Inhibitory effects of Asterina pectinifera extracts on melanin biosynthesis through tyrosinase activity

MIN-HO JEONG, KWANG-MO YANG, JUNG-KI KIM, BYUNG-HYOKK NAM, GI-YONG KIM, SANG-WHA LEE, SU-YEONG SEO and WOL-SOON JO

1Research Center, Dongnam Institute of Radiological and Medical Sciences; 2Test and Certification Team, Marine Bio-Industry Development Center; 3Department of Microbiology, College of Medicine, Dong-A University, Busan, Republic of Korea

Received August 17, 2012; Accepted September 24, 2012

DOI: 10.3892/ijmm.2012.1181

Abstract. The control of melanogenesis is an important strategy in the treatment of abnormal skin pigmentation for cosmetic purposes. The aim of the present study was to investigate the anti-melanogenic effect of Asterina pectinifera (A. pectinifera) extracts by cell-free mushroom tyrosinase assay, cellular tyrosinase assay, melanin content assay and the analysis of related protein expression in melan-a cells. A. pectinifera was extracted with 80% methanol (80-MAP) and further fractionated with hexane (He-AP) and ethyl acetate (EA-AP). In addition, the enzyme extract (En-AP) of A. pectinifera, to which protease was added, was processed. EA-AP and En-AP among A. pectinifera extracts showed strong inhibitory activity against the cell-free mushroom tyrosinase activity. EA-AP and En-AP induced significant inhibition of melanin production and cellular tyrosinase activity. In the action of EA-AP and En-AP on melanogenesis, they reduced the expression of melanogenic genes and proteins including tyrosinase, tyrosinase-related protein-1 (TRP-1) and dopachrome tautomerase (Dct). These results suggested that EA-AP and En-AP inhibited melanogenesis by reducing tyrosinase activity and melanin production via subsequent downregulation of tyrosinase-related proteins. The overall results suggest that EA-AP and En-AP among A. pectinifera extracts may be promising candidates for the treatment of hyperpigmentation disorder and useful for self-tanning cosmetic products.

Introduction

Melanogenesis is a physiological process resulting in the synthesis of melanin pigments, which are secreted by melanocytes in the basal layer of the epidermis. Melanin is principally responsible for skin color and plays an important role in the prevention of skin injury under normal physiological conditions. However, abnormal pigmentation such as freckles, age spots and melasma could indicate skin problems. The pigmentary disorders are caused by various factors, including UV radiation, inflammation, estrogens and genetic disorders. Melanin synthesis is mediated by melanocyte-specific enzymes such as tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 or dopachrome tautomerase (Dct). On the sequential pathway to melanin formation, tyrosinase is a rate-limiting enzyme that catalyzes tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and further oxidizes it to dopaquinone. Therefore, melanin production mainly depends on the expression and activation of tyrosinase. The modulation of melanogenesis is one of the significant strategies to treat abnormal skin pigments through medication and cosmetics.

To satisfy the desire for decreased melanogenesis, several cosmetic companies are developing melanogenesis inhibitors and discovering skin-whitening cosmetic preparations. In cosmetic preparations, inhibitors such as kojic acid, arbutin, ascorbic acid and licorice extracts have been used as whitening ingredients. In particular, tyrosinase inhibitors may not only be clinically useful for the treatment of some dermatological diseases associated with melanin hyperpigmentation, but they may also be important in cosmetics for depigmentation. A great deal of interest has recently focused on deriving tyrosinase inhibitors from a natural source. Several chemical compounds have been reported from plant origins as tyrosinase inhibitors, such as ellagic acid, oxysrexeratrol, chlorophorin and norartocarpanone and the most common natural antitowering agent is ascorbic acid. However, the effect of ascorbic acid against enzymatic oxidation is temporary since it is chemically oxidized to the nonfunctional form, dehydroascorbic acid. These problems prompted us to search for safer and more effective melanin formation inhibitors from natural sources.

Marine organisms are rich sources of structurally novel and biologically active metabolites with valuable industrial potential. Therefore, in recent years, numerous marine resources have attracted attention as researchers search for bioactive compounds to develop new cosmetics, drugs and health food. In particular, some efforts have sought to develop new depigmentation agents using biologically active compounds.
obtained from marine organisms (14). Among the marine organisms, the starfish has gradually increased in fish or shellfish farms and can cause serious damage to fish, shellfish, ark shell, abalone, little clam, scallop, that inhibit farms of coastal area (15). It is known that a starfish has very strong reproductive-power, i.e., cutting part of the body as well as the whole body can develop into new starfish. Starfish grow in the sea to the east of Korea; they are known to cause serious damage to shellfish farms, and are known as Asterias amurensis and Asterina pectinifera (A. pectinifera) (16). Some crude extracts of Acanthaster planci, Asterias forbesi and A. pectinifera among starfish are active against influenza B virus in embryonic chicks. It was reported that some extracts obtained from A. pectinifera had antimicrobial and anticancer activities (17) and also exhibited antimicrobial capacities against B. subtilis and S. aureus (18,19). Moreover, we previously showed that the methanolic extract from A. pectinifera had strong anti-inflammatory activity (20). However, little is known about the inhibitory effects of starfish extracts on melanin synthesis via tyrosinase activity.

In this study, we found the candidate fraction which contained potential bioactive compounds such as tyrosinase inhibitor from A. pectinifera extracts and investigated the inhibitory activity of the candidate fraction on melanin biosynthesis through tyrosinase activity in melan-a cells.

Materials and methods

Chemicals and reagents. L-Tyrosine, L-DOPA, mushroom tyrosinase, phorbol 12-myristate 13-acetate (TPA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ascorbic acid, arbutin and other chemical reagents were purchased from Sigma (St. Louis, MO, USA). RPMI-1640, penicillin/streptomycin solution and trypsin were obtained from Gibco (Gaithersburg, MD, USA).

Preparation of A. pectinifera extracts. For the 80% methanolic extract (80-MAP), the powdered A. pectinifera (100 g d.w.) was first soaked with 80% methanol at room temperature and was ultrasonicated (Sonics Dismembrator; Fisher Scientific Inc., Pittsburgh, PA, USA) for 30 min. Then, it was incubated at room temperature for 24 h and methanolic extracts were filtered by Whatman filter paper (particle retention, 5 µm) and the filtrates were made into powder by the vacuum rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 40˚C (Fig. 1A). To give a hexane-soluble fraction (He-AP), hexane (n-C6H14; 1:1 ratio, w/v) was added into 80-MAP for 24 h at room temperature (Fig. 1A). The hexane-insoluble residue was partitioned between ethyl acetate-water (2:1 ratio, w/v) to give an ethyl acetate soluble fraction (EA-AP) and a water soluble fraction. The ethyl acetate fraction was dried in a rotary evaporator at 40˚C to yield a dry extract. The 80-MAP, He-AP and EA-AP were dissolved in dimethyl sulphoxide (DMSO; Sigma). The enzyme extract (En-AP) of A. pectinifera was treated with protease (Protamex™; Novo Nordisk Co., Bagsvaerd, Denmark) in a dried powder of A. pectinifera. The powdered A. pectinifera (100 g d.w.) was ultrasonicated with distilled water (1:2 ratio, w/v) for 30 min and was supplemented with 1% of protease (to dried weight of sample). The enzyme reactant was incubated at 50˚C for 3 h and centrifuged at 10,000 x g for 20 min. The supernatant was adjusted at pH 3.0 with L-tartaric acid until the supernatant was a transparent solution. Following centrifugation at 10,000 x g for 20 min, the supernatant was readjusted to pH 6.0 with calcium carbonate. The sample was filtered with Whatman filter paper (particle retention, 5 µm) and then was filtered with 0.2 µm membrane filter (Advantec MFS, Inc., Dublin, CA, USA) (Fig. 1B). The stock solutions were diluted appropriately with buffer or media at the time of testing and the final concentration of DMSO in test wells was 1% for cell-free assay and 0.1% for cell-based assay.

Mushroom tyrosinase assay. In vitro mushroom tyrosinase assay was performed with L-tyrosine and L-DOPA as substrate for tyrosinase activity. Inhibitory activity of each extract against tyrosinase catalysed oxidation of L-tyrosine was determined according to the methods of Chang et al (21) in the presence of each crude extract of A. pectinifera. A volume of 40 µl of 1.5 mM substrate (L-tyrosine) dissolved in 0.1 M phosphate buffer (pH 6.8) and 120 µl of 0.1 M phosphate buffer, were mixed with 20 µl of different concentrations from each extracted sample (80-MAP, He-AP, EA-AP and En-AP). Then, 20 µl of mushroom tyrosinase (2,000 U/ml in phosphate buffer) were added to initiate the reaction. The assay mixture was incubated at 37˚C for 15 min. The increase in absorbance at 475 nm caused by the formation of dopachrome was monitored using a microplate reader (Opsys MR; Dynex Technologies, Ltd., Frankfurt, Germany). The inhibitory effect of each extract on mushroom tyrosinase in L-DOPA oxidation was determined according to Masamoto et al (22) with some modifications. A volume of 100 µl of 0.1 M phosphate buffer was mixed with 20 µl of different concentrations from each extracted sample (80-MAP, He-AP, EA-AP and En-AP). Then, 20 µl of mushroom tyrosinase (2,000 U/ml in phosphate buffer) were added to initiate the reaction. The mixture was incubated at 37˚C for 5 min and added to 40 µl of L-DOPA (4 mM in 0.1 M phosphate buffer). The mixture was incubated for 10 min at 37˚C and the absorbance at 475 nm of the reaction mixture was recorded. Ascorbic acid (500 µg/ml) as a positive control was used for assay. The percentage inhibition of tyrosine or L-DOPA oxidation was calculated as follows: % inhibition = 100 - (B/A x 100), where A = ∆OD475 in 10 min without sample, and B = ∆OD475 in 10 min with tested sample.

Cell cultures and treatment. Murine melan-a melanocytes were originally derived from C57BL/6 J (black, a/a) mice and were obtained from David Kalenberg (St. George’s University of London, UK). Melan-a cells were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 200 nM of phorbol 12-myristate 13-acetate (TPA) at 37˚C in 10% CO2. The culture medium was changed every 2 days. The cells were harvested by trypsinization when they were approximately 70% confluent, counted with a haemocytometer and seeded at the appropriate numbers into wells of cell culture plates for further experiments.

Cell viability assay. The number of viable cells was determined by the ability of mitochondria to convert MTT to formazan dye. Melan-a cells were cultured overnight in 96-well plates, at
a density of 2x10^4 cells/200 µl in each well. The next day, the
cells were coincubated with various concentrations of EA-AP
and En-AP from A. pectinifera for 24 h. Following incubation,
the medium was removed and the cells were supplemented
with 10 µl of 10 mg/ml MTT into each well. Following
incubation for another 4 h at 37˚C in a humidified 10% CO₂
atmosphere, the MTT was removed, and cells were lysed with
150 µl DMSO. The absorbance was measured at 550 nm using
a microplate reader.

**Measurement of melanin content.** Determination of the
amount of melanin content was performed using a modified
method of Hosoi et al. (23). Briefly, melan-a cells were seeded
onto a 24-well plate at a density of 1x10^5 cells/well. Following
overnight incubation, the medium was replaced with a medium
containing EA-AP and En-AP at different concentrations and
incubated for a further 72 h. Arbutin (250 µg/ml) as a positive
control was used for the assay. The medium was then removed,
the cells washed twice with phosphate-buffered saline (PBS)
and harvested by trypsinization using 0.25% trypsin/0.02%
EDTA in PBS. The harvested cells were pelleted and solubi-
lized in 1 N NaOH. After centrifugation at 3,000 x g for 10 min,
the optical density at 450 nm of the resulting supernatant was
measured by a microplate reader. The melanin contents per
well were calculated, and were expressed as a percentage of
the control.

**Cellular tyrosinase assay.** Cellular tyrosinase activity was
measured using the method of Pomerantz (24) with a slight
modification. Cells were pretreated with TPA 200 nM for 72 h
and harvested. The cells were then washed with sodium PBS
(pH 6.8) and lysed with M-PER mammalian protein extraction
reagent (Pierce, Rockford, IL, USA). The lysates were then
clarified by centrifugation at 13,000 x g for 15 min at 4˚C. The
protein concentration was determined by the Bradford method
(Bio-Rad Laboratories, Inc., Hercules, CA, USA) using bovine
serum albumin (BSA, St. Louis, MO, USA) as the standard.
These proteins were used as a tyrosinase source. A volume of
100 µl of 0.1 M phosphate buffer was mixed with 20 µl of
different concentrations from EA-AP and En-AP. Then, 20 µl
of the reaction mixture consisting of 40 µg protein (adjusted to
100 µl with 0.1 M PBS, pH 6.8) was added to initiate the reac-
tion. The mixture was incubated at 37˚C for 5 min and added
to 40 µl of L-DOPA (4 mM in 0.1 M phosphate buffer). The
mixture was incubated for 10 min at 37˚C and the absorbance
at 475 nm of the reaction mixture was recorded. Ascorbic
acid (500 µg/ml) as a positive control was used for assay. The
percentage inhibition of tyrosine or L-DOPA oxidation was
calculated as follows: % inhibition = 100 - (B/A x 100), where
A = ∆OD_475 in 10 min without sample, and B = ∆OD_475 in
10 min with tested sample.

**RNA isolation and real-time PCR.** Melan-a cells (1x10^5 cells/ml)
were plated on 100 mm culture dishes and incubated in the
presence of TPA 200 nM. Then, the cells were treated with
various concentrations of EA-AP and En-AP for 24 h. Arbutin
(250 µg/ml) as a positive control was used for the assay. The
cells were harvested and washed twice with ice-cold PBS. Total
cellular RNA was prepared using TRIzol solution (Invitrogen,
Paisley, UK) according to the manufacturer’s instructions.
RNA was then precipitated with isopropanol and dissolved
in diethylpyrocarbonate-treated distilled water. First-strand
cDNA was generated with the oligo(dT) adaptor primers by
reverse transcriptase (Takara Bio, Inc., Otsu, Japan). Each
specific primer (accession no. of tyrosinase, Mm.238127; access-
ion no. of TRP-1, Mm.30438; accession no. of Dct, Mm.19987; and accession no. of GADPH, Mm.304088) was
designed using primer express software from TaqMan® Gene
expression array (Applied Biosystems, Carlsbad, CA, USA).
GAPDH was used as the invariant control. The real-time PCR
reaction (10 µl) contained 10 ng of reverse transcribed RNA,
200 nM each of forward and reverse primers, and a PCR
Master mixture. The reaction was performed using the CFX96 real time system (Bio-Rad, Hercules, CA, USA). All reactions were conducted in triplicate.

Immunoblotting. Melan-a cells (1x10⁵ cells/ml) were plated on 100 mm culture dishes and incubated in the presence of TPA 200 nM. The cells were treated with various concentrations of EA-AP and En-AP for 72 h. Arbutin (250 µg/ml) as a positive control was used for the assay. The cells were harvested and washed twice with ice-cold PBS. Then, the cells were resuspended in 200 µl ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, 1 ml protease inhibitor cocktail) and incubated at 4°C for 40 min. The lysates were centrifuged at 14,000 x g for 20 min. Protein concentrations of cell lysates were determined by the Bradford method. Equal amounts of protein were subjected to 7.5-15% SDS-PAGE for tyrosinase, TRP-1, Dct and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), respectively, and transferred to a nitrocellulose membrane. Immunostaining with antibodies was performed using Super-Signal West Pico enhanced chemiluminescence substrate and detected with LAS-3000PLUS (Fuji Photo Film Co., Kanagawa, Japan).

Statistical analysis. The data are expressed as the means ± standard deviation (SD). The evaluation of statistical significance was performed using Student's t-test or one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 18.0 (SPSS, Chicago, IL, USA). P<0.05 was considered to indicate statistically significant differences.

Results

A. pectinifera extracts inhibit mushroom tyrosinase activity. To investigate whether A. pectinifera extracts showed any direct inhibitory effect against the key enzyme in the whole melanogenesis, in vitro cell-free mushroom tyrosinase assay was carried out. The effects of each extract (80-MAP, He-AP, EA-AP and En-AP) on mushroom tyrosinase activity are shown in Fig. 2. We observed the inhibitory effect of all extracts on the oxidation of tyrosine and L-DOPA by mushroom tyrosinase in a dose-dependent manner. However, 80-MAP and He-AP showed less inhibitory activity of mushroom tyrosinase than EA-AP or En-AP. A positive control, ascorbic acid, showed strong tyrosinase inhibition, as expected. The EA-AP and En-AP showed less inhibitory activity of mushroom tyrosinase than ascorbic acid at the maximum concentration. In addition, the IC₅₀ of EA-AP and En-AP was 250 and 500 µg/ml for L-tyrosine and 500 µg/ml for L-DOPA, respectively (Table I). Ascorbic acid substantially inhibited the enzyme activity with an IC₅₀ value of 63 µg/ml for L-tyrosine and 175 µg/ml for L-DOPA and also showed inhibitory activity >95% at 500 µg/ml when compared to untreated control in L-tyrosine and L-DOPA. Taken together, our results demonstrated that EA-AP and En-AP among A. pectinifera extracts have the highest inhibitory effect on mushroom tyrosinase activity.

EA-AP and En-AP induce cell cytotoxicity in melan-a cells. Based on the results of the enzymatic assay, we determined EA-AP and En-AP among A. pectinifera extracts for effective anti-melanogenic activity. Thus, to exclude the possibility that inhibitory effects of EA-AP and En-AP on melanogenesis might be caused by the inhibition of melan-a cell growth, we compared the number of cell growth in the presence and absence of these extracts. We observed the cell viability with MTT assay after treatment of EA-AP and En-AP with different concentrations. After treatment, 50% of cell viability presenting concentration (IC₅₀ values) of EA-AP was 250 µg/ml while the IC₅₀ of En-AP was over 500 µg/ml at tested maximum concentrations (Fig. 3 and Table II). A positive control, the IC₅₀ of arbutin, was 1,041 µg/ml and it induced no cytotoxicity at
below 500 µg/ml (data not shown). These results showed that EA-AP induced more cell cytotoxicity than En-AP. Therefore, in this study, we tested further experiments such as melanin synthesis, cellular tyrosinase activity, related gene and protein expression in melan-a cells at concentrations below IC\textsubscript{50} of EA-AP and En-AP.

**EA-AP and En-AP reduce melanin synthesis and cellular tyrosinase activity in melan-a cells.** To evaluate the inhibitory effect of EA-AP and En-AP on melanin content and cellular tyrosinase activity, melan-a cells were treated with different concentrations of these extracts. The inhibitory effect of EA-AP and En-AP on melanin content is shown in Fig. 4. The levels of melanin in the melan-a cells were reduced significantly as a result of the EA-AP (Fig. 4A) and En-AP treatment (Fig. 4B) when compared to the TPA-treated control group. In addition, to measure cellular tyrosinase activity, instead of using mushroom tyrosinase, cell lysates prepared from the melan-a cells treated with TPA were used as a tyrosinase source. The EA-AP and En-AP showed an inhibitory effect on cellular tyrosinase activity in a dose-dependent manner. Arbutin and ascorbic acid as a positive control showed a significant reduction of the melanin secretion and cellular tyrosinase activity ($P<0.05$) (Figs. 4 and 5), respectively. These results suggest that the inhibitory effect of EA-AP and En-AP on melanin synthesis appear to be well-correlated with the measurement of mushroom tyrosinase activity and cellular tyrosinase activity.

**EA-AP and En-AP suppress melanogenesis-related gene and protein expression.** To investigate whether EA-AP and En-AP affect the expression of melanogenesis-related proteins including tyrosinase, TRP-1 and Dct, these proteins levels were examined in melan-a cells in the presence of TPA using western blot analysis after treating with various concentrations of EA-AP and En-AP for 72 h. Levels of melanogenesis-related genes and proteins were reduced significantly when treated with EA-AP and En-AP.

**Table II. Effects of EA-AP and En-AP on melanin production and cellular tyrosinase activity of melan-a cells.**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cytotoxicity IC\textsubscript{50} (µg/ml)$^a$</th>
<th>Melanin synthesis IC\textsubscript{50} (µg/ml)$^b$</th>
<th>Cellular tyrosinase activity IC\textsubscript{50} (µg/ml)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA-AP</td>
<td>125</td>
<td>31.3</td>
<td>31.3</td>
</tr>
<tr>
<td>En-AP</td>
<td>&gt;500</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Arbutin</td>
<td>1,041</td>
<td>63</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-</td>
<td>-</td>
<td>250</td>
</tr>
</tbody>
</table>

$^a$50% cell cytotoxicity concentration; $^b$50% inhibitory concentration.
protein, tyrosinase, TRP-1 and Dct were reduced after EA-AP (Fig. 6A) and En-AP (Fig. 6B) treatment in a dose-dependent manner. To examine whether the inhibition of tyrosinase-related protein expression by EA-AP and En-AP was due to decreased level transcription, we tested real time PCR using specific primers. The mRNA level of tyrosinase, TRP-1 and Dct was significantly decreased by treatment with EA-AP (P<0.05) (Fig. 7A) and En-AP (P<0.05) (Fig. 7B). Arbutin as a positive control showed a significant reduction of gene and protein levels of tyrosinase, TRP-1 and Dct in a dose-dependent manner while only TPA-treated cells markedly increased gene and protein levels in tyrosinase, TRP-1 and Dct (Figs. 6 and 7). These results indicate that the suppressive activity of EA-AP and En-AP on melanogenesis is linked to the downregulation of tyrosinase expression signaling pathways.

Discussion

In Asian countries, women are concerned with skin whitening as having whiter skin is often seen to be a superior standard of beauty (25). As a number of women worry about skin pigmentation, effective agents for the improvement of hyperpigmentation have been researched for skin whitening products (26). These foregoing attributes prompted the present hypothesis that a marine natural product might be
valuable as a cosmetic component to improve the appearance of hyperpigmentation. We intended in this study to find new whitening materials from *A. pectinifera*, a marine organism that would also have significance insofar as we would obtain bioactive materials by using starfish which would be discarded after collection from the sea. Therefore, we investigated the potential whitening effect of *A. pectinifera* extracts (80-MAP, He-AP, EA-AP and En-AP) and also demonstrated the effect of each extract on melanin biosynthesis through tyrosinase activity which is a standard model for assessing regulators of melanogenesis since tyrosinase is the key enzyme in melanogenesis, initiating a cascade of reactions which convert tyrosine to the biopolimer melanin (27).

Tyrosinase (polyphenol oxidase) plays rate-limiting roles in the production of melanin by melanocytes. Melanin pigment, responsible for visible skin color, is formed through a series of oxidative reactions involving the amino acid, tyrosine. Tyrosinase catalyzes three main steps in melanogenesis; the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), the oxidation of L-DOPA to dopaquinone, and the additional oxidation of 5,6-dihydroxyindole to indol-quinone (28). Almost all factors affecting melanin production exert their action either directly or indirectly via stimulation of tyrosinase and the most common target for skin-lightening activities is tyrosinase inhibition (29). Indeed, recently, a great deal of interest has been on deriving tyrosinase inhibitors from plant origin and anti-melanogenesis activities of several herbal medicines have been evaluated by their ability to inhibit tyrosinase (30).

In this study, several fractions of powdered dried *A. pectinifera* were extracted with solvents of different polarity and tested for their possible anti-melanogenesis or skin-whitening properties using the inhibition of mushroom tyrosinase activity in a cell-free system as screening assays. Our data revealed that the methanolic extract (80-MAP) and enzymatic extract (En-AP) inhibited mushroom tyrosinase activity among *A. pectinifera* extracts and particularly the separated ethyl acetate fraction (EA-AP) from 80-MAP had a strong suppression of tyrosinase activity. However, hexane fraction (He-AP) showed less inhibitory activity than EA-AP (Fig. 2 and Table I). In addition, EA-AP and En-AP inhibited cellular tyrosinase activity as well as melanin production in melan-a cells (Figs. 4 and 5). These results suggest that EA-AP and En-AP among *A. pectinifera* extracts may contain potential skin-whitening materials that could induce reduction of melanogenesis in melan-a cells by reduction of tyrosinase activity.

Dooley (31) previously speculated that a desirable skin-whitening agent should inhibit melanin synthesis in melanocytes by acting specifically to reduce the synthesis or activity of tyrosinase with little or no cytotoxicity. In this study, En-AP appeared to have little cytotoxic as well as anti-melanogenic activity among *A. pectinifera* extracts while EA-AP induced some cytotoxicity under TPA-stimulated melan-a cells (Fig. 3). Accordingly, EA-AP at higher concentration levels (250 and 500 µg/ml) was not used further due to its greater cytotoxicity on the melan-a cells. These data demonstrate that En-AP contains safer materials for potential skin-whitening activity than EA-AP due to the slight cytotoxic effects of EA-AP at the higher concentrations. In mammals, melanogenesis occurs in melanocytes after differentiation of the nonpigmented precursors, melanoblasts. Three melanocyte-specific enzymes, including tyrosinase, TRP-1 and Dct (TRP-2), are involved in the enzymatic process that converts tyrosine to melanin pigments (32). In particular, there are two tyrosinase-related proteins, TRP-1 and Dct (TRP-2), which are structurally related to tyrosinase and share approximately 40% amino acid homology, suggesting that they originated from a common ancestral gene (33). As TRP-1 forms a complex with tyrosinase, it is possible that TRP-1 plays a role in tyrosinase activation and/or stabilization (34). TRP-1 also plays a role in melanosomal biogenesis, as suppression of TRP-1 expression is associated with structurally abnormal melanosomes (35). TRP-2 complexes with tyrosinase and also with TRP-1 (36). TRP-2 converts DOPAchrome to the carboxylated derivative dihydroxyindole-2-carboxylic acid (DHICA) during one of the later stages of melanin biosynthesis (37). Indeed, TRP-1 and TRP-2 have been demonstrated to increase tyrosinase stability (38) and are responsible for the induction of melanin synthesis (29,39,40). Both EA-AP and En-AP reduced melanogenic proteins, tyrosinase, TRP-1 and Dct (TRP-2) (Fig. 6), and also the inhibitory effects of EA-AP and En-AP on melanogenesis likely resulted from suppressed gene expression of tyrosinase, TRP-1 and Dct (TRP-2) (Fig. 7).

In conclusion, we suggest that EA-AP and En-AP from *A. pectinifera* extracts inhibit melanin synthesis via subsequent downregulation of tyrosinase-related proteins. Therefore, EA-AP and En-AP may be useful candidates for the development of potential therapeutic agents for hypopigmentation treatment, and may be an effective component in whitening and lightening cosmetics. We will further separate single compounds from EA-AP fraction of *A. pectinifera* and explore the inhibition of melanin synthesis via MITF downregulation and related signaling pathways.

Acknowledgements

We thank Dr Dorothy C. Bennett and Dr David Kallenberg (St. George’s University of London) for their helpful discussions and gifts of melan-a. This research was supported by the 2012 National R&D Program through the Dongnam Institute of Radiological and Medical Sciences (DIRAMS) funded by the Ministry of Education, Science and Technology (50493-2012) and the Technology Development Program for Agriculture and Forestry (610003-03-1-SB110), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

References