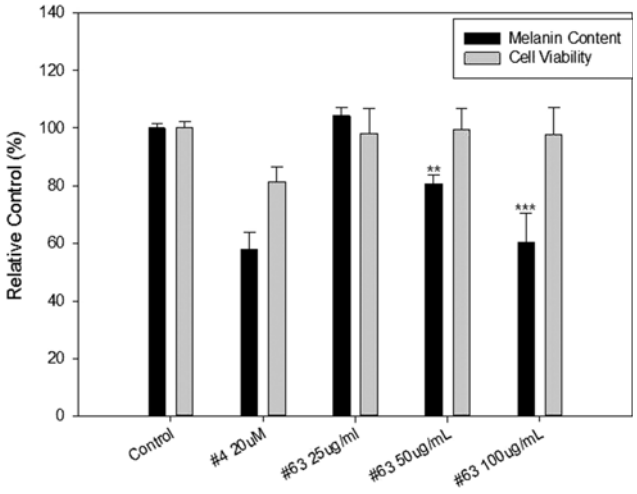

Fig. 1. Showing the dose-dependent inhibition of melanin content after the treatment of methanol extract of *Prunus davidiana* (Stem-Bark) without affecting the cell viability. Data are the mean±standard deviation. *p<0.05, **p<0.001, ***p<0.0001; Student's t-test.

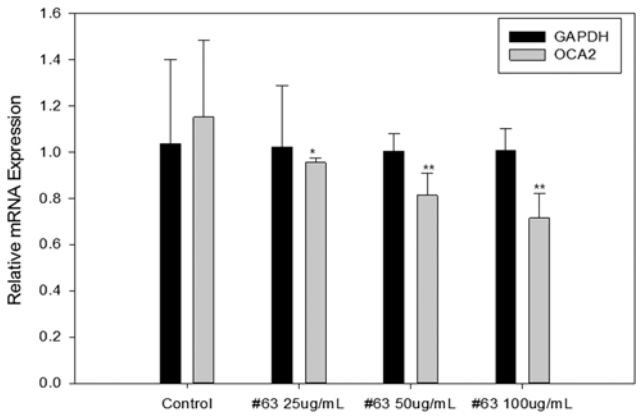

Fig. 2. Showing the dose-dependent down-regulation of mRNA expression of the p-protein after the treatment of methanol extract of *Prunus davidiana* (Stem-Bark) without affecting the constitutive mRNA (GAPDH) taken as control. Data are the mean±standard deviation. *p<0.05, **p<0.001, ***p< 0.0001; Student's t-test.

to be less toxic to the melan-a cell. However, no further inhibition in melanin content was observed beyond 100 μg/mL concentration. At a 100 μg/mL concentration of the methanol extract of *P. davidiana* about 40% inhibition in melanin synthesis was observed. Therefore, 100 μg/mL concentrations were used for further study.

3. Expression Profiling of P-protein

To investigate the effect of extract of *P. davidiana* on p-protein, RT-PCR was performed. mRNA expression profile of the p-protein in response to treatment of methanol extract of *P. davidiana* was observed as down-regulated and a clear dose-dependent pattern was obtained (Fig. 2). It was clearly shown in Fig. 2 that the down-regulation of mRNA expression of the p-protein was dose-dependent. However, the constitutive mRNA (GAPDH) expression remain unaffected (Data are the mean±standard deviation. *p<0.05, **p<0.001, ***p<0.0001; Student's t-test).

4. Effect of *P. davidiana* on Expression Profile of Melanosomal Proteins

The expression profile of melanosomal key protein was evalu-

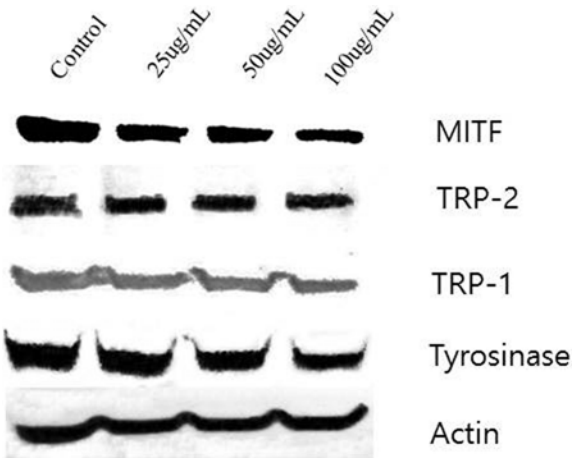

Fig. 3. Showing the effect of the methanol extract of *Prunus davidiana* (Stem-Bark) on the expression profile of the melanosomal proteins after treatment with different concentration on melan-a cells. Lane 1 control, lane 2 25 μg/mL, lane 3 50 μg/mL and lane 4 100 μg/mL. β-Actin was served as loading control.

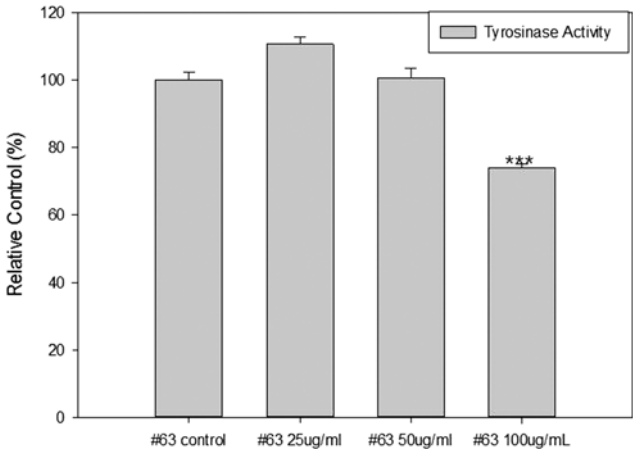

Fig. 4. Showing the effect of methanol extract of *Prunus davidiana* (Sterm-Bark) on the cellular tyrosinase activity. Data are the mean±standard deviation. *p<0.05, **p<0.001, ***p<0.0001; Student's t-test.

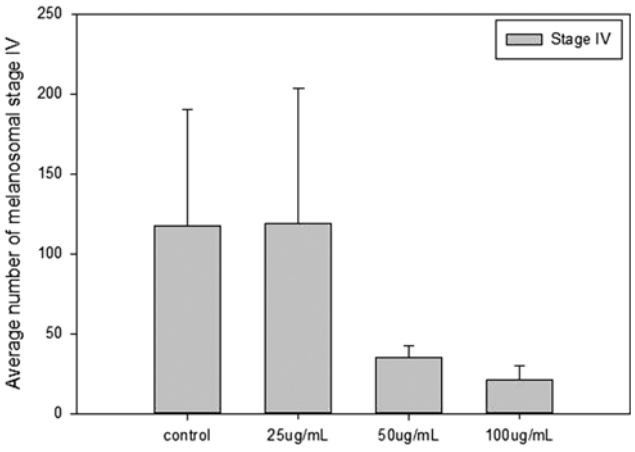

Fig. 6. Average number of melanosomes counted (per unit area) in normal and *Prunus davidiana* treated melan-a cells.

ated by western blot. The tyrosinase was inhibited in a dose-dependent manner in response to methanol extract of *P. davidiana*, in melan-a cells (Fig. 4). The effect of methanol extract on tyrosinase, MITF, TRP-1 and TRP-2 was also examined. The results show methanol extract has a significant effect on the expression of these proteins (Fig. 3).

5. Effect on Cellular Tyrosinase Activity

The melan-a cell was subjected to methanol extract of *P. davidiana* to determine the effect on cellular tyrosinase, and it was revealed that *P. davidiana* lowered the activity of cellular tyrosinase as well (Fig. 4). Although, the effect was observed with 100 μg/mL concentration.

6. Effect on Melanosomal Structure

To ensure the effect of methanol extract of *P. davidiana* on cell morphology and growth pattern of melan-a cells in culture condition, the transmission electron microscope (TEM) view of the cells was compared (Fig. S1). TEM was performed to analyze the structure, stages and number of melanosomes in response to methanol extract of *P. davidiana*. Ultra-structure of normal melan-a cells revealed melanosomes at different resolutions such as grid view, normal melan-a cell, pre-melanosomes, mature melanosomes etc. (Fig. S1). The results showed the embedded (in resin) cells revealed both the control and treated cells were structurally similar except the melanosomes. The dose-dependent effect on melanosome stages was clearly revealed in Fig. 6. It easily distinguished the lower number of stage III and IV melanosomes (Fig. 5).

Stage IV melanosome was predominantly observed in melan-a cell than the methanol extract treated melan-a cell. In methanol extract, treated melan-a cells of the stage II and III of melanosome were higher than the stage IV (Fig. 5). Stage II melanosomes had elongated vesicles and distinct fibrillar structures, whereas in stage III melanosomes melanin synthesis was initiated and the pigment was deposited uniformly (Fig. S1(e)). Significant reduction in the number of stage IV melanosomes was also found in methanol-extracted cells (Fig. 5). However, the melanosomes were found to be slightly lower and spherical in the treated cells (Fig. S1). It was found that the average number of the melanosomes was less in methanol extract treated cells than normal melan-a cells (Fig. 6). When stage I and stage II melanosomes were counted together, the treated cells had higher percentage than the non-treated cells

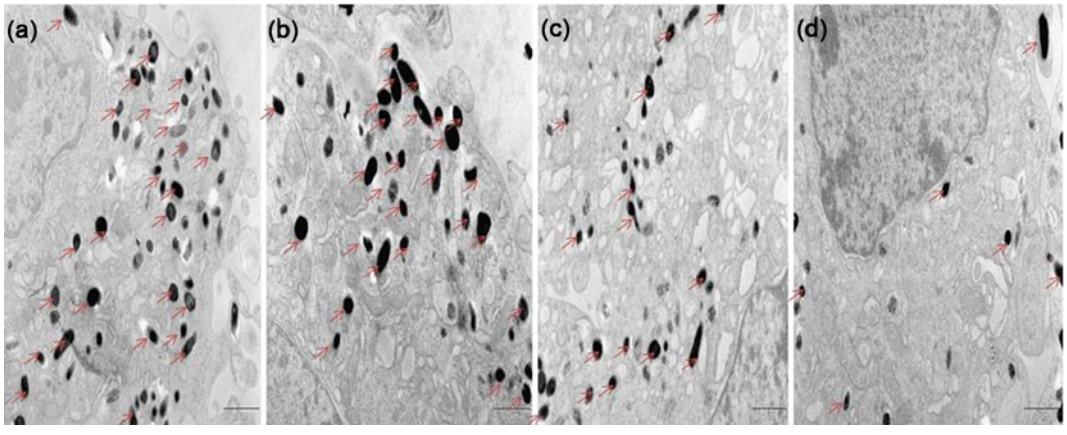

Fig. 5. Comparison of melanosome stage IV in between normal melan-a cells (a), 25 μg/mL treated cell (b), Cell treated with 50 μg/mL (c) and Cell treated with 100 μg/mL (d).

(data not shown). The percentage of melanosome stages III was found to be slightly decreased in treated cells. However, the percentage of stage IV melanosomes was significantly low in treated cells (Fig. 6).

DISCUSSION

Many natural and synthetic anti-melanogenic bioactive fractions have been reported as well as used as cosmetic additives for the treatment of hyper-pigmented skin disorders [1,2]. However, many of them have a limited effect, cause reactions or side effects during long-term use, and are difficult to formulate. Therefore, many efforts have been devoted to screen putative depigmenting agents from natural products. As it has been mentioned, the aim of this study was to find a p-protein inhibitor to develop novel cosmetics. Therefore, in this study a comparative and comprehensive study among different plant extract was carried out. Among these extracts, *P. davidiana* had a significant inhibition of melanin content without any subsidiary toxicity, while treated at concentration of 100 μg/ml. However, it is well known that the mutation or deletion of p-protein in melanocyte directly affects melanin synthesis [3,18, 19]. It was observed that the OCA-2 gene expression was down regulated in a dose-dependent manner, and melanin synthesis was reduced up to 40%. The melan-a cells which showed a down regulation of p-protein mRNA (Fig. 2) also showed melanin inhibition (Fig. 1). P-protein was reported as tyrosine transport facilitator, and mutation or deletion in this protein may affect availability of tyrosine in melanosome, a limiting factor of melanin synthesis [2,19]. Conversely, other reported functions of p-protein may also affect the gross melanin synthesis. The *P. davidiana* mediated down regulation of the p-protein causing significant inhibition of the melanin synthesis without affecting the growth of melan-a (Fig. 1). In present study, tyrosinase expression was down regulated in response to *P. davidiana* on melan a cells in dose-dependent manner (Fig. 3). Previous studies on p-mutant also reported the diminution in tyrosinase expression [20,21]. Total cellular tyrosinase activity was measured to evaluate the effect of methanol extract of *P. davidiana* on melan-a cells. The results from cellular tyrosinase assay showed that cellular tyrosinase activity was significantly inhibited after treatment with the methanol extract at the concentration of 100 μg/mL (Fig. 4). At this concentration, the cellular tyrosinase activity was inhibited up to 30% in melan-a cell. Naturally, the mutated cell was also reported with a lowered activity of cellular tyrosinase [5,20,21].

The morphological behavior of the melan-a cell was not affected by methanol extract of *P. davidiana* as revealed by microscopic examination of the cultured cells (data not shown). The effect of p-protein down regulation, using *P. davidiana*, on structural and numerical alterations of melanosomes in melanocytes, was analyzed by TEM. Results showed that *P. davidiana* significantly changed the number of melanosome in treated cells (Fig. S1, 5). Not only was the average number of stage IV melanosome found to be lowered, but the immature melanosome, i.e., stages I and II, was found to be approximately double in treated cells than the normal melan-a cells. During the study of melanosome stages, the normal melan-a cells were highly pigmented with stage IV melanosomes, whereas 100 μg/mL treated cells contained stage I, II melanosomes predominantly. The melanosomes were slightly lowered and spherical in shape in treated cells. The shape of melanosome was reported oval or elliptical in most of the previous studies [3,22]. The percentage of stage IV melanosomes was significantly low in treated cells. These results showed that *P. davidiana* has an effect on the maturation and number of melanosomes. A study conducted on melanocyte form OCA-2 patient showed the abnormal shape of melanosome and a diminution in the number of melanized melanosomes. The studies on melan-p1 (null at p-locus) cells stated that the numbers of immature melanosome were higher than the mature melanosomes. The melan-p1 cells have no p-protein and lower the number of mature melanosome reflects that the p-protein have a significant role in melanosome maturation. These results were supported by previous studies on p-mutant cells [3,4,19].

The methanol extracts of *P. davidiana* has a regulatory effect on expression of tyrosinase and TRP-1, while TRP-2 remains unchanged. This shows that the other proteins were affected by the p-protein down regulation. These results are similar to the previously reported studies on melan-p1 cells in comparison to melan-a cells [19]. These results support the study which stated that p-Protein regulates the post-translational processing of tyrosinase [10]. However, methanol extracts of *P. davidiana* did not show a significant effect on the expression of TRP-2 protein. The methanol extract lowered the expression of tyrosinase and TRP-1. However, the expression of TRP-2 remained unchanged. This shows that the other proteins were affected by the p-protein down regulation. These results are similar to the previously reported studies on melan-p1 cells in comparison to melan-a cells [19]. The reports suggest that p-Protein regulates the melanin formation through MITF.

This study demonstrates the the inhibitory effect of *P. davidiana* on melanin synthesis and melanosomal biology in melan-a cell may provide a novel agent for skin cell pigmentation. This study concluded that the *P. davidiana* has a significant role in inhibition of melanin synthesis and melanosomal biology. The results revealed that down regulation of p-Protein could obstruct the biogenesis of melanosomes by methanol extract of *P. davidiana*.

However, further characterization and identification of active ingredient (compound/s) is needed for development of *P. davidiana* based cosmetics.

The present study reveals that *P. davidiana* shows low cytotoxicity and high depigmenting activity on melan-a cell. It is revealed that the mechanism of melanin inhibition occurs via p-Protein down-regulation. P-Protein is one of the key melanosomal membrane proteins which has a vital role in melanosome structure and controlling the synthetic pathways of melanin biogenesis. *P. davidiana* has been used as folkloric medicine, and the stem-bark methanol extract can be explored as new whitening agent in cosmetics.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is

available via the Internet at http://www.springer.com/chemistry/journal/11814.

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Supporting Information

Anti-melanogenic effect of *Prunus davidiana* extract in melan-a melanocyte through regulation of OCA-2, TRP-1 and tyrosinase

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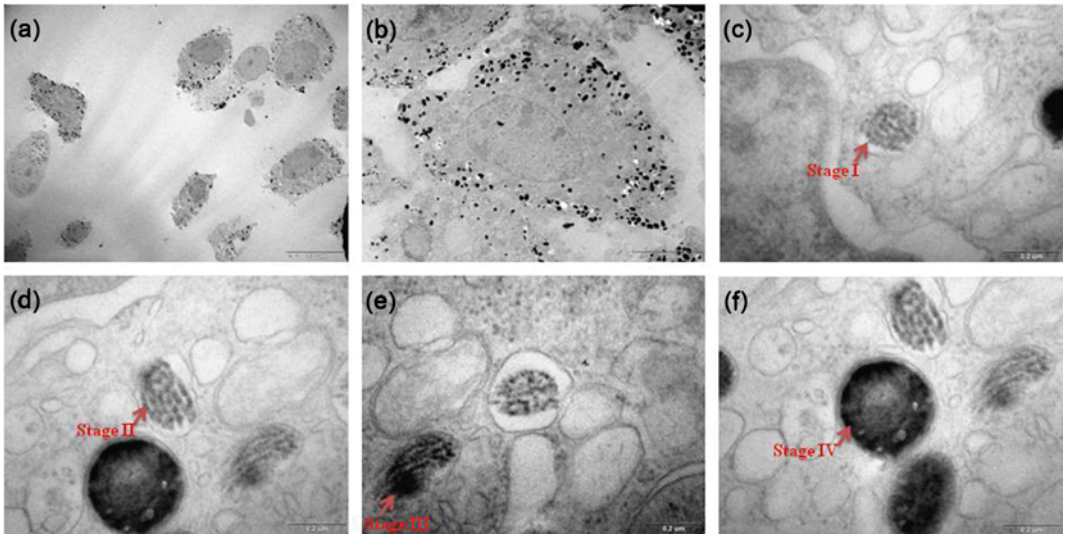

Fig. S1. Normal melan-a cell and their ultrastructure's showing melanosomes viewed under different magnification, (a) grid view (b) normal melan-a cells (c) stage I melanosome (d) stage II melanosome (e) stage III melanosome (f) stage IV melanosome.