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ORIGINAL ARTICLE

Melanogenesis Inhibition by Palm Tocotrienol Rich Fraction in Cellular Ageing

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ABSTRAK

Melanin merupakan pigmen yang menentukan warna kulit. Sintesis melanin dimangkinkan oleh enzim tirosinase dan melibatkan pengawalaturan oleh gen *TYR, TYRP1* dan *TYRP2*. Dalam kajian ini, fraksi kaya tokotrienol (TRF) telah digunakan sebagai perencat sintesis melanin. TRF mengandungi 75% alfa-tokotrienol dan 25% tokoferol. Objektif kajian ini adalah untuk menentukan kesan fraksi kaya tokotrienol sawit terhadap sintesis melanin melalui penentuan kandungan aras melanin, aktiviti enzim tirosinase dan ekspresi gen yang mengawalatur sintesis melanin di dalam melanosit kulit iaitu *TYR, TYRP1* dan *TYRP2*. Sintesis melanin ditentukan melalui pengukuran kandungan melanin dan asai aktiviti tirosinase. Ekspresi gen diukur dengan menggunakan kaedah kuantitatif PCR transkripsi berbalik (real time RT-PCR). Kultur primer sel melanosit kulit dibahagikan kepada dua kumpulan iaitu kumpulan kawalan dan kumpulan perlakuan dengan TRF pada dos 500 µg/ml selama 24 jam. Hasil kajian menunjukkan terdapat penurunan kandungan melanin dan aktiviti tirosinase pada sel melanosit kulit yang diperlakukan dengan TRF berbanding sel kawalan ($p<0.05$). Ekspresi gen *TYRP2* menunjukkan penurunan apabila melanosit diperlakukan dengan TRF berbanding dengan sel kawalan ($p<0.05$) . Oleh itu, dapat disimpulkan bahawa fraksi kaya tokotrienol sawit bertindak sebagai agen anti pigmentasi semasa penuaan kulit iaitu ia merencat sintesis melanin melalui perencatan aktiviti tirosinase dan menurunkan ekspresi gen *TYRP2*.

Kata kunci: sintesis melanin, ekpresi gen, fraksi kaya tokotrienol, melanosit kulit

ABSTRACT

Melanin is the pigment that determines skin color. Melanin synthesis is catalysed by the enzyme tyrosinase and is controlled by *TYR, TYRP1* and *TYRP2* genes. In this study, tocotrienol rich fraction (TRF) was used to inhibit melanin synthesis. TRF contains 75% α-tocotrienol and 25% tocopherol. The objective of this study was to determine the effect of tocotrienol rich fraction (TRF) on melanin synthesis by determining melanin content and expression of genes involved in the regulation of

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melanin synthesis in skin melanocytes. Melanin synthesis was studied by determining melanin level and tyrosinase enzyme activity, while expression of *TYR, TYRP1* and *TYRP2* genes was determined by quantitative real time reverse transcriptase polymerase chain reaction (real time RT-PCR). Primary culture of skin melanocytes were divided into two groups; control and cells that were treated with 500 µg/ml tocotrienol rich fraction for 24 hours. Our results showed that there was a reduction in melanin content and tyrosinase activity in skin melanocytes treated with tocotrienol rich fraction compared to the control ($p<0.05$). Expression of *TYRP2* gene in melanocytes treated with tocotrienol rich fraction was also decreased ($p<0.05$) compared to the control. In conclusion, palm tocotrienol rich fraction has anti pigmentation property that inhibits melanin synthesis in cellular aging by inhibiting tyrosinase activity and down regulating *TYRP2* gene expression.

Key words: melanin synthesis, gene expression, tocotrienol rich fraction, skin melanocytes

INTRODUCTION

Skin is the largest and outermost organ of the human body. The skin is frequently and directly exposed to a pro-oxidative environment, including ultraviolet radiation (UV), drugs, and air pollutants. Besides external inducers of oxidative attack, the skin has to cope with endogenous generation of reactive oxygen species (ROS) and other free radicals, which are continuously produced during physiological cellular metabolism.

Balance between oxidants and antioxidants is needed to minimize molecular, cellular, and tissue damage. However, if the balance is upset in favour of the oxidants, oxidative stress could occur and results in oxidative damage. Reactive oxygen species (ROS) are known to cause oxidative modification of DNA, proteins, lipids and small cellular molecules (Kang et al. 2005). To counteract the harmful effects of ROS, the various compartments of the skin (epidermis, dermis, subcutis) are equipped with layerspecific antioxidant systems to maintain an equilibrium between ROS and antioxidants and thus prevent oxidative stress (Thiele and Ekanayake-Mudiyanselage 2007).

Skin aging is defined as structural changes of skin as the age increase. Aging is indicated as inability to balance the important functions of skin leading to cell death. The free radical theory of aging explains the importance of antioxidants in reducing pigmentation in older people (Chandrosoma & Taylor 2001).

The human pigmentary system is dependent on the production of the light absorbing biopolymer, melanin, within epidermal, ocular and follicular melanocytes (Nordlund et al. 1998). Melanocytes within the skin are situated on the basal layer between the dermis and epidermis and have a number of dendritic processes that interdigitate with the surrounding keratinocytes. While pigment synthesis occurs within the melanocytes, the majority of pigment within the skin is found in melanin laden vesicles known as melanosomes located within the keratinocytes (Sturm et al. 2001).

Melanin synthesis is also known as melanogenesis where the pigment melanin is formed. The melanin pigments are of no fixed molecular weight but are all derived by enzymatic oxidation of the amino acid tyrosine and eventually produce two types of melanin in mammalian skin which are feomelanin and eumelanin

(Prota 1992; Nordlund et al. 1998). Tyrosinases catalyze two distinct reactions in melanin synthesis: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone (Tripathi et al. 1992). The eumelanins are derived from the metabolites of DOPAchrome, whereas the pheomelanins are derived from metabolites of 5-S-cysteinylDOPA. The isomerization of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) is catalysed by DOPAchrome tautomerase and the oxidation of DHICA is performed by a DHICA-oxidase enzyme (Sturm et al. 2001).

Important genes involved in melanin synthesis pathway are *TYR, TYRP1* and *TYRP2.* Tyrosinase is encoded by the TYR or c-locus that maps to chromosome 11q14–21 in humans (Barton et al. 1988) and chromosome 7 in mice, respectively. It is composed of five exons and four introns (Jimbow et al. 2000). The gene for TYRP1 is 37 kb long and contains eight exons separated by seven introns (Nordlund et al. 1998). The gene for TYRP2 is 60 kb long and contains eight exons and seven introns; all eight exons encode the final protein (Budd and Jackson 1995). Gene and protein structures of tyrosinase, *TYRP1* and *TYRP2* consist of NH_2-terminal domain of tyrosinase which comprises of the NH_2-terminal signal peptide that is important for intracellular trafficking and processing, the EGF-like/cysteine-rich domain, two histidine-rich regions binding copper with a cysteine-rich region between them (the important catalytic domain), as well as the COOH-terminal hydrophobic transmembrane segment and cytoplasmic tail (Kwon 1993). The transmembrane and cytoplasmic domains are necessary for targeting the enzyme to the melanosome (Jimbow et al. 2000; Selaturi 2000) while the NH_2 terminus cysteine-rich region may serve as a protein binding/regulatory domain unrelated to enzymatic function.

Tocopherols and tocotrienols are subfamilies of vitamin E (Brigelius-Flohe & Traber 1999). Both tocopherols and tocotrienols have isomers, designated as -α, -β,-δ and -γ which differ by the number and position of the methyl groups on the chromanol ring (Machlin 1991). Tocotrienol rich fractions are vitamin E that consists of 75% alpha-tocotrienol and 25% tocopherol (Mutalib et al. 2003). Tocotrienol rich fractions are found in barley, oats, palm, and commercial rice brans (Qureshi & Qureshi 1993).

In this study, the anti pigmentation properties of palm tocotrienol rich fraction was evaluated. Primary culture of skin melanocytes was treated with tocotrienol rich fraction to evaluate the effects of tocotrienol rich fraction on melanin synthesis by determining melanin content and tyrosinase enzyme activity. Determination of gene expression involved in the regulation of melanin synthesis such as *TYR, TYRP1* and *TYRP2* was also carried out.

MATERIALS AND METHODS

Cell culture

Human melanocytes obtained from the foreskin of nine to 12 year-old boys were grown in Medium 254, which is a basal medium containing essential and non essential amino acids, vitamins, organic compounds, trace minerals, and inorganic salts (Cascade Biologics, USA). The medium was supplemented with Human Melanocyte Growth Supplement-2 (HMGS-2), which contains 0.5% fetal bovine serum, 3 ng/ml basic fibroblast growth factor (human recombinant), 0.2% bovine pituitary extract, 3 µg/ml heparin, 0.18 µg/ml hydrocortisone, 5 µg/ml insulin, 5 µg/ml transferrin, and 10 ng/ml phorbol 12- myristate 13-acetate. Written consent was obtained from parents or guardians of all subjects.

Cell viability assay

Cell viability was assessed with CellTiter 96* Aqueous Non-Radioactive Cell Proliferation Assay (MTS, Promega, USA). The MTS assay employs 3-(4,5-dimethylthiazol-2-yl)-5-carboxymethoxyphenyl) 2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron coupling agent phenazine methosulphate (PMS). The MTS compound is reduced by the dehydrogenase enzymes found in metabolically active cells into a formazan product that is soluble in the medium. The amount of colored formazan product is proportional to the number of viable cells. Briefly, 20 µl MTS solution was added to each well and incubated in a humidified incubator at 37^0C in 5% CO_2 for 2 - 4 hours. The quantity of formazan product present was determined by measuring the absorbance at 490 nm with a microtiter plate reader (VeraMax Molecular Devices, USA).

Determination of melanin content

Melanin content was determined according to Huang et al. (2008) with slight modification. The cells (10^5) were treated with 500µg/ml TRF (Sime Darby Bhd, Malaysia) for 24 hours. Cell pellets were dissolved in 1 ml of 1 N NaOH at 37 °C overnight and centrifuged for 10 minutes at 10,000 x g. The optical density (OD) of the supernatants was measured at 450nm using the µQuant microplate reader. Melanin concentration was calculated by comparing the OD at 450 nm of unknown samples with a standard curve (Figure 2).

Determination of cellular tyrosinase activity

Cellular tyrosinase activity was measured according to Lin et al. (2007), with slight modification. Melanocytes (10^5) were cultured in 24-well plates for 24 hours followed by 24 hours treatment with 500µg/ml TRF (Sime Darby Bhd, Malaysia). Cells were then washed and lysed with potassium phosphate-buffered saline (PBS) pH 6.8 containing 1% Triton X-100 and ruptured by freezing at -80°C and thawing in a water bath. Cell lysates were then clarified by centrifugation at 10,000 x g for 10 minutes. Protein content was determined using Bradford assay. Protein concentrations were adjusted with lysis buffer until each lysate contained the same amount of protein (40 µg). The final reaction mixture in each well contained the cell lysate, 10 µl 2.5 mM L-dopa, and 0.1 M PBS pH 6.8. Absorbance was then measured at 475 nm using the µQuant microplate reader after incubation at 37°C for 1 hour.

RNA extraction

Total RNA from fibroblast cells in different groups were extracted using TRI Reagent (Molecular Research Centre, Cincinnati OH) according to the manufacturer's instruction. Polyacryl Carrier was added in each extraction to precipitate the total RNA. Extracted RNA pellet was then washed with 75% ethanol and dried before being dissolved in RNase and DNase free distilled water (Gibco Invitrogen Corp.). Total RNA was stored at -80°C immediately after extraction. Yield and purity of the extracted RNA was determined by Nanodrop (Thermo Scientific).

Primer design

Primers for human GADPH and gene of interest were designed with Primer 3 software and blasted with Genebank database sequences. The sequence of the primers is shown in Table 1.

Real time RT-PCR

Gene expression of *TYR, TYRP1, TYRP2* was quantitatively analysed with real time RT-PCR technique. The ex-

Table 1 : Primer sequences for quantitative gene expression analysis

Gene	Accession numbers	Primer sequence (5'→3')	PCR product size (bp)
GAPDH	BC020308	F: tcc ctg agc tga acg gga ag R: gga gga gtg ggt gtc gct gt	217
TYR	NM_000372.3	F: gatgagtacatgggaggtcagc R: gtactcctccaatcggctacag	102
TYRP1	NM_000550.1	F: gctccagacaacctgggata R: tcagtgaggagaggctggtt	185
TYRP2	NM_001922.2	F: agattgcctgtctctccagaag R: cttgagaatccagagtcccatc	116

pression level of each targeted gene was normalized to GAPDH. Primers for human *GAPDH, TYR, TYRP1, TYRP2* were designed with Primer 3 software and blasted with GeneBank database sequences in order to obtain primers with high specificity. The efficiency and specificity of each primer set were confirmed with standard curve (Ct value versus serial dilution of total RNA) and melting profile evaluation. Real time RT-PCR reaction was performed with 100 ng of total RNA, 400 nM of each primer and iScript One-Step RT-PCR kit with SYBR Green (Biorad) according to the manufacturer's instruction. Reactions were run using Bio-Rad iCycler with reaction profile as follows; cDNA synthesis for 30 min at 50°C; pre-denaturation for 2 min at 94°C; PCR amplification for 38 cycles with 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. This was followed by a melt curve analysis to determine the reaction specificity. Agarose gel electrophoresis was performed for confirmation of the PCR product. Expression level of each targeted gene was normalized to *GAPDH*.

Statistical analysis

Each experiment was carried out in triplicates with at least 3 independent cultures with comparable results. Data are reported as mean ± SD of at least three experiments. Comparison between groups was made by Student's *t*-test (two-tailed). $P<0.05$ was considered statistically significant.

RESULTS

Effects of Tocotrienol Rich Fraction on cell viability

Incubation of melanocytes with different concentrations of tocotrienol rich fraction (100, 200, 300, 400, 500 ug/ml) for 24 hours caused an increase in the number of viable cells (Figure 1). The percentage of the viable cells was increased with increased concentration of tocotrienol rich fraction. Tocotrienol rich fraction at 500 ug/ml was used in this study to determine its effects on melanin synthesis.

Effects of Tocotrienol Rich Fraction on melanin content and tyrosinase activity.

Melanin concentration was calculated by comparing the OD at 450 nm of unknown samples with a standard curve (Figure 2). Results showed that treatment with tocotrienol rich fraction caused a significant reduction in melanin content (Figure 3) ($p<0.05$) as compared to the untreated control. Tyrosinase activity was significantly lower ($p<0.05$) in melanocytes treated with tocotrienol rich fraction (Figure 4) compared to the untreated control.

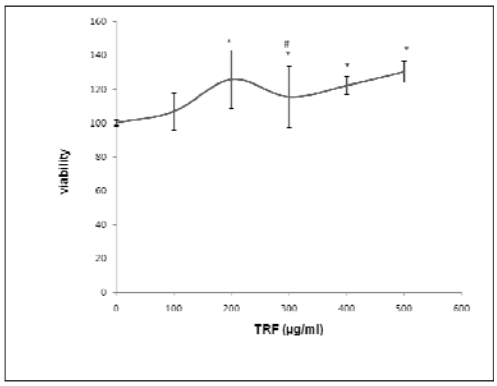


Figure 1: Effects of palm tocotrienol rich fraction (TRF) on melanocyte proliferation as assessed by MTS assay. Percent MTS reduction corresponds to the viable cell number. Melanocytes were incubated with increasing concentrations of TRF for 24 hours at 37°C. Incubation with TRF caused a significant increase in the number of cell viable. * Denotes $p<0.05$ compared to untreated control, # $p<0.05$ compared to previous concentration. Data is presented as means ± SD, n = 3.

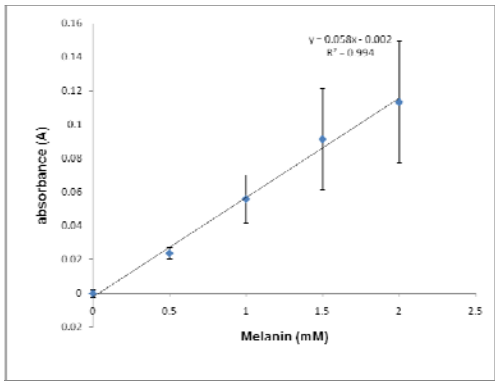


Figure 2: Standard curve for synthetic melanin

Effects of tocotrienol rich fraction on the expression of TYR, TYRP1 and TYRP2 genes

The agarose gel electrophoresis showed a single band of PCR product indicating that the primers were specific (Figure 5a). The melting curve analysis showed single and narrow peak as an indication of primer specificity (Figure 5b-e)

Results showed that treatment with tocotrienol rich fraction caused a significant downregulation of *TYRP2* gene ($p<0.05$) compared to the untreated control (Figure 6). Expression of *TYR* and *TYRP1* genes was not significant from the untreated control.

DISCUSSION

Previous study reported that compounds with redox properties or (antioxidants) may have anti pigmentation effects by interacting with o-quinones or interacting with copper at the tyrosinase active site, thus avoiding the oxidative polymerization of melanin intermediates. Redox agents could inhibit second messengers which were able to stimulate epidermal melanogenesis either directly or indirectly by scavenging reactive oxygen species generated in the skin following UV exposure (Karg et al. 1993). Our findings showed that melanin content and tyrosinase activity were significantly reduced in melanocytes treated with 500 µg/ml tocotrienol rich fraction.

It has been reported that α-tocopherol derivatives could inhibit tyrosinase *in vitro* (Shimizu et al. 2001) and inhibit melanogenesis in epidermal melanocytes (Ichihashi et al. 1999). The antioxidant properties of α-tocopherol (25% in tocotrienol rich fraction), which interfere lipid peroxidation of melanocyte membranes and increased intracellular glutathione content, could explain its depigmenting effect (Marmol et al. 1993). Other antioxidants such as ascorbic acid interfere with the different steps of melanization by interacting with copper ion at the tyrosinase active site (Gukasyan 2002) or/and reducing dopaquinone as well as blocking DHICA oxidation (Ros et al. 1993) thus causing reduction in pigmentation.

Tyrosinase activity was an important and significant parameter to measure melanogenesis in pigment cell culture (Hu

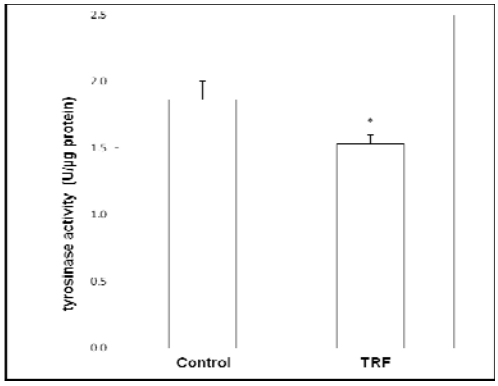


Figure 3: Anti pigmentation effects of palm tocotrienol rich fraction (TRF) as shown by reduction in melanin content (ng/cell) in TRF-treated skin melanocytes. Primary culture of skin melanocytes was treated with 500 ug/ml TRF for 24 hours at 37°C. *Denotes $p<0.05$ compared to untreated control. Data is presented as mean ± SD, n=3.

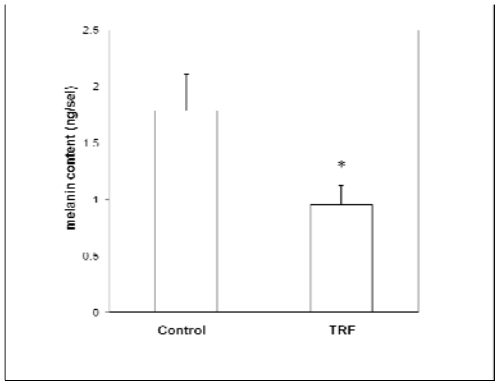


Figure 4: Anti pigmentation effects of palm tocotrienol rich fraction (TRF) as shown by reduction in tyrosinase activity (U/ug protein) in TRF-treated skin melanocytes. Primary culture of skin melanocytes was treated with 500 ug/ml TRF for 24 hours at 37°C. * Denotes $p<0.05$ compared to untreated control. Data is presented as mean ± SD, n=3.

2008). The significant reduction in tyrosinase activity and melanin content in the tocotrienol rich fraction-treated melanocytes in this study showed inhibition of melanogenesis in these cells. Similar findings were reported in previous studies that used active constituents from formosan apple (Lin et al. 2007), aloesin (Jones et al. 2002), picnogenol (Kim et al. 2008) and anemonin (Huang et al. 2008).

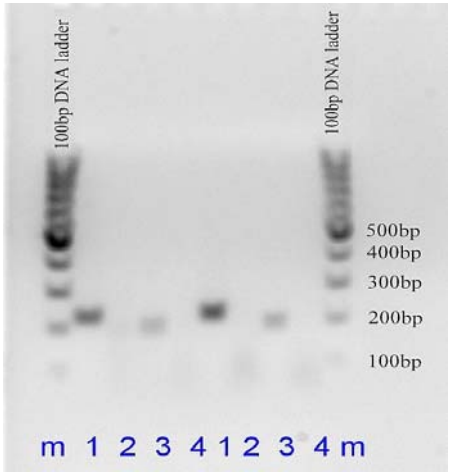
Melanin synthesis was affected by many factors such as the presence of TRP-1 and TRP-2 proteins, UV exposure, prostaglandin, vitamins, growth factors, interleukin, interferon and hormones such as alpha-melanocyte stimulating hormone, adrenocortropic hormone and endothelin-1 (Fang et al. 2002). Genetic control was also involved in melanin synthesis (Rinchik et al. 1993).

Our results showed that *TYRP2* gene expression was significantly reduced in tocotrienol rich fraction-treated melanocytes while *TYR* and *TYRP1* genes decreased insignificantly. These findings showed *TYRP2* expression was affected by tocotrienol rich fraction treatment but not for *TYR* and *TYRP1* genes expression. A previous study reported that antioxidants caused reduction in tyrosinase activity but did not affect the tyrosinase related protein itself (Khatib et al. 2005; Kim et al. 2006).

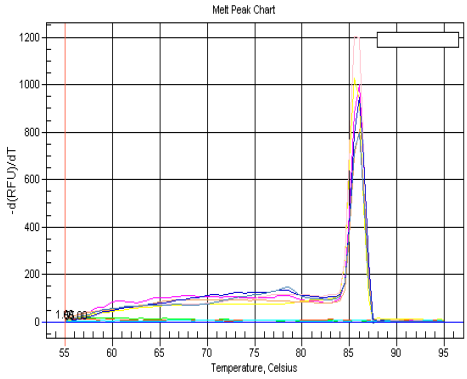
Huang et al. (2008) reported that anemonin, a natural bioactive compound, could regulate tyrosinase-related proteins and mRNA in human melanocytes. Eberle et al. (2001) showed that catechol could inhibit gene expression encoded for TRP-2 protein which was *TYRP2*.

TYR is controlled by a quantitative rheostat-like switch which allows continuous transcription of *TYR* meanwhile *TYRP1* is controlled by binary switch that would allow transcription of genes only at certain time. Therefore *TYR* has variable control mechanisms such as regulation at transcription level, and post translational mechanism as well as affected by mRNA stability (Hazily et al. 2002).

TYR, TYRP1 and *TYRP* genes have been cloned and extrinsic factors regulating their expression were recently identified. It was reported that *TYRP1* and *TYRP2* genes may act together to



(a)



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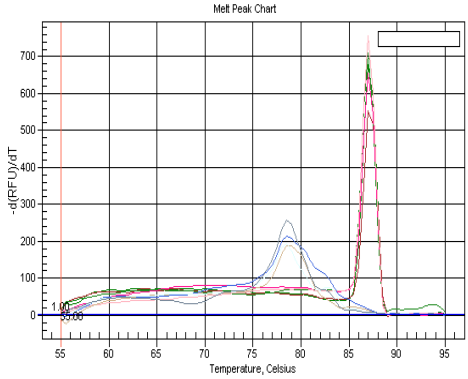

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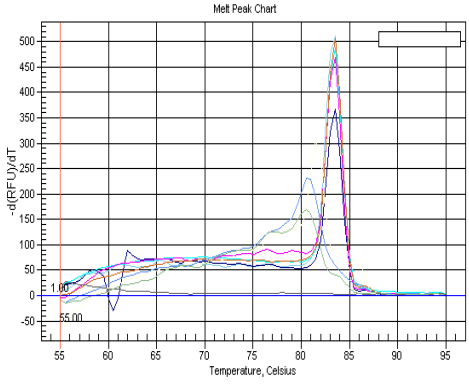

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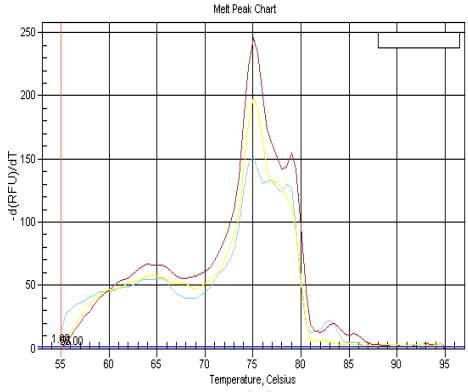

(e)

Figure 5: Agarose gel electrophoresis for *TYR, TYRP1, TYRP2* primers in human skin melanocytes. Primers were specific to gene of interest (a). Specificity of primer was shown by melting curve graph. Single and narrow peak indicated primers were specific for GAPDH gene (b), *TYR* gene (c), *TYRP1* gene and *TYRP2* gene (e).

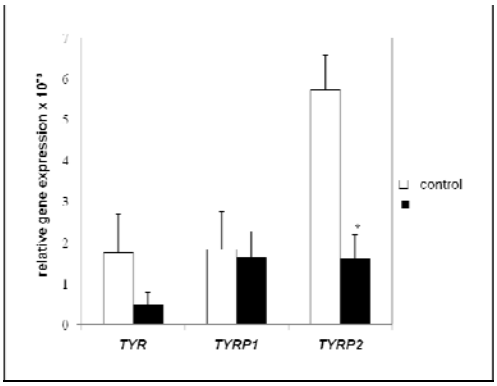

Figure 6: Effects of palm tocotrienol rich fraction (TRF) on *TYR*, *TYRP1*, *TYRP2* mRNA expression in TRF-treated skin melanocytes. Primary culture of skin melanocytes was treated with 500 ug/ml TRF for 24 hours at 37°C. * Denotes $p<0.05$ compared to untreated control. Data is presented as mean ± SD, n=2.

modulate *TYR* activity (Manga et al. 2000). This may explained the down regulation of *TYRP2* gene in this study that affect the activity of tyrosinase enzyme.

CONCLUSION

In conclusion, the significant reduction in tyrosinase activity and melanin content in melanocytes treated with tocotrienol rich fraction and down regulation of *TYRP2* gene confirmed the anti pigmentation property of palm tocotrienol rich fraction, an invaluable finding in the cosmetic industry.

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