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Full Length Research Paper

Modulation of melanin synthesis and its gene expression in skin melanocytes by palm tocotrienol rich fraction

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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. The objective of this study was to evaluate the anti pigmentation property of palm tocotrienol rich fraction by determining melanin synthesis and expression of genes involved in its regulation in skin melanocytes. Palm tocotrienol rich fraction (TRF) which contains 75% α-tocotrienol and 25% tocopherol was used to inhibit melanin synthesis which was determined by determining melanin level and tyrosinase enzyme activity. 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 was divided into two groups; untreated control and cells that were treated with 500 µg/ml tocotrienol rich fraction for 24 h. Our results showed that there was a reduction in tyrosinase activity and melanin content in melanocytes treated with tocotrienol rich fraction compared to control ($p < 0.05$). Expression of *TYRP2* gene in melanocytes treated with tocotrienol rich fraction was also decreased ($p < 0.05$) compared to control. In conclusion, palm tocotrienol rich fraction has an anti pigmentation property that inhibit melanin synthesis by inhibiting tyrosinase activity and down regulating *TYRP2* gene expression.

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

INTRODUCTION

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 three types of melanin in mammalian skin which are pheomelanin, eumelanin and neuromelanin as well as mixed melanin (Ito, 2003). The essential enzyme in melanin biosynthetic pathway is tyrosinase. Tyrosinase is multifunctional, glycosylated, copper-containing oxidase with a molecular weight of approximately 60 - 70 kDa. In mammals, it is exclusively found in melanin cells. It is therefore a good specific

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marker for these cells (Slominski et al., 2004). Tyrosinase is the rate-limiting enzyme and it catalyzes three different reactions in the biosynthetic pathway of melanin. Therefore the quantity of melanin synthesized is thus proportional to the amount of tyrosinase activity present in the cell (Tripathi et al., 1992).

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). 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). The protein structures of tyrosinase, *TYRP1* and *TYRP2* are homologues which consist of NH_2-terminal domain of tyrosinase which comprises 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.

Vitamin E is the major chain-breaking antioxidant preventing the propagation of oxidative stress, especially in biological membranes (Sebastian et al., 2005). Vitamin E also has revealed many important molecular properties, such as the scavenging of ROS to prevent oxidative damage that is associated with many diseases, or the modulation of signal transduction and gene expression in antioxidant and non-antioxidant manners (Zingg, 2007). The other biological functions of vitamin E that are unrelated to its antioxidant properties include its roles in cellular signaling, gene expression, immune response, and apoptosis are now considered to be of importance (Azzi et al., 2002; Brigelius-Flohe et al., 2002). In the vitamin E group, α-tocopherol is considered to be the most active form. However, recently, tocotrienols have gained increasing scientific interest due to their eminent antioxidant effects and a nonantioxidant activity profile that differs somewhat from that of tocopherol (Sebastian et al., 2005). Tocotrienols are identical in structure to tocopherols except for the degree of saturation in their side chain. Tocopherol has a saturated phytyl tail, while tocotrienols posses an unsaturated isoprenoid side chain. The prenyl side chain of tocotrienol has been postulated to be responsible for the differential membrane distribution and metabolism of tocotrienols when compared with tocopherols. α-Tocotrienol was observed *in vitro* to possess a remarkably higher antioxidant activity against lipid peroxidation than α-tocopherol. This was due to a more uniform distribution in the membrane lipid bilayer providing a more efficient interaction of the chromanol ring with lipid radicals (Kamat et al., 1997). Tocotrienols penetrate rapidly through skin, and its topical application is an efficient means with which to enrich skin with vitamin E (Traber et al., 1998). If skin is exposed to oxidative stress produced by ultraviolet or ozone after the topical application of vitamin E, the increased antioxidant content is sufficient to combat oxidative stress (Weber et al., 1997). Tocotrienol rich fractions are vitamin E that consists of 75% α-tocotrienol and 25% tocopherol (Mutalib et al., 2003). Tocotrienol rich fractions were found in barley, oats, palm, and commercial rice brans (Qureshi and Qureshi, 1993).

In this study, the anti pigmentation property 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 genes expression involved in the regulation of melanin synthesis such as *TYR, TYRP1* and *TYRP2* was also carried out. The study has been approved by the Research and Ethical Committee, National University of Malaysia.

MATERIALS AND METHODS

Cell culture

Human melanocytes obtained from foreskin of 9 - 12 year-old boys were grown in Medium 254, which is a basal medium containing essential and non essential amino acids, vitamins, other 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 study was assessed with Cell Titer 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, stock solution of TRF (Sime Darby Bhd, Malaysia) was freshly prepared in 100% ethanol and stored at -20°C for not more than 1 month. Immediately before use, TRF was incubated with FBS for an overnight (37°C). Then, culture medium and 100% ethanol were added to give the final concentration. Dilutions of TRF (100, 200, 300, 400, 500 µg/ml) were made in 50% ethanol. Cells were plated at 2×10^4 in 96-well plate and incubated overnight. The media was replaced with the new media containing various concentration of TRF and incubated for 24 h at 37°C, and 5% CO_2. After incubation, 20 µl MTS was added and cells were incubated for another 2 h. The absorbance of

Table 1. Primer sequences for quantitative gene expression analysis.

Gene	Accession No.	Primer sequence (5'→3')	PCR product size (bp)
GAPDH	BC020308	F: TCC CTG AGC TGA ACG GGA AG	217
		R: GGA GGA GTG GGT GTC GCT GT	
TYR	NM_000372.3	F: GAT GAG TAC ATG GGA GGT CAG C	102
		R: GTA CTC CTC CAA TCG GCT ACA G	
TYRP1	NM_000550.1	F: GCT CCA GAC AAC CTG GGA TA	185
		R: TCA GTG AGG AGA GGC TGG TT	
TYRP2	NM_001922.2	F: AGA TTG CCT GTC TCT CCA GAA G	116
		R: CTT GAG AAT CCA GAG TCC CAT C	

resulting MTS formazan was measured 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 500 µg/ml TRF for 24 h. Cell pellets were dissolved in 1 ml of 1 N NaOH at 37℃ overnight and centrifuged for 10 min at 10,000 x g. The optical density (OD) of the supernatants was measured at 450 nm 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 h followed by 24 h treatment with 500 µg/ml TRF. 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℃ and thawing in a water bath. Cell lysates were then clarified by centrifugation at 10,000 x g for 10 min. 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℃ for 1 h.

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℃ 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.

Quantitative Real time RT-PCR

Gene expression of *TYR, TYRP1 and TYRP2* was quantitatively analysed with real time RT-PCR technique. The expression level of each targeted gene was normalized to GAPDH. Primers for human *GAPDH, TYR, TYRP1 and 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℃; pre-denaturation for 2 min at 94℃; PCR amplification for 38 cycles with 30 s at 94℃, 30 s at 60℃ and 30 s at 72℃. 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 h 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

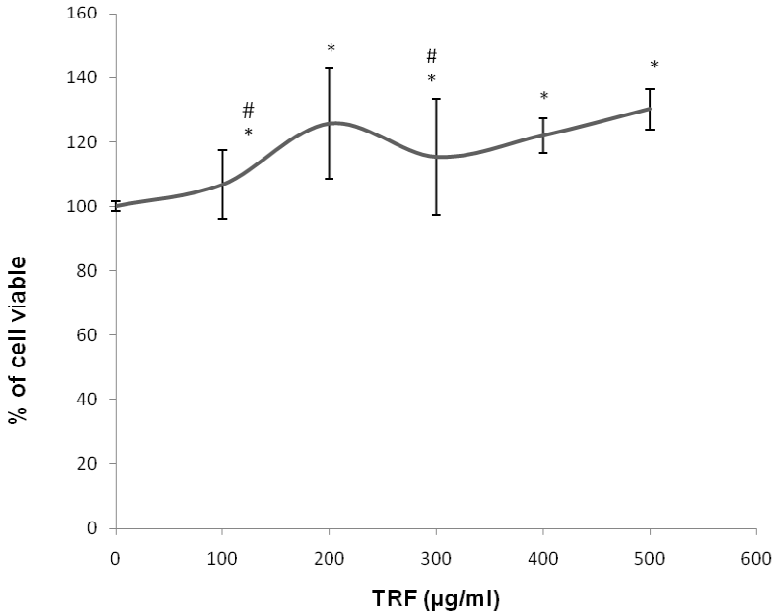

Figure 1. Effects of palm tocotrienol rich fraction (TRF) on melanocytes viability as assessed by MTS assay. Percent MTS reduction corresponds to the viable cell number. Melanocytes were incubated with increasing concentrations of TRF for 24 h at 37°C. Incubation with TRF caused a significant increase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

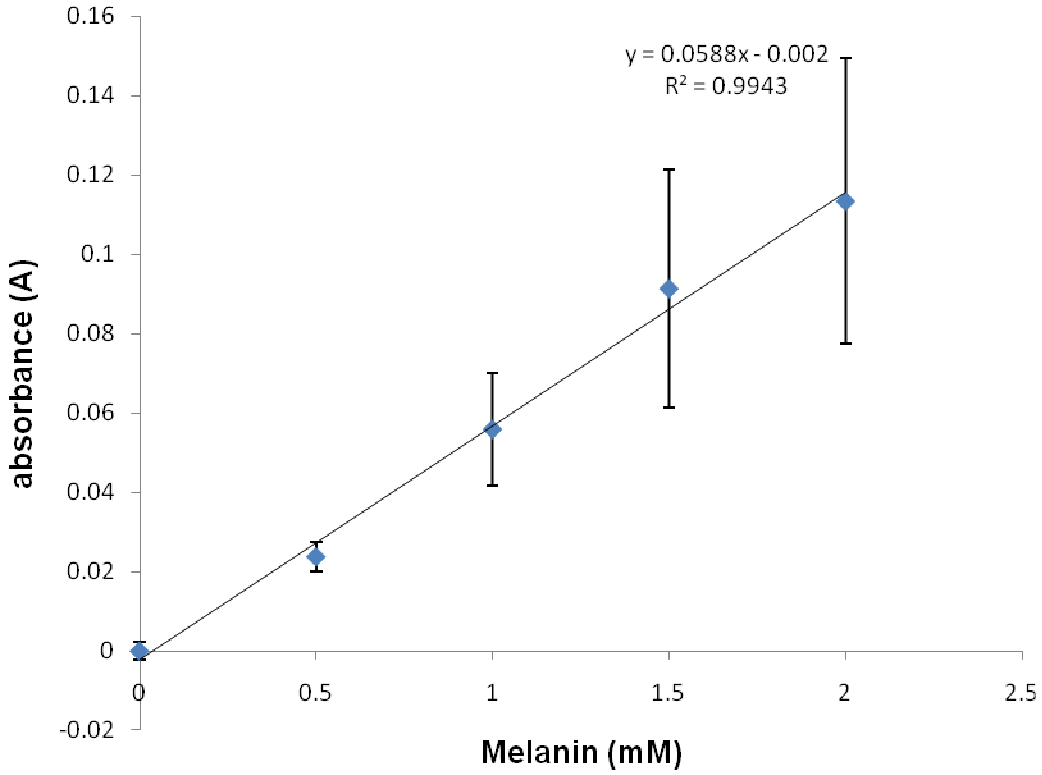

Figure 2. Standard curve for synthetic melanin.

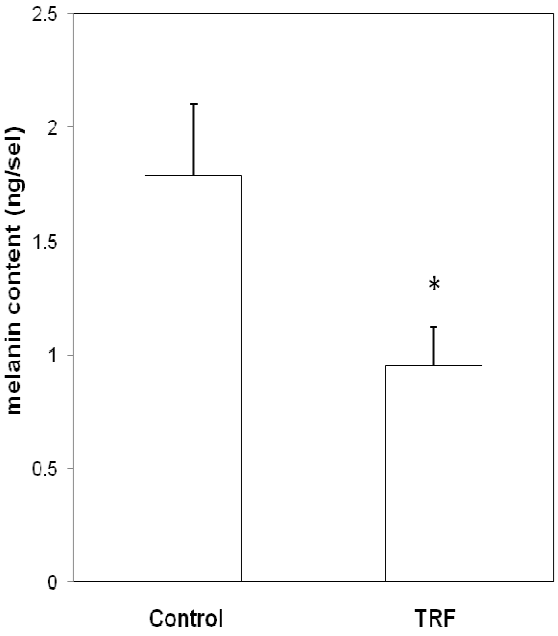

Figure 3. Anti pigmentation effects of palm tocotrienol rich fraction (TRF) as shown by reduction in melanin content (ng/cell) in TRF-treated skin melanocytes. Primary culture of skin melanocytes was treated with 500 ug/ml TRF for 24 h at 37°C. *Denotes $p < 0.05$ compared to untreated control. Data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

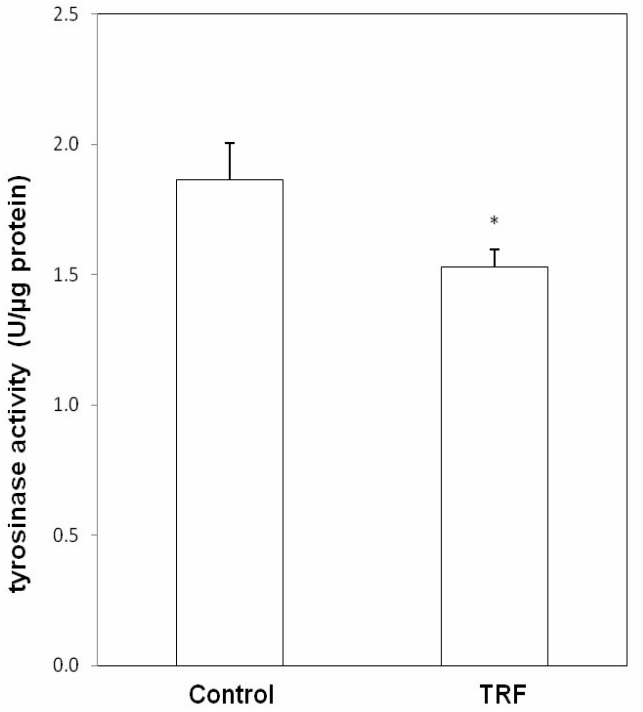

Figure 4. Anti pigmentation effects of palm tocotrienol rich fraction (TRF) as shown by reduction in tyrosinase activity (U/ug protein) in TRF-treated skin melanocytes. Primary culture of skin melanocytes was treated with 500 ug/ml TRF for 24 h at 37°C. *Denotes $p < 0.05$ compared to untreated control.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

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.

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 found to decrease but not significant compared to the untreated control.

DISCUSSION

Previous study reported that compounds with redox properties (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). Other previous study reported that the antioxidant properties of α-tocopherol, which interfered with lipid peroxidation of melanocyte membranes and increased intracellular glutathione content, could explain its depigmenting effect (Marmol et al. 1993). The findings from this study however showed tocotrienol rich fraction which contains both tocotrienol and tocopherol has an anti pigmentation property that inhibit melanin synthesis by inhibiting tyrosinase activity.

Other antioxidants such as ascorbic acid interfered 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 pigmen-

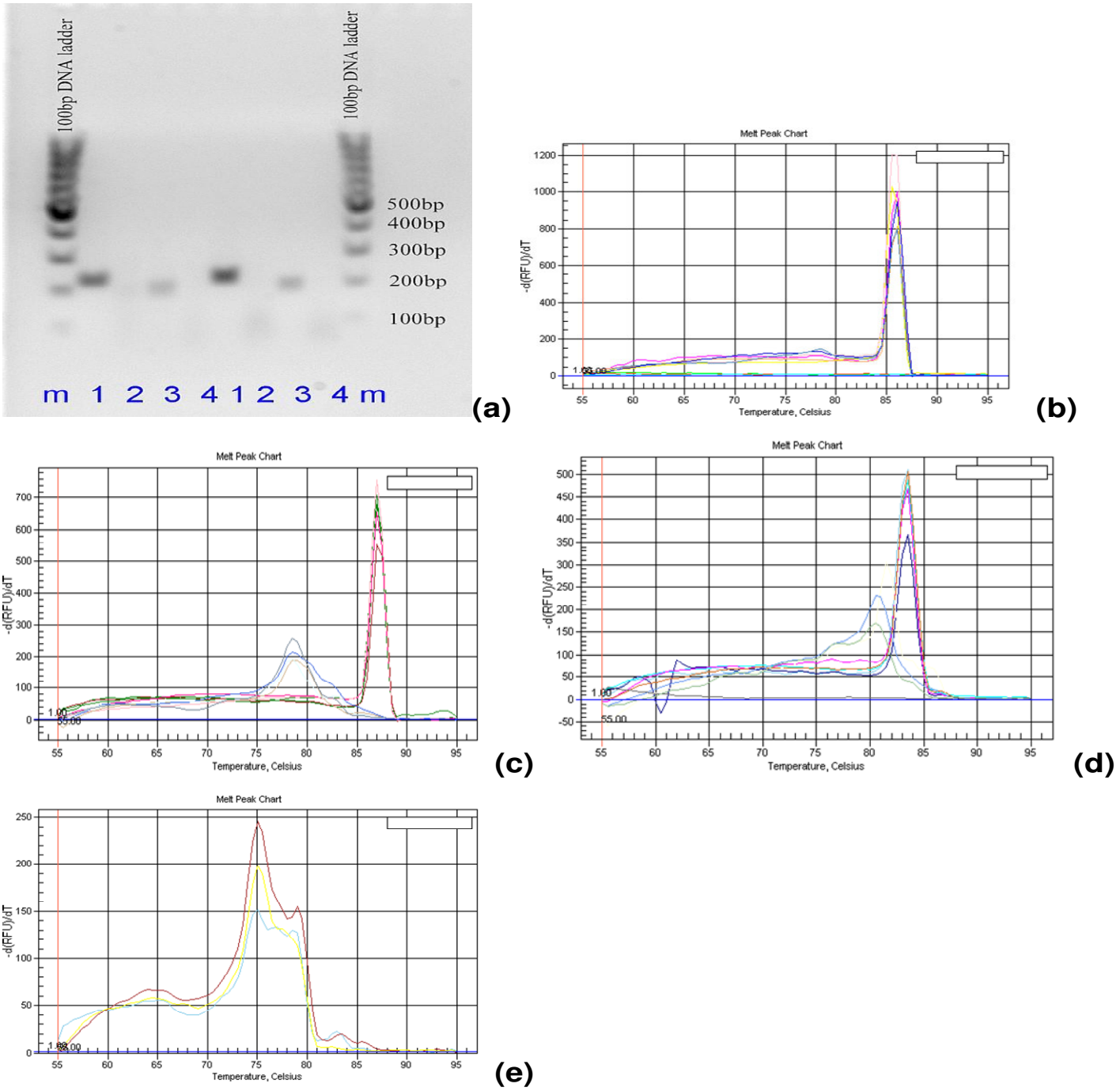

Figure 5. Agarose gel electrophoresis for *TYR, TYRP1, TYRP2* primers in human skin melanocytes. Primers were specific to gene of interest (a). Specificity of primer was shown by melting curve graph. Single and narrow peak indicated primers were specific for GAPDH gene (b), *TYR* gene (c), *TYRP1* gene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

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

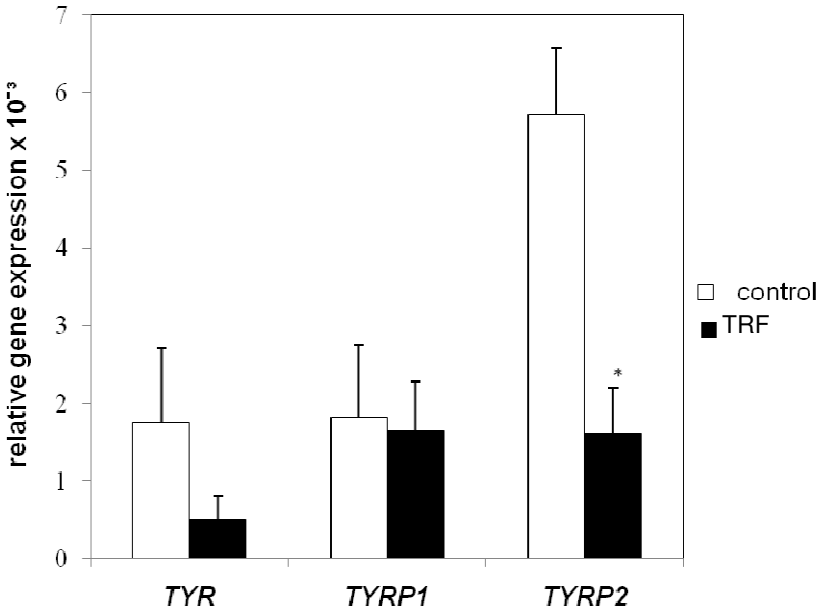

Figure 6. Effects of palm tocotrienol rich fraction (TRF) on *TYR*, *TYRP1*, *TYRP2* mRNA expression in TRF-treated skin melanocytes. Primary culture of skin melanocytes was treated with 500 ug/ml TRF for 24 h at 37°C. * Denotes $p < 0.05$ compared to untreated control. Data is presented as mean ± SD, n = 3.

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. This finding showed *TYRP2* expression was affected by tocotrienol rich fraction treatment but not for *TYR* and *TYRP1* genes expression. Previous study reported that certain antioxidant 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 which was 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 modulate *TYR* activity (Manga et al. 2000). Therefore down regulation of these genes may affect the activity of tyrosinase enzyme. This may explained the decreased in tyrosinase activity with down regulation of the *TYRP2* gene reported in this study.

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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