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In Vitro EVALUATION OF MELANOGENESIS INHIBITION BY TOCOTRIENOL RICH FRACTION

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ABSTRACT

Melanogenesis is a process of melanin production, the leading cause of pigmentation in human skin. However, ultraviolet (UV) irradiation can stimulate melanocytes and enhance melanin production. This study aimed to evaluate the effects of tocotrienol-rich fraction, a potent antioxidant, on UVA-induced melanogenesis in melanocytes. Melanocytes were treated with different concentrations of study materials (TRF, alpha-tocopherol (α-TP) and kojic acid (KA)) to determine the cell viability and the effect of TRF on melanin synthesis. Then, the optimum concentration of study materials was used to assess their effect on melanin synthesis in the UVA-irradiated melanocytes. Treatment of melanocytes with different concentrations of TRF and α-TP increased the number of viable cells. The optimum concentration of 62.5 µg mL^{-1} for study materials was used to determine their effects on melanin synthesis. After seven days, the irradiated and untreated melanocytes showed a significant 10.8% increase in melanin content, indicating that UVA irradiation directly influences melanogenesis. Melanocytes treated with TRF and irradiated with UVA showed a slight decrease in melanin content of about 4% compared to the negative control (irradiated), indicating that TRF may effectively protect cells from UVA irradiation. From findings, TRF could be a useful therapeutic agent for treating skin hyperpigmentation and a practical component to support skin lightening in skincare products.

Keywords: melanin content, melanocytes, TRF, UVA.

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INTRODUCTION

Melanogenesis is a highly regulated process in human skin. Melanogenesis occurs within melanosomes (Hida *et al*., 2020), the specialised lysosome-related organelles of melanocytes located in the basal layer of the epidermis (Le, 2020; Maranduca *et al*., 2019). Melanocytes are dendritic cells that synthesise melanin in melanosomes and transfer it to surrounding keratinocytes, where the cells are protected from DNA damage (Ramsden and Riley, 2014). Melanosomes protect epidermal cells from damage by limiting the penetration of UV radiation through the epidermal layers of the skin and scavenging reactive oxygen species (ROS) that are generated by UV radiation (Yi *et al*., 2018).

UV exposure is recognised as a well-known external risk factor for melanoma development (Sample and He, 2018), and a better strategy to prevent UV-induced melanoma is to inhibit both UVA and UVB cell damage pathways (Sun *et al*., 2020). Studies have shown that UVA strongly influences skin melanogenesis by activating the oxidative stress pathway involving ROS generation (Dumbuya *et al*., 2020). It is well established that UVA irradiation, especially UVA (340-400 nm), induces pigmentation and oxidative stress to a greater extent than UVB (290-320 nm) irradiation and is also more prominently associated with oxidative stress (Lohan *et al*., 2016; Nahhas *et al*., 2018). In another study, Chen *et al*. (2021) reported a link between oxidative stress and UVA-mediated exacerbation of melanogenesis, due to the overproduction of reactive oxidants.

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Plants are a rich source of biologically active substances that significantly affect human skin. Plants can exhibit a variety of properties, both medicinal for certain skin diseases and beneficial for skin health, including antioxidant effects (Michalak, 2020). Several botanical extracts have been found to have high antioxidant capacity and the ability to protect the skin from UV-induced effects (Dunaway *et al*., 2018). The ability to scavenge free radicals and the antioxidant properties of plants are associated with the presence of components such as polyphenols, tocopherols, carotenoids and ascorbic acid (Song *et al*., 2020). Tocopherols and tocotrienols are isoforms of vitamin E that inhibit the formation of new free radicals and neutralise existing free radicals.

Oil palm is the most efficient oil crop in the world, contributing to 31.6% (76.39 million tonnes) of global total oil and fats (Parveez *et al*., 2022). Palm oil is a rich source of vitamin E which comprises tocopherols and tocotrienols. It plays a pivotal role as an essential, fat-soluble nutrient that functions as an antioxidant in the human body. Both tocopherols (-TP) and tocotrienols (-T3) have isomers, designated as alpha (α-), beta (β-), gamma (γ-) and delta (δ-), which differ by the number and position of methyl groups on the chromanol ring. The mechanism of antioxidant activity of tocopherols and tocotrienols is partly related to the presence of a hydroxyl group in the chromanol ring, which donates a hydrogen atom to reduce free radicals (Adwas *et al*., 2019). In addition, tocotrienols have a beneficial effect on various chronic diseases, such as cardiovascular and neurodegenerative (Montagnani Marelli *et al*., 2016). It also has an anti-proliferative effects on human breast cancer cells (Loganathan *et al*., 2021).

Tocopherol is commonly and widely used in anti-ageing and skin-whitening products, although most scientific studies evaluating the potential benefits of tocopherol have been conducted in animals (Makpol *et al*., 2014). α-TP of vitamin E is a well-known antioxidant, reported to be the most abundant non-enzymatic, lipid-soluble antioxidant in human tissues. Topically applied α-TP can inhibit UV radiation-induced DNA damage in human keratinocytes *in vitro* pre and post-UVA exposure (Delinasios *et al*., 2018).

Kojic acid (KA) is an organic metabolite produced by fungi that inhibits tyrosinase activity in the formation of melanin (Saeedi *et al*., 2019). KA is a well-known depigmenting agent often used as a positive control in screening new components or extracts that successfully inhibit melanin production (Wang *et al*., 2022). Despite the numerous advantages of using KA in topical products, there are some drawbacks, such as contact dermatitis and potential skin photodamage (Saeedi *et al*., 2019). Tocopherols and tocotrienols, on the other hand, are widely used in dermatology and cosmeceutical for their antioxidant and UV protective properties that provide photoprotection to the skin.

There are many reports on the effects of vitamin E isomers on the inhibition of skin cancer cells, reduction of tyrosinase enzyme and melanin production. In mouse B16 melanoma cells, γ-TP inhibited up to 39% of melanin synthesis and 45% of tyrosinase activity. δ-T3 inhibited melanin synthesis significantly in B16 melanoma cells (Yap *et al*., 2010). A study done by Ng *et al*. (2014) showed that the inhibitory effects of δ-T3 on melanogenesis are mediated by the activation of extracellular signal-regulated kinase signaling, thus resulting in downstream suppression of melanogenesis-related proteins and melanin production. The potent antioxidant property of δ-T3 decreased the melanin levels in murine B16 melanocyte cells by preventing the oxidative reactions of tyrosinase (Michihara *et al*., 2010; Yap *et al*., 2010).

TRF consists of 70% tocotrienols (T3) and 30% tocopherols (TP) (Sen *et al*., 2010). It has been reported that treatment with palm TRF (500 $\mu g\ mL^{-1}$) possessed anti-melanogenic properties and may be useful in improving skin pigmentation caused by UVA (Makpol *et al*., 2014). However, the exact compositions of TRF used were not provided. Moreover, there are limited studies reporting the use of TRF to inhibit melanogenesis.

Therefore, the present work aimed to determine the effects of TRF (70% tocotrienols, 30% tocopherols) on UVA-induced melanogenesis. The optimum concentration of TRF, α-TP and KA was assessed by analysing cell viability, measuring melanin content and UVA-induced melanogenesis using human epidermal melanocytes. We hypothesised that TRF has beneficial effects as an anti-melanogenic agent to protect the skin from UVA-induced melanogenesis.

MATERIALS AND METHODS

Reagents

Ethanol with a purity of 99% was obtained from RCI Labscan, Thailand. TRF with a purity of 70% was obtained from Sime Darby, Malaysia. Kojic acid (KA), alpha-tocopherol (α-TP), thiazolyl blue tetrazolium bromide (MTT) and synthetic melanin were purchased from Sigma-Aldrich, US. Isopropanol, potassium phosphate buffer (potassium dihydrogen phosphate and dipotassium hydrogen phosphate) and sodium hydroxide (NaOH) were purchased from Merck, Germany. Normal human epidermal melanocyte, reagents and Dulbecco's

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Phosphate Buffered Saline (PBS) were purchased from Sciencell Research Laboratories (Carlsbad, CA, US).

Cell Culture

Melanocytes were cultured in a complete medium containing melanocyte medium, supplemented with a low percentage of Fetal Bovine Serum (FBS) (0.5%), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and melanocyte growth supplement. The cells were grown in a humidified chamber containing 5% CO_2 at 37°C.

Cell Viability Assay

When 90% confluency of melanocyte was attained at passage four, trypsinisation and seeding were done in 96-well plates ($2x10^4$ cells/well) in a complete medium. After overnight incubation, the medium was replaced with a new medium containing cells treated with various concentrations of TRF, α-TP and KA (7.8, 15.6, 31.3, 62.5, 125, 250 and 500 µg mL^{-1}) and incubated for 24 hr at 5% CO_2 at 37°C. After incubation, the wells were washed with PBS to remove debris and dead cells. The fresh medium was replaced with 10 µL of MTT (5 mg mL^{-1} in PBS), and MTT was added to each well and incubated for 4 hr at 5% CO_2 at 37°C. The solution in each well was replaced with dimethyl sulfoxide to solubilise the formed formazan crystals, and the wells were further incubated at room temperature for ten minutes. The absorbance of each well was measured at 540 nm using a BioTek Synergy™ H1 Reader (BioTek Instrument, Winooski, VT, US). The cell viability assay was performed to determine the optimum dose of TRF for subsequent experiments. The medium for untreated cells (control) was changed in parallel to the treated cells.

Melanin Content Measurement

Melanin content was determined using the method described by Zaidi *et al.* (2016) with slight modifications. A total of $1x10^5$ cells/well were seeded in 24-well plates. After overnight incubation, the medium was refreshed with a new medium. TRF, α-TP and KA at a concentration of 62.5 µg mL^{-1} were added and incubated for 24, 48, 72 hr and seven days. Melanocyte cells were harvested with EDTA/trypsin after treatments with TRF, α-TP and KA at each period for 1 hr. Then melanocyte cells were centrifuged at 10 000 g for 10 min. The supernatants were discarded, while pellets were dissolved in one normality (N) sodium hydroxide (NaOH) and incubated at 80°C for 1 hr. After incubation, the solution was centrifuged at 10 000 g for 10 min. The supernatants were transferred into 96-well plates in triplicate, and optical density (OD) was measured at 450 nm using BioTek Synergy™ H1 Reader.

Determination of Optimum Dose for UVA Irradiation

The optimum dosage for UVA irradiation was determined by exposing the skin melanocytes to UVA for seven consecutive days at doses of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 $J.cm^{-2}$. UVA irradiation was carried out according to Makpol *et al.* (2014) with slight modification. Melanocytes were exposured to UVA stress with one stress per day for seven consecutive days. After an optimised UVA dosage was determined, $1x10^5$ melanocytes at passage five were directly exposed to 0.4, 0.6 and 0.8 $J.cm^{-2}$ UVA in a thin layer of PBS (pH 7.4) in an uncovered 12-well plate. The irradiation was carried out at room temperature using a light UVL-56 Handheld UV lamp (UVP, USA) which emits UVA at 365 nm. The emitted radiation was checked using a UVX-digital radiometer with UVA 365 nm sensor (UVP, USA). After irradiation, the PBS was replaced with a medium, and cells were cultured under standard culture conditions.

Determination of Melanin in UVA-induced Melanogenesis

A total of $1x10^5$ melanocytes were incubated with palm TRF, KA and α-TP at 62.5 µg mL^{-1} per sample before UVA irradiation. The culture plates were placed at a distance of 20 cm from the UVA lamp and exposed for 6, 9, or 12 minutes to achieve a single dose of 0.4, 0.6 and 0.8 $J.cm^{-2}$, respectively. The UVA irradiation was performed once a day for seven consecutive days. The control cells were maintained in the same culture conditions without treatment with test samples and UVA exposure. After irradiation, PBS was discarded, and cells were incubated in the growth medium for 24 hr and analysed for melanin content.

Melanin content was measured spectrophotometrically at 450 nm after heating the cell lysates for 1 hr with one normality NaOH at 80°C as described by Zaidi *et al.* (2016) with slight modification. Melanin content was expressed as the percentage of the controls (Chatatikun *et al.*, 2019).

Statistical Analysis

The results were presented as means ± standard deviation. One-way analysis of variance ANOVA (SPSS version 16.0) was performed, and sequential differences between the means were identified at the level of $P<0.05$ using Bonferroni post-test analysis.

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RESULTS

Cell Viability

Incubation of melanocytes with different concentrations of TRF (7.8, 15.6, 31.3, 62.5, 125, 250.0 and 500.0 μg mL^{-1}) caused an increase in the number of viable cells (*Figure 1*).

After 24 hr of treatment, increasing the concentration of TRF and α-TP increased the percentage of viable cells. In contrast, increasing the concentration of KA did not significantly affect cell viability. The survival rate of melanocyte cells treated with TRF and α-TP was found to be high, indicating that TRF and α-TP promoted melanocyte cell proliferation. Therefore, 62.5 μg mL^{-1} of TRF was used in the subsequent experiment to determine its effect on melanocyte cells. Prolonged treatment of seven days using the optimal dose (62.5 μg mL^{-1}) of TRF and α-TP on melanocyte cells improved cell viability (*Figure 2*).

Melanin Content

Melanocyte cells were treated with TRF, α-TP and KA for 24, 48, 72 hr and seven days at 62.5 μg mL^{-1}. The melanin concentration was determined

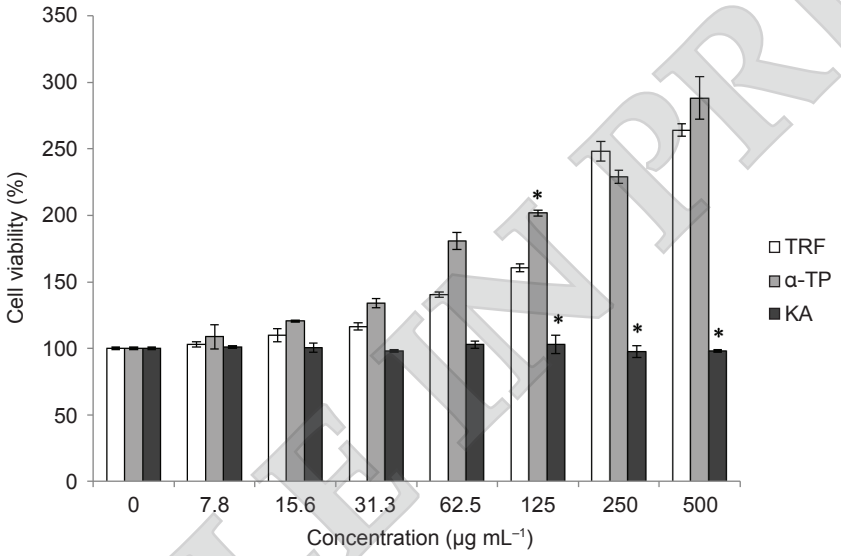

*Figure 1. Effects of TRF, α-TP and KA on melanocyte proliferation at 24 hr of treatment, as assessed by MTT assay. Data are presented as mean ± SD, n=3. Statistical significance was set at P<0.05 (ANOVA+Dunnett). * Significant difference with TRF within the same concentration.*

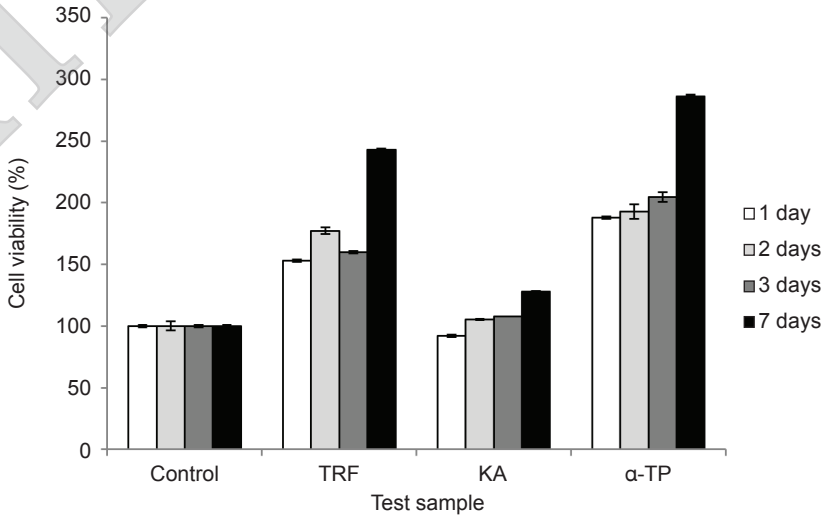

Figure 2. Effect of TRF, KA and α-TP at dose 62.5 μg mL^{-1} on cell viability within seven days.

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by comparing the optical density of TRF, α-TP and KA at OD_{450nm}. *Figure 3* shows a standard curve of melanin concentration which was highly correlated with the OD_{450nm}, where R^2 was 0.999.

Effect of Different Doses of UVA on Cell Viability

Figure 4 shows the effects of increasing UVA (0-10 J.cm^{-2}) on the viability of melanocyte cells after seven days of treatment. After daily exposure to UVA for seven consecutive days at different doses, the percentage of viable melanocytes decreased compared to the control (non-irradiated cells). In this study, the UVA dose of 0.6 J.cm^{-2} was less toxic than the other doses. It was observed that at doses beyond 0.6 J.cm^{-2}, the melanocytes were swollen or enlarged cell bodies and ruptured membranes compared to non-irradiated cells and cells exposed to lower doses of UVA. The results of our study showed that cell viability was increased in melanocytes exposed to 0.6 J.cm^{-2} UVA and produced the highest melanin content, which is consistent with the report of Makpol *et al*. (2014). Melanocytes with high melanin content showed increased resistance to UVA cytotoxicity and those with low melanin content showed low resistance to UVA cytotoxicity.

Figure 5 shows the melanin level of melanocyte cells exposed to single UVA stress daily for seven consecutive days at a dose of 0 to 1 J.cm^{-2}. Exposure to increasing amounts of UVA increased the level of melanin content compared to cells without exposure

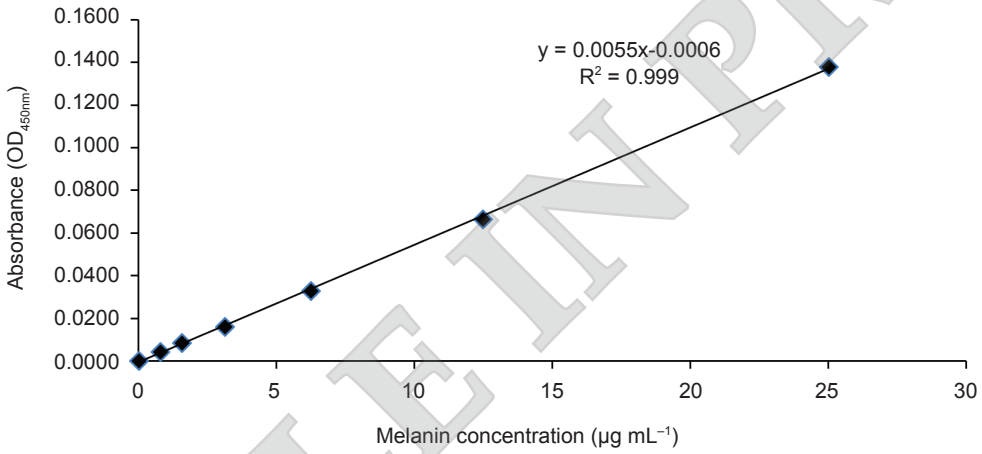

Figure 3. Standard curve for synthetic melanin at concentration ranges between 0-25 µg mL^{-1}.

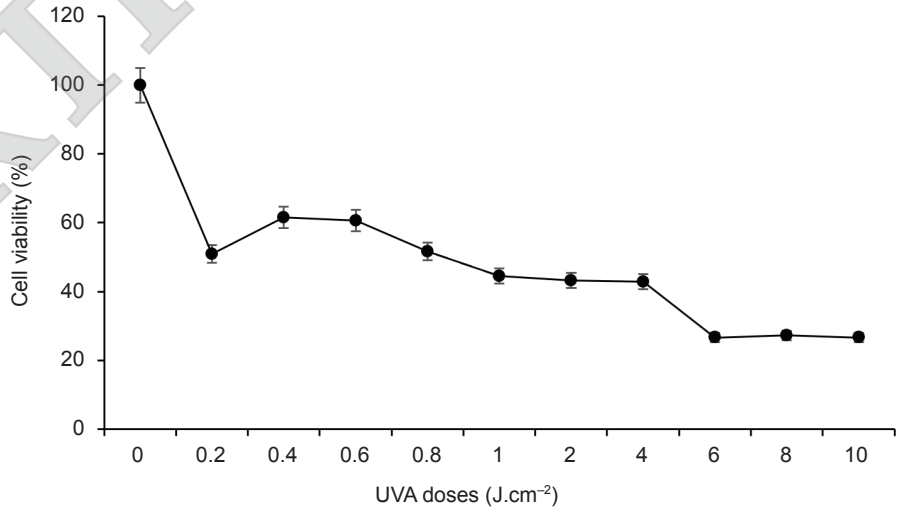

Figure 4. Effect of different doses of UVA on melanocyte cell viability.

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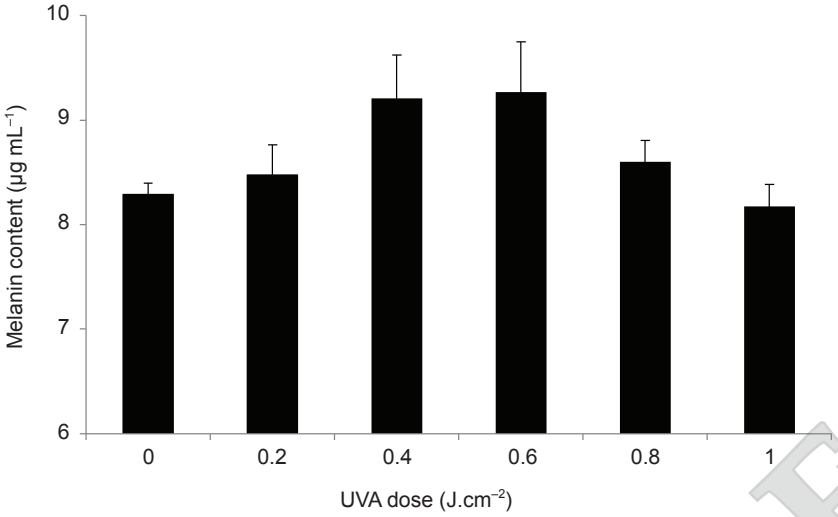

Figure 5. Effect of different doses of UVA on melanin level.

to UVA. The melanin content of melanocytes exposed to UVA reached a maximum level at 0.6 J.cm^{-2} and decreased beyond 0.6 J.cm^{-2}. Thus, further study on the photoprotective effect of test samples against UVA irradiation was limited to the UVA dose of 0.6 J.cm^{-2}.

Photoprotective Effect of Palm TRF

Figure 6 shows the melanin content of melanocyte cells treated with TRF, KA and α-TP (62.5 µg mL^{-1}) in the absence of UVA irradiation. After seven days, there was no significant difference in melanin content for negative control and melanocyte cells treated with TRF and KA compared to day one. However, there was a significant increase (P<0.05) in melanin content when melanocyte cells were treated with α-TP compared to day one. This may be due to the ability of melanocyte cells to proliferate when treated with α-TP, thus increasing the melanin level as well. However, in the presence of UVA irradiation, melanocyte cells treated with KA (known as a universal whitening agent) showed significantly lower (P<0.05) melanin content than TRF and α-TP at day seven. Meanwhile, the melanin content of melanocyte cells treated with TRF significantly decreased (P<0.05) compared to α-TP on day seven. This data was supported by a study by Yap *et al.* (2010) which stated that treatment with tocotrienol isomers (δ- and γ-) was able to lower melanin content in cells but not α-TP.

Melanogenesis is intensified in melanocytes in response to harmful conditions, including UVA radiation (Rok *et al.*, 2018). Thus, the photoprotective effect of TRF on UVA-irradiated melanocyte cells was evaluated. The melanocyte cells were irradiated with UVA light with a single UVA dose of 0.6 J.cm^{-2} for seven consecutive days. After seven days, the irradiated and untreated melanocyte cells showed a 10.8% increase in melanin content, indicating that UVA irradiation directly influences melanogenesis. Melanocytes treated with TRF and irradiated with UVA showed a slight 2.8% increase in melanin content, indicating that TRF may effectively protect the cells against UVA irradiation. KA-treated and UVA-irradiated melanocyte cells did not indicate any significant increase in melanin content. However, melanocytes treated with α-TP showed a 16.6% increase in melanin content. A previous study reported that a UVA dose of 0.6 J.cm^{-2} was identified as the non-toxic dose (Makpol *et al.*, 2014).

DISCUSSION

In this study, we investigated the potential of TRF on melanogenesis, including inhibition of melanin synthesis and UV-induced melanogenesis in melanocytes.

We found that treatment with increasing concentrations of TRF and α-TP caused a significant increase in the number of viable cells for skin melanocytes. The viable cells' percentage increased at doses of 62.5 to 500.0 µg mL^{-1} (*Figure 1*). A previous study by Khor *et al*. (2016) reported that TRF and α-TP could increase cell viability and improve cellular morphology at a concentration of 50.0 µg mL^{-1}. Also, treatment with both TRF and α-TP for 24 hr promoted higher cell proliferation than KA. Data was supported by Khor *et al*. (2016), wherein TRF increases cell viability and enhances the proliferation capacity of senescent myoblasts. These findings proved the potential of TRF in improving the proliferation capacity of melanocytes

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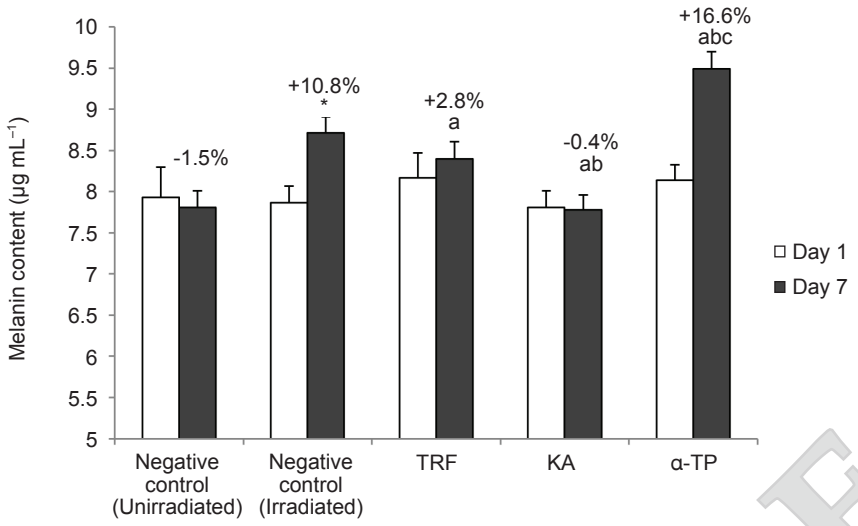

*Figure 6. Evaluation of photoprotective properties of melanocyte cells treated with TRF, KA and α-TP and irradiated with UVA 0.6 J.cm^{-2}. *P<0.05 compared to Day 1; [a]P<0.05 compared to control at Day 7; [b]P<0.05 compared to TRF at Day 7; [c]P<0.05 compared to KA at Day 7.*

in culture. Also, treatment with both TRF and α-TP for 24 hr promoted higher cell proliferation than KA. Prolonged treatment for seven days using the dose of 62.5 µg mL^{-1} improved cell viability in melanocyte cells (*Figure 2*). In this study, the data indicated that TRF and α-TP not only increase cell viability but also enhance the proliferation capacity of melanocyte cells. Vitamin E can act as a cellular antioxidants and promote cell growth. Tocotrienol has also been reported to promote proliferation, differentiation, and mineralised nodule formation in osteoblastic cells (Xu *et al*., 2018). In another study it was reported that low doses (50.0 µg mL^{-1}) of TRF stimulated the proliferative capacity of myoblasts (Lim *et al*., 2019). Recently, treatments with α-T3, δ-T3 or γ-T3 significantly increased the viability of the murine splenocytes. These results suggest that tocotrienol isoforms do not have cytotoxic effects on the murine splenocytes, *i.e.*, normal primary cells and tocotrienols may have less impact on the proliferation of normal cells (Loganathan *et al*., 2021). From this study, it was evident that TRF improves the proliferative capacity of melanocytes in culture without being cytotoxic.

Figure 4 shows that the percentage of viable cells decreased when exposed to an increasing dose of UVA as compared to the control (non-irradiated cells). The UVA dose of 0.6 J.cm^{-2} was less cytotoxicity than doses beyond 0.6 J.cm^{-2} in primary human melanocytes. This is in agreement with what was reported by Makpol *et al*. (2014). Meanwhile, *Figure 5* shows the melanin content in melanocytes at different doses of UVA. Exposure to increasing doses of UVA (up to 0.6 J.cm^{-2}) raised the level of melanin content compared to cells without exposure to UVA (*Figure 5*). This is because UVA-induced ROS oxidises proteins and lipids, disrupting normal cellular signaling and possibly promoting abnormal cell proliferation (Dunaway *et al*., 2018). The presence of melanin may protect against the effects of direct UVA exposure, and it is possible that melanin may have heterogeneous effects and may simultaneously absorb UVA photons and intracellular ROS.

Melanocytes treated with TRF and irradiated with UVA showed a significant reduction in melanin content (+2.8%) compared to untreated control melanocytes (+10.8%), as shown in *Figure 6*. In this study, melanocytes treated with α-TP and irradiated with UVA showed the highest melanin content (+16.6%), indicating that α-TP is ineffective in protecting the melanocytes compared to TRF. The significant difference in melanin content in melanocytes treated with TRF or tocopherol may be due to the difference in molecular structure. Tocotrienols have a unique structure with three unsaturated double bonds in their side chains, while tocopherols have a saturated phytyl tail. The unsaturated side chain of the tocotrienol molecule allows for better penetration into the cell membrane compared to tocopherols (Serbinova *et al*., 1991; Wong and Radhakrishnan, 2012). The presence of these double bonds in tocotrienols allows them to interact more effectively and neutralise free radicals. Free radicals are highly reactive molecules that cause oxidative damage to cells (Adwas *et al*., 2019; Drotleff and Ternes, 2001; Farouk Musa, 2021). This unique property increases the efficiency of tocotrienols in reducing the formation of ROS in melanocytes when irradiated with UVA and indirectly reduces the formation of melanin, as observed in this experiment compared to melanocytes treated with

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tocopherol. Another study showed that TRF protects the cells and thus reduces oxidative damage caused by UV radiation (Sample and He, 2018; Solano, 2020).

Vitamin E and its derivatives act as a melanogenesis inhibitor in epidermal melanocytes *in vitro* and inhibit tyrosinase activity, a key enzyme in melanisation. Moreover, the study found that tocotrienols effectively reduce skin redness and pigmentation after UV exposure (Yap, 2018).

KA is a well-known skin whitening agent and a potent tyrosinase inhibitor (Masum *et al*., 2019) and in this study, KA was used as the positive control. As shown in *Figure 6*, there was a significant reduction in melanin levels of melanocyte cells treated with KA after UVA radiation compared to the control on day seven. This is because KA is a naturally occurring metabolite produced by fungi that can inhibit tyrosinase activity in synthesis of melanin. Tyrosinase is composed of copper ions. When exposed to UV light, the copper ion induced tyrosinase to become more active. The copper ion is captured by KA, preventing it from activating the tyrosinase. By inhibiting tyrosinase activity, KA can also prevent melanin formation (Saeedi *et al*., 2019).

Vitamin E has been proven for its antioxidant and UV protection effects, providing photoprotection (Aparecida Sales de Oliveira Pinto *et al*., 2021), moisturising and delaying skin aging (Ijaz and Akhtar, 2020). Different studies have shown that supplementation with vitamins C and E significantly reduces UV-induced skin damage than supplementation with vitamin C alone (Pullar *et al*., 2017), possibly due to vitamin E regeneration. Vitamin E protection against UV-induced cytotoxicity has been linked to the extent of its uptake into cells. It also has been reported that vitamin E interacts with endogenous antioxidant enzymes and plays a crucial role in recycling the antioxidant defence system in human cells (Miyazawa *et al*., 2019).

Our findings revealed that TRF protects the melanocyte cells from UVA irradiation. Inhibition of melanin synthesis might be the possible mechanism of TRF as an anti-melanogenic agent that protects against UVA-induced melanogenesis.

CONCLUSION

In conclusion, palm TRF can inhibit melanin synthesis and thus may act as an anti-melanogenic agent that protects against UVA-induced melanogenesis. Interestingly, this study found that the inhibitory effect of TRF on melanin synthesis was higher than KA and α-TP. Therefore, TRF may potentially be developed as an anti-melanogenic agent for topical skin care applications.

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