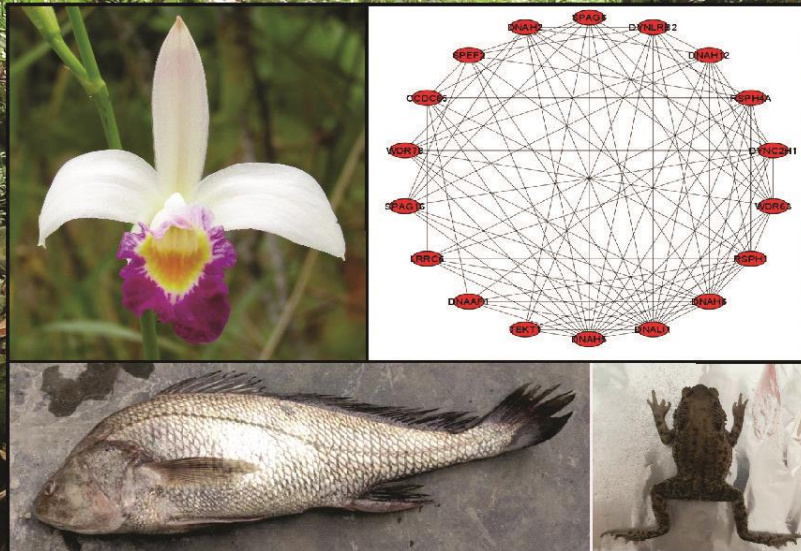


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# Inhibition of UVB-mediated Oxidative Stress in Immortalized HaCaT Keratinocytes by n-hexane Terpenoid Rich *Canarium odontophyllum* Extract (TRCO) as Evinced by Markers of Photodamage

AHMAD ROHI GHAZALI<sup>1</sup>, YOGABAANU ULAGANATHAN<sup>1</sup>, MUHAMMAD WAHIZUL HASWAN ABDUL AZIZ<sup>1,2\*</sup> & DAYANG FREDALINA BASRI<sup>3</sup>

<sup>1</sup>Centre for Toxicology and Health Risk Studies (CORE), Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM), Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Federal Territory of Kuala Lumpur; <sup>2</sup>Department of Para-clinical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak (UNIMAS); <sup>3</sup>Centre for Diagnostic, Therapeutic & Investigative Studies (CODTIS), Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM), Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Federal Territory of Kuala Lumpur

\*Corresponding author: [amwhaswan@unimas.my](mailto:amwhaswan@unimas.my)

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## ABSTRACT

Acute exposure of eukaryotic cells to ultraviolet-B (UVB) radiation leads to a number of detrimental effects, one such prominent effect of UVB exposure is increased production of free radicals which can lead to oxidative damage. Although, the human skin is well equipped with endogenous antioxidant defence system, often increased levels of free radicals lead to oxidative damage in skin. Skin inflammation, accelerated skin aging, and formation of wrinkles are all consequences of UVB induced photodamage. Hence, it is posited that supplementation of an exogenous antioxidant derived from natural products could prevent and reduce oxidative damage in skin cells. This study set forth to investigate the antioxidative role of terpenoid rich *Canarium odontophyllum* Miq. (Dabai) extract on acute UVB-induced photodamage human keratinocyte cells (HaCaT). We first evaluated the antioxidative capacity of increasing concentrations of crude extracts of TRCO Dabai extracts (62.50 µg/mL, 125 µg/mL, 250 µg/mL, and 500 µg/mL) through FRAP assay. We found all the tested TRCO extract exhibited antioxidative capacity in dosage dependent manner. We further investigated the effects of pre-treatment 250 µg/mL and 500 µg/mL TRCO on UVB-induced photodamaged HaCaT cell by measuring oxidative stress markers of lipid peroxide (LPO content), protein carbonyl (PC) content, glutathione peroxidase (GSH-Px) and glutathione-S-transferase (GST) activities. Both 250 µg/mL and 500 µg/mL TRCO extract pre-treated UVB-induced HaCaT cell group exhibited significantly reduced lipid peroxides content and GST activity compared to the positive control ( $p < 0.05$ ). Pre-treatment of 250 µg/mL TRCO extract significant enhanced GSH-Px activity ( $p < 0.05$ ). However, no significant difference in protein carbonyl content could be established across all tested groups. Therefore, our results suggest that TRCO extract can offer protection against oxidative damages caused by UVB exposure, and said protective effects can be attributed by its antioxidant properties.

Keywords: *Canarium odontophyllum*, exogenous antioxidants, keratinocyte, oxidative photoaging, skin

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## INTRODUCTION

The epidermis layer of human skin forms the outermost protective barrier which defends against xenobiotic, environmental and pathogenic stressors (Grice & Segre, 2011). The epidermal layer provides physical separation between organisms and the external environment (Ghazali *et al.*, 2020) which is important in negating effects of environmental stressors. However, the protective mechanism of skin is predominantly compromised by skin aging. Skin aging is influenced by both intrinsic and

extrinsic factors which results in loss of cellular integrity thus resulting in altered functional roles (Landau, 2007). Intrinsic aging is a normal physiological occurrence that gradually leads loss of skin firmness, thickness as well as reduced capacity in skin repair mechanisms while extrinsic aging is caused due to environmental aspects like cosmetics, pollution, poor nutrition and UV irradiation, resulting in pre-mature skin aging, coarser skin, skin laxity and loss of cell elasticity (Zhang & Duan, 2018). UV irradiations are well-documented to be the primary external factor to accelerate

skin aging (Wang *et al.*, 2019).

There are three types of UV radiations namely UVA (315- 400 nm), UVB (280 to 320 nm) and UVC (100 to 280 nm) except for UVC, two other ultraviolet radiations penetrate into skin in wavelength dependent manner. UVA radiation penetrates through epidermis and dermis layer while UVB radiation does not penetrate deeper than the epidermis layer (You *et al.*, 2001). Although both UVA and UVB impose damaging effects to the skin, UVB radiation is considered more detrimental, as UVB radiations are shorter and can be directly absorbed by DNA causing mutations in pyrimidine bases of DNA leading to initiation of skin cancer (Mahendra *et al.*, 2021). In particular, UVB ultraviolet irradiation accelerates the generation of free radicals (Wondrak *et al.*, 2006). When the levels of free radicals exceed threshold levels, oxidative damage occurs, thus, leading to detrimental effects to cellular nucleic acids, cell membranes, cellular proteins and lipids (McDaniel *et al.*, 2018).

Fortunately, skin cells are equipped with elaborate antioxidant defence system composing of enzymatic and non-enzymatic antioxidants which work in cohort to protect cells against oxidative damage. The roles of endogenous antioxidants against UV induced oxidative damage are classified into four major mechanisms; i) scavenging and quenching reactive species, ii) ending free radical chain reactions, iii) repairing molecular damages caused by radicals and iv) sequestration of transition metal ions (Aguilar *et al.*, 2016). These functions are essential in inhibiting oxidant reactivity and in safe-guarding from cellular oxidative stress. However, the capacity of these endogenous antioxidants is not unlimited. Overexposure to UV can overwhelm the antioxidant defence system (Steenvoorden & Beijersbergen van Henegouwen, 1997). Therefore, additional photoprotection approaches are essential to maintain redox balance in cells thus avoiding further oxidative injury.

## MATERIALS AND METHODS

### Plant Material

Fresh leaves of *Canarium odontophyllum* Miq were collected in December 2019 from

(1°26'03.2"N 110°25'52.1"E) Kuching, Sarawak. The specimen was brought in with export and research and development permits obtained from Sarawak Biodiversity Centre (Permit No: SBC-2020-EP-58-MWH & SBC-2019-RDP-20-MWH) by Dr. Muhammad Wahizul Haswan Aziz and Associate Prof Dr. Dayang Fredalina Basri, University Kebangsaan Malaysia (UKM). The specimen was deposited in UKM Herbarium with voucher number ID ID028/2020.

### Preparation of leaf extract of *Canarium odontophyllum*

Solid-liquid solvent extraction method was employed to extract *Canarium odontophyllum* Miq. (Dabai) leaves extract. In the ratio of 1:10, 40g of *C. odontophyllum* air-dried and coarsely grounded leaves were soaked in 400 mL of n-hexane solvent for 48 hr at room temperature. After 48 hours, the mixture was filtered by using Whatman No.1 filter paper to collect the filtrate. The filtrate was then concentrated under reduced pressure using rotary evaporator until crude were formed. The obtained crude was allowed to air-dry under fume hood for 24 hr to remove remaining solvent. The resultant terpenoid rich crude was weighed. Terpenoid rich *C. odontophyllum* (TRCO) stock (1mg/mL) were prepared by diluting 1 mg of the crude in 1000mL of DMSO and kept at 4°C until further use. *C. odontophyllum* test concentration ranging from 62.50 µg/mL, 125 µg/mL, 250 µg/mL and 500 µg/mL were prepared by diluting the 1mg/mL of *C. odontophyllum* stock in distilled water and sterilised using 0.22 µm Millipore syringe filter.

### Determination of *In Vitro* Antioxidant efficacy of *Canarium odontophyllum* Miq. Leaves Extract via Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power of TRCO were evaluated in accordance to (Benzie and Strain 1996). Essentially, in the presence of antioxidants, colorless ferric ion  $[\text{Fe}^{3+}-(2,4,6\text{-Tris}(2\text{-pirydy})\text{-s-triazine})_2]^{3+}$  is reduced to insoluble Prussian blue ferrous ion-TPTZ complex  $[\text{Fe}^{2+}-(\text{TPTZ})_2]^{2+}$  in acidic medium. Firstly, FRAP working reagent were prepared in 10:1:1 ratio by mixing 30 mL of acetate buffer (30 mM, pH 3.6), 3 mL of  $\text{FeCl}_3$  (20 mM) and 3 mL of TPTZ solution (10 mM). The FRAP

working reagent wrapped in aluminium foil and placed in 37°C water bath until use. A calibration curve using iron (II) sulphate  $\text{FeSO}_4$  calibration is prepared with serially diluted concentrations that range from 100 to 1,000  $\mu\text{M}$ . Ascorbic acid as positive control with concentrations ranging from 3.125  $\mu\text{g/mL}$  to 50  $\mu\text{g/mL}$  were also prepared. The *C. odontophyllum* Miq. test concentrations of 62.50  $\mu\text{g/mL}$ , 125  $\mu\text{g/mL}$ , 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  were also prepared. For the assay, 50  $\mu\text{L}$  of  $\text{FeSO}_4$ , ascorbic acid solution and *C. odontophyllum* Miq. test extracts were added into respective wells in a 96-well plate in which each concentration consisted of quadruplicate ( $n=4$ ). Then, 175  $\mu\text{L}$  of pre-warmed FRAP reagent were added to the wells. Next, the plates were incubated at 37 °C for 5 min. Finally, absorbance readings were taken at 595 nm using microplate reader. The FRAP values were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) (Serbessa, 2019).

### Cell culture

The immortalized human keratinocyte (HaCaT) cell lines were purchased from Elabscience USA, catalogue number EP-CL-0090). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing glucose, L-glutamine and sodium pyruvate (HiMedia, India), 1% penicillin-streptomycin mixture (Nacalai Tesque, Japan) (Pen-Strep, 10 000 IU/mL) and supplemented with 15% foetal bovine serum (FBS) (Sigma-Aldrich®). The cells were maintained under standard cell culture conditions of 37°C, 5%  $\text{CO}_2$  and 95% humidity.

### UVB Treatment of HaCaT Cells

HaCaT cells were seeded in 6 wells cell-culture plates at a density of  $4 \times 10^4$  cell/mL. Cells were maintained in DMEM media supplemented with 1% pen-strep and 15 % FBS until 80% confluency were reached. The media was removed and cells were rinsed with sterile phosphate-buffered saline (PBS). Then, cells were divided into 250  $\mu\text{g/mL}$  treatment group, 500  $\mu\text{g/mL}$  treatment group, negative control, and positive control groups. The 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  treatment groups were pre-treated with 1 mL of 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  *C. odontophyllum* extract prepared in DMEM media. Whereas, both positive and negative control groups were added with 1 mL DMEM

media only. Next, cells were incubated at 37 °C, 5%  $\text{CO}_2$  and 95% humidity for 30 min. Upon incubation, 250  $\mu\text{g/mL}$ , 500  $\mu\text{g/mL}$  *C. odontophyllum* extracts treatment groups and positive control group were exposed to UVB radiation at 30  $\text{mJ/cm}^2$  without culture plate cover. Negative control group was not exposed UVB irradiation. Then, cells were washed twice with 1 mL ice-cold PBS for cell lysate preparation.

### Cell lysates Preparation

Cell lysates were prepared by adding 400  $\mu\text{L}$  of ice-cold RIPA lysis buffer to cells. Next, cells were incubated on ice for 5 min. Upon incubation, cells were gently scrapped and collected in micro-centrifuge tubes. The lysates were incubated in ice for 30 min on constant agitation. After incubation, cell lysates were centrifuged at 16128 RCF/g for 10 min at 4°C to remove cell debris. Supernatant of cell lysates were carefully collected and protein estimation was made based on BCA method.

### Determination of Lipid Peroxides (LPO) Content, Protein Carbonyl (PC) Content, Glutathione Peroxidase (GPx) and Glutathione-S-Transferase (GST) Activity

The lipid peroxide (LPO) and protein carbonyl (PC) content as well as glutathione peroxidase (GPx) and glutathione-s-transferase (GST) activities of cell lysate samples were determined using relevant commercial kits from Elabscience® in accordance to manufacturer's instruction.

### Statistical analysis

Data values are expressed in mean  $\pm$  standard error of measurement (SEM) based on experiments performed in triplicates ( $n=3$ ). One-way ANOVA test was carried out to compare means of various treatment groups. Significant level was set to 0.05 wherein results were considered statistically significant if  $p < 0.05$ . IBM SPSS Statistics Version 23 and GraphPad Prism Version 9 were used for statistical analysis.

## RESULTS

### Antioxidant Capacity of n-hexane extract of *C. odontophyllum* Miq

The reducing capacity of TRCO were through FRAP Assay. The FRAP values were expressed as ascorbic acid equivalent antioxidant capacity (AAEAC) in the unit of  $\mu\text{g AA}$  (ascorbic acid)/g *C. odontophyllum* Miq leaves extract (Serbessa, 2019). TRCO extract with concentration of range of 62.50  $\mu\text{g/mL}$ , 125  $\mu\text{g/mL}$ , 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  were tested for its antioxidative capacity. Significant difference in FRAP values

between all the tested extract concentrations were observed ( $p < 0.05$ ). The highest FRAP value was exhibited by 500  $\mu\text{g/mL}$  with  $6.75 \pm 0.08 \mu\text{gAA/g}$  (Table 1), followed by 250  $\mu\text{g/mL}$  expressing  $4.27 \pm 0.06 \mu\text{gAA/g}$ , 125  $\mu\text{g/mL}$ , with  $1.66 \pm 0.03 \mu\text{gAA/g}$  while the lowest concentration of 62.50  $\mu\text{g/mL}$  exhibited  $1.11 \pm 0.07 \mu\text{gAA/g}$  ( $n=4$ ) (Fig 1). All the FRAP values were statistically significant at  $p < 0.05$ .

**Table 1.** Ascorbic Acid equivalent FRAP values of different concentrations of Terpenoid rich *Canarium odontophyllum* (TRCO) extracts

Concentration ( $\mu\text{g/mL}$ )	Ascorbic acid Equivalent FRAP values ( $\mu\text{gAA/g}$ )
62.5	$1.11 \pm 0.07$
125	$1.66 \pm 0.03$
250	$4.27 \pm 0.06$
500	$6.75 \pm 0.08$

#### Effects of TRCO Extract on Lipid Peroxides (LPO) Content on UVB-irradiated HaCaT Cells

In the present study, we assessed the effects of TRCO on lipid peroxides content in acute UVB exposed keratinocyte cells. We found that the highest concentration of lipid peroxides was exhibited in UVB only exposed HaCaT cells (Figure 2), whereas the control group (non-UVB exposed) exhibited lower lipid peroxide concentration. A statistically significant difference between UVB exposed and control group in lipid peroxide concentration was observed ( $p < 0.05$ ). Our result further demonstrated that pre-treatment with TRCO extracts prior to UVB exposure effectively reduces lipid peroxide content in HaCat cells (Figure 2). Notable lower lipid peroxide content was observed in both TRCO extracts pretreated UVB-exposed groups compared to UVB only exposed group. Both 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  TRCO treatment groups significantly reduced generation lipid peroxides in comparison to UVB only treated group ( $p < 0.05$ ). However, no significant difference in lipid peroxide content was observed between 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  TRCO. extracts pre-treated UVB exposed HaCat cells ( $p < 0.05$ ). Both 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  TRCO extracts pre-treated UVB exposed HaCat cells show no significant difference in LPO content compared to negative control group (non-UVB exposed group) ( $p < 0.05$ ).

#### Effects of TRCO Extract on Protein Carbonyl (PC) Content on UVB-irradiated HaCaT Cells

We assessed the effects of TRCO extracts in protein carbonyls formation in UVB exposed HaCaT cells. Highest protein carbonyl content was evident in UVB only exposed group ( $134.70 \pm 15.21 \text{ nmol/mgprot}$ ) (Figure 3). However, when comparing protein carbonyl levels between UVB only exposed group to negative control group ( $108.20 \pm 10.82 \text{ nmol/mgprot}$ ), no significant difference could not be established ( $p < 0.05$ ). We notice that the negative control group exhibited relatively high protein carbonyl content albeit the absence of UVB exposure. Pre-treatment of 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  TRCO extracts exhibited relatively lower protein carbonyl content compared to UVB exposed group. However, only 500  $\mu\text{g/mL}$  TRCO extract pre-treatment group exhibited significant difference in PC content when compared to UVB only exposed group ( $p < 0.05$ ).

#### Effects of TRCO Extract on Glutathione Peroxidase (GPx) activity on UVB-irradiated HaCaT Cells

We investigated GPx activity in UVB irradiated HaCaT cells pre-treated with TRCO extracts. UVB exposed HaCaT cells exhibited significant depletion (Figure 4) in GPx activity ( $246.70 \pm 21.45 \text{ U/mgprot}$ ) compared to control group ( $635.50 \pm 10.38 \text{ U/mgprot}$ ) ( $p < 0.05$ ). The 250  $\mu\text{g/mL}$  TRCO extract pre-treatment group

exhibited no loss of GPx activity, in fact it exhibited increased GPx activity than UVB only exposed group ( $p < 0.05$ ). GPx activity in 250  $\mu\text{g/mL}$  TRCO extract pre-treated group were significantly higher than negative control group ( $p < 0.05$ ). However, we notice that 500  $\mu\text{g/mL}$  TRCO extract pre-treated group exhibited significant depletion in GSH-Px activity compared to all other tested groups ( $p < 0.05$ )

### **Effects of TRCO Extract on Glutathione-S-Transferase (GST) activity on UVB-irradiated HaCaT Cells**

In our study GST activity in UVB only exposed HaCaT cells resulted in highest activity at  $0.38 \pm 0.29$  U/mgprot (Figure 5). The negative control group revealed significantly lower GST enzyme activity  $0.09 \pm 0.10$  U/mgprot) in comparison to UVB only exposed HaCaT cells ( $p < 0.05$ ). Both 250  $\mu\text{g/mL}$  and 500  $\mu\text{g/mL}$  TRCO extract pre-treatment groups expressed significantly reduced GST activity compared to UVB only exposed group ( $p < 0.05$ ).

## **DISCUSSION**

UVB is one of the main causes of oxidative stress in skin. The duration and length of UVB exposure determines the severity of the effects. UVB radiation induces alterations in skin's biochemical compositions and mechanical functionality due to oxidative stress. Accelerated skin aging, formation of wrinkles and impoverished skin firmness are all signs of photodamage (Mishra *et al.*, 2011). Although, the human skin is well equipped with endogenous antioxidant defence system comprising of a myriad of antioxidant molecules, often the increased levels of free radicals lead to depletion of said endogenous antioxidants (Babiarz *et al.*, 2002). Therefore, application of exogenous antioxidants on skin may help negate ill effects caused by excessive free radicals during UVB exposure.

*C. odontophyllum* Miq (Dabai) is one such plant which is known for its nutritional and antioxidative values. *C. odontophyllum* Miq or colloquially known as "Dabai" is an indigenous plant to Borneo, Sarawak, Sabah, Brunei, Kalimantan, and Philippines. Many studies had been carried out on various parts of the Dabai plant to assess its nutritional and antioxidative values. *C. odontophyllum* plant extracts are

recognized for its antioxidative activity against free radicals (Azlan *et al.*, 2010), as well as for targeted cytotoxicity effects against many types solid cancers without harming normal cells (Latif *et al.*, 2018). *C. odontophyllum* fruits are reported to exhibit high antioxidant capacity (Chew *et al.*, 2011). The skin in particular is shown to contain polyphenols, phenolic and flavonoid compounds which is thought to be responsible for the antioxidative capacity (Chew *et al.*, 2011). Various other studies have reported antioxidative activity in various parts of *C. odontophyllum* plants such as bark (Basri *et al.*, 2016), pulp (Basri, 2014), peel (Yang *et al.*, 2003; Chew *et al.*, 2011) fruits and leaves (Basri, 2014; Basri, 2015).

To date, there are no published data available on the antioxidative capacity of n-hexane extracted *C. odontophyllum*, thereof, these results serve as the first reported data on antioxidative capacity of n-hexane extracts of *C. odontophyllum*.

The antioxidative properties in *C. odontophyllum* leaves extract is of particular interest because leaves are constantly exposed to UVB radiation stress and thereby, it is hypothesized to be equipped with photoprotective molecules composed of antioxidants in order to mitigate the formation of reactive oxygen species observed (Chu *et al.*, 2008). Protective antioxidants such as terpenes, flavonoids, tannins and polyphenols are well characterized in UVB induced leaves (Czegeny *et al.*, 2016). *C. odontophyllum* leaves extracts were found to exhibit promising antioxidative values (Basri, 2014; Basri, 2015; Budin *et al.*, 2018). These extracts were found to contain terpenoids, tannins and flavonoids. Previous gas chromatography (GC-MS) analysis on the n-hexane extracts of *C. odontophyllum* leaves wherein the major type of terpenoids were identified as spathulenol and phytol (Basri *et al.*, 2022). The FRAP assay revealed antioxidative capacity in all the tested n-hexane *C. odontophyllum* leaves extracts. The antioxidative capacity in n-hexane *C. odontophyllum* leaves extracts could be contributed by the high presence of terpenoids. Studies on spathulenol and phytol terpenoids reveal antioxidative activities (do Nascimento *et al.*, 2018, Islam; Ali *et al.*, 2018)



Studies have confirmed that the presence of terpenoids exhibit high reducing activities and that, as the content of terpenoids increase the ferric reducing capacities also increases (Das *et al.*, 2011, Greeshma & Murugan, 2018). Therefore, as previously mentioned by Abdul Aziz *et al.*, (2022), terpenoid rich *C. odontophyllum* (TRCO) leaf extract can offer antioxidative therapeutic effects.

In our study, n-hexane extracts of *C. odontophyllum* were found to exhibit protective effects when topically applied on human keratinocyte cells prior to acute UVB irradiation exposure. UVB is a potent generator of reactive oxygen species (ROS) in skin. Under normal circumstances, ROS are neutralized through the network of endogenous enzymatic and non-enzymatic antioxidants. However, when exposed to external stimuli such like UVB radiation, ROS levels are highly elevated, this occurrence results in overwhelmed endogenous antioxidant system which leads to oxidative stress. UVB irradiation is reported to not only elevate ROS levels but also most worryingly, it is responsible for the depletion of endogenous antioxidants such as glutathione peroxidase (GSH-Px) and Glutathione-S-Transferases (GST).

Glutathione peroxidase and glutathione-S-transferase are part of endogenous antioxidant defence system Jablonska *et al.*, (2015) which help to preventing reactive oxygen species (ROS) induced oxidative stress in cells. Glutathione peroxidases are cytoprotective antioxidant selenoenzymes that plays two crucial roles; i) the primary function of GSH-Px is to catalyse the breakdown of hydrogen peroxides ( $H_2O_2$ ), lipidic or non-lipidic peroxides to non-toxic products (e.g. water and oxygen) through oxidation of reduced glutathione (GSH), this activity of GSH-Px protects cells against lipid peroxidation and ii) to maintain redox balance between reduced glutathione (GSH) and oxidized glutathione (GSSG) in cells (Arthur, 2001; Xianyong *et al.*, 2017)

Pre-treatment of n-hexane extract of *C. odontophyllum* at a low dose on acute UVB exposed human keratinocyte cells (HaCaT) resulted in high GSH-Px activity compared with untreated-UVB exposed groups. This could be due antioxidative activity of TRCO in maintaining redox balance by reducing

formation free radicals upon UVB exposure. The increase in GSH-Px activity in exogenous antioxidant treated cells is similarly observed in many studies including (Arthur, 2001; Xu *et al.*, 2018; Biernacki *et al.*, 2021; Kunchana *et al.*, 2021). The enhancement of GSH-Px activity also corroborates with the low levels of lipid peroxides observed. However, pre-treatment of a higher dose of n-hexane extract of *C. odontophyllum* revealed exhibited significant depletion in GSH-Px activity compared to all other tested groups ( $p < 0.05$ ). Instead of further enhancing or maintaining GSH-Px activity, 500  $\mu\text{g/mL}$  TRCO dose depleted GSH-Px activity to even lower than UVB only exposed group. The reason for this contradictory behaviour of 500  $\mu\text{g/mL}$  TRCO dose could indicate the depletion of reduced glutathione (GSH) in the UVB exposed cells. Xenobiotics are capable of inducing oxidative stress in cells which eventually leads to depletion of endogenous antioxidants in the process of maintaining cell redox homeostasis. Reduced glutathione (GSH) is part of endogenous antioxidant system which plays major role in the detoxification and neutralization of reactive oxygen species (ROS). GSH is also major substrate for the activity of GSH-Px (Eren & Selami, 2020). Therefore, depletion of GSH could lead to decreased GSH-Px activity. We presume that the depletion of GSH at 500  $\mu\text{g/mL}$  TRCO extract is what affects the non-significant lipid peroxides and protein carbonyl content when compared with 250  $\mu\text{g/mL}$  TRCO extract.

At higher concentration, it can be presumed that TRCO further aggravates the oxidative damage in cells which leads to severe depletion in GSH. This process eventually leads to the loss of activity in GSH-Px activity as observed in this study. Depletion of GSH can also mean the cells are activating cell death machinery which leads to apoptosis. Increased oxidative stress is linked to cellular damage and induction of cell death. Previous study by Abdul Aziz *et al.* (2022) on cell viability of HaCaT cells tested at 500  $\mu\text{g/mL}$  TRCO provide evidences of decreased cell survivability with increment of TRCO concentration. Therefore, the severe depletion of GSH-Px activity may indicate cell death due to the increased oxidative damage. Studies have shown that the concentration of terpenoids determines the behaviour of terpenoids. At higher concentration, terpenoids act as pro-oxidants but at much lower concentrations it is

found to exert antioxidative effects (González & Gómez, 2012) Antioxidants such as ascorbate can function both as antioxidant and pro-oxidant on certain circumstances (Podmore *et al.*, 1998). Beneficial antioxidants such as resveratrol, curcumin, coenzyme Q10,  $\alpha$ -lipoic acid (He *et al.*, 2017) is also known to act as pro-oxidants and produce overshooting of desired effects resulting fluctuations in redox balance.

GST are part of phase II detoxification enzymes (Townsend & Tew, 2003) which are largely responsible for detoxification of xenobiotics and electrophilic free radicals through conjugation with GSH. The mechanism of GST conjugation involves pairing xenobiotics with electrophilic centres to nucleophilic portions of GSH to detoxify and prevent toxic injuries to cells and tissues (Ishikawa, 1992).

TRCO extract pre-treated groups expressed significantly reduced GST activity compared to UVB only exposed group ( $p < 0.05$ ). This trend was similarly reported in (Monga *et al.*, 2014). Reduced activity of GST in the TRCO pre-treated group compared to the untreated group could point out lower formation of free radicals and toxic products associated with UVB exposure. The reduced free radicals present can be attributed to antioxidative capacity of TRCO in scavenging free radicals thus leading overall reduction in GST activity. In the event of oxidative stress, antioxidative defence system are activated to reduce, eliminate, and detoxify free radicals to prevent oxidative damage to cells. In this case, treatment of exogenous antioxidant TRCO resulted in the overall reduction in free radicals and its associated oxidative stress products thus, not necessitating induction of high GST activity. Therefore, when there is a cellular redox balance, the need for elevated detoxification activity is lower (Benincasa *et al.*, 2019). GSTs are not the only detoxification enzyme responsible for eliminating free radicals, but there are various other mechanisms involved in the elimination of free radicals as well which were not explored in this study. Many factors also affect the activity of GST enzyme for example the availability of GSH for the conjugation process which could be rate-limiting factor for GST activity. Therefore, further studies are needed to elucidate the involvement of major enzymatic and non-enzymatic antioxidants in the elimination of

UVB induced free radicals in the presence of TRCO.

Lipid peroxidation on epidermal layers is a direct consequence of UVB irradiation exposure. Lipid peroxidation occurs due to the oxidative deterioration lipids by free radicals. It is initiated when ROS attacks and abstracts hydrogen from the methylene groups of lipids, resulting in lipid radicals. Elevated amount of ROS poses two consequences: i) impairing cell components and ii) activating specific signalling pathways (Finkel & Holbrook, 2000). The overwhelming amount of free radicals inflicts direct damage to lipids and leads to the production of lipid peroxidation products such as lipid peroxides, malondialdehyde (MDA), and 4-hydroxyalkenals (HAE/HNE). Most of these lipid peroxidation products are known to be highly toxic and mutagenic (Esterbauer *et al.*, 1990) which causes injury in cells, tissues and organs (Ayala *et al.*, 2014). Lipid peroxidation products are also strongly associated with photodamage and pre-mature skin aging (Alvarez & Stratton, 2008). Lipid peroxides are prominent indicators of lipid peroxidation which formed due to oxidative degradation of polyunsaturated fatty acids (PUFA) by free radicals.

In our study, we found that the highest concentration of lipid peroxides ( $p < 0.05$ ) was exhibited in UVB only exposed HaCaT cells, whereas the control group (untreated and non-UVB exposed) exhibited lower lipid peroxide concentration. The reason for the increased LPO formation upon UVB exposure can be majorly attributed to the lipid rich matrix of epidermal keratinocytes (Alvarez & Stratton, 2008). As we know, lipid peroxides are product of free radicals induced oxidation of polyunsaturated fatty acids. Skin epidermis is composed of polyunsaturated fatty acids such as linoleic acids which are found abundantly in ceramides, while arachidonic acid (AA) being the second most abundant (PUFA) (Knox & O'Boyle, 2021) and omega 3 fatty acids (Ziboh *et al.*, 2000). These lipids could be primary targets of free radical species such as oxyl radicals, hydroxyl radicals and peroxy radicals thus leading to increased formation of LPO. Our result is in accordance with Townsend and Tew (2003) that reported higher secretion of 8-isoprostane, an end product of lipid peroxidation in UVB- irradiated HaCaT cells compared control group and as well as

(Ishikawa, 1992) which reported similar inclination of increased lipid peroxidation product levels in UVB exposed group. Similar trend was also observed in few studies which evaluated effects of UVB radiation on lipid peroxidation products in keratinocyte cells (Chapkin *et al.*, 1990; Afaq *et al.*, 2007; Piao *et al.*, 2013; Chen *et al.*, 2015; Fehér *et al.*, 2016; Fernando *et al.*, 2016; Luangpraditkun *et al.*, 2020; Lee *et al.*, 2020).

We demonstrated that TRCO extracts of 250 µg/mL and 500 µg/mL were able significantly reduce the generation of lipid peroxidation products in HaCaT cells exposed to acute UVB exposure. The reduction in lipid peroxides formation is presumed to be due to terpenoid antioxidant content in TRCO extracts. Antioxidants are capable of scavenging free radicals which are formed due to acute and chronic UVB exposure. When HaCaT cells are pretreated with exogenous antioxidants such as TRCO extract, the generation of lipid peroxides are suppressed. Supplementation of exogenous antioxidant helps to scavenge excessive free radicals that are generated due to UVB exposure, thus, resulting in minimized lipid peroxide formation.

Similar to lipids and DNA, ROS induced oxidative stress also induces structural and functional modifications to cellular proteins due to protein oxidation. These structural modifications of protein molecules inevitably lead to loss of biological functions. Another prominent modification of proteins caused by ROS is the conversion of proteins to carbonyl derivatives (Sitte, 2003). Protein oxidation is a natural consequence of aerobic life, however environmental stimuli such as UV, chemotherapeutic drugs and hyperthermia can generate elevated amounts of ROS (Sitte, 2003). High amount of protein carbonyls were reported on keratinocytes cells that are exposed to UV radiation (Ogura *et al.*, 2011). This is because UV-induced ROS reacts with amino acids of proteins in dermis and epidermis of skin to generate protein carbonyls (Yamawaki *et al.*, 2019). Although the consequence of protein carbonyl accumulation in skin physiology remains unclear, studies have reported changes in skin colour (yellow-dark), alteration in collagen and extracellular matrix associated with photoaging (Uehleke, 2010), decreased moisture-holding capacity that leads to skin

dryness and trans-epidermal water loss in stratum corneum as a result of elevated protein carbonyl levels (Baraibar, 2018; Yamawaki *et al.*, 2019) and accelerated skin aging by amplifying decomposition of elastic fibres and collagens (Yamawaki *et al.*, 2019). Even though, the body's innate antioxidant defence system is able to quench and eliminate ROS, excessive amount of ROS can lead to irreparable protein misfolding, to a large extent, which leads to selective proteolysis (Sitte, 2003). Therefore, strengthening the endogenous antioxidants with additional exogenous antioxidants in neutralizing the harmful effects of ROS could be a good strategy in reducing protein carbonyl induced skin damages.

This study assessed the effects of TRCO extracts in protein carbonyls formation in UVB exposed HaCaT cells. We found that pretreatment of 250 µg/mL and 500 µg/mL TRCO extracts exhibited relatively lower protein carbonyl content compared to UVB exposed group. However, when compared to UVB only exposed group, no significant difference in protein carbonyl content can be established ( $p < 0.05$ ). In fact, when comparing protein carbonyl levels between UVB only exposed group to control group, no statistical significance could not be established ( $p < 0.05$ ). We notice that the negative control group exhibited relatively high protein carbonyl content independent of any UVB exposure. This could be attributed to other oxidation processes including formation of carbonylated proteins through aldehyde and amino residues reaction due to lipid peroxidation (Chevion *et al.*, 2000; Togni *et al.*, 2019). The negligible induction of protein carbonyl content between the control group and UVB exposed group could be ascribable to few reasons; firstly, single exposure to UVB at low dose of 30 mJ/cm<sup>2</sup> may not be sufficient to induce notable increase in protein carbonyls. Study by Luangpraditkun *et al.* (2020) revealed enhanced protein carbonyl levels when HaCaT cells were exposed to single exposure of UVB at 60 mJ/cm<sup>2</sup>. Secondly, possibly frequency of exposure to UVB may influence protein carbonyl content in keratinocytes. HaCaT cells which were exposed to repeated dose UVB irradiation were shown to express higher protein carbonyls content by Lee *et al.* (2020). Thirdly, a study by Ramachandran and Prasad (2008) provided that the extent of protein oxidation in living epidermal layer of skin is far less than

dermal layer due to high antioxidant capacity in the epidermis layer, thus, we presume that keratinocyte cells which is the major constituent of epidermal layer may also similar activities. Finally, the generation of protein carbonyls upon UVB exposure may require longer period of time to exhibit any substantial changes. Time-dependent fluctuations in reactive species upon UVB exposure is possible as different radical species act at different times (Sitte, 2003; Ogura *et al.*, 2011). Study by Lee *et al.* (2013) reported higher protein carbonyl concentration when measured 2 hr after exposing HaCat cells to 30 mJ/cm<sup>2</sup>, whereas in our study, cell lysates for protein carbonyl measurement were measured within 30 mins upon UVB exposure as we wanted to examine the immediate effects of UVB exposure on keratinocyte cells. Even though, 500 µg/mL TRCO extract significantly reduced protein carbonyl content in comparison to UVB only exposed HaCat group (p<0.05), the veritableness of this effect could not be established as both positive and negative controls indicate non-significant difference in protein carbonyl levels. Therefore, further studies are needed to establish the effects of TRCO in protein carbonyl content in UVB induced HaCat cell.

## CONCLUSION

To summarise, UVB induced oxidative stress is a key factor in the onset and progression of photodamage and photoaging of the skin. Supplementation of exogenous antioxidants such as TRCO extracts as demonstrated in this study provide antioxidative and photoprotective effects against UVB. Specifically, TRCO gets involved in the prevention of UVB-mediated oxidative damage in HaCaT cells by lowering lipid peroxides levels, lowering glutathione-s-transferase activity (GST) and increasing glutathione peroxidase activity (GSH-Px). Taken together, TRCO appears to be a promising candidate as exogeneous antioxidant for protection against photoaging and photodamage.

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## Conflicts of Interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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