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POTENTIAL SKIN CANCER CHEMOPREVENTIVE EFFECTS OF TERPENOID-RICH *CANARIUM ODONTOPHYLLUM* (TRCO) LEAF EXTRACT IN UVB-IRRADIATED HUMAN KERATINOCYTES (HaCaT)

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Skin cancer is a prevalent form of cancer, primarily driven by the harmful effects of ultraviolet-B (UVB) rays from the sun. Cancer chemoprevention seeks to prevent cancer development in the ongoing battle against the disease. *Canarium odontophyllum* Miq., recognised as "dabai" is a native plant found in Sarawak, Malaysia. Our study investigated the chemopreventive activity of the terpenoid-rich extract of *C. odontophyllum* Miq. leaves (TRCO) in skin cancer model in vitro. The terpenoid profiling was done using gas chromatography-mass spectrometry (GC-MS). A skin cancer model using human keratinocytes (HaCaT) was used (induced with 30 mJ/cm^2 UVB for 6 passages and pretreated with 500 and 1000 µg/mL TRCO). Results demonstrated that the extract was rich in terpenoids, particularly phytol and spathulenol. Pre-treatment with TRCO significantly increased ($p < 0.05$) the level of tumour protein p53 (TP53), reduced proliferative protein KI-67 (KI67) and vascular endothelial growth factor (VEGF) compared to the UVB-only group. The abundance of terpenoids in TRCO functioned as potent exogenous antioxidants, countering free radicals, reducing inflammation and minimising cell and tissue damage. TRCO exhibited promising chemopreventive activity and held great potential as a chemopreventive agent against UVB-induced skin cancer by promoting cell repair and suppressing proliferation and angiogenesis levels. In conclusion, the findings may facilitate the exploitation of the endemic *C. odontophyllum* Miq. of Sarawak as a possible cancer chemoprevention agent.

Keywords: Dabai, *Canarium odontophyllum*, skin cancer, chemopreventive, UVB

INTRODUCTION

The silent disease known as cancer is typified by unplanned and unregulated cell division. According to Demir et al. (2016), the diverse illness results in normal cells deviating from the regular cell cycle and forming tumours. The incidence of skin cancer is rising globally, according to newly diagnosed case reports each year (Kong & Xu 2020, Ziaj et al. 2020 & Sinikumpu et al. 2022). The carcinogenesis of skin cancer is largely influenced by environmental variables, including prolonged exposure to UVB radiation (Al-Sadek & Yusuf 2024). UVB is recognised as a carcinogenic agent and poses a bodily risk. When it comes to different types of cancer diagnosis, prognosis, and treatment, cancer biomarkers are crucial. The primary tumour suppressor gene that typically mutates in human cancer is the p53 protein tumour (TP53), which serves as the primary regulator of genotoxic responses (Kastenhuber & Lowe 2017).

Furthermore, the status of the TP53 mutation has been associated with the microenvironment associated with cancer (Xu et al. 2020). Antigen Kiel 67 (KI67) is a marker of cell proliferation and is commonly used as a prognostic marker in breast cancer (Feeley et al. 2014). It has also been studied in other types of cancers, such as skin cancer (Dos Anjos et al. 2019) and cervical cancer (Zhao et al. 2023).

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In cervical cancer, the expression of KI67 has been found to decrease with the effectiveness of neoadjuvant therapy. In addition to TP53 and KI67, the vascular endothelial growth factor (VEGF) is the main controller of angiogenesis and is associated with tumour growth and metastases. Some types of skin cancer, such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are known to produce VEGF in excessive amounts. Increased production of VEGF by cancer cells can encourage the development of new blood vessels around the tumour, providing the blood supply and nutrients needed for the growth of the tumour.

Figure 1 *Canarium odontophyllum* Miq. leaf

Canarium odontophyllum Miq. or locally known as dabai by Sarawak locals is categorised under the Burseracea family and can be found mainly in Sarawak. According to Basri et al. (2015), the most prevalent active ingredient in *C. odontophyllum* leaves (Figure 1) is terpenoid, as evidenced by the positive results that appeared as a reddish-brown color after the leaf extract was dissolved in chloroform ($CHCl_3$) and sulfuric acid (H_2SO_4). Terpenoids comprise five carbon compounds (isoprene units) with numerous functional groups and a basic skeleton (Mansuri et al. 2021). Terpenoids are a derivative of terpenes (which are modified by removing a methyl group by adding oxygen to hydrocarbon) and are mostly active against many diseases biologically such as cancer, malaria, viral and bacterial diseases, and inflammation (Mansuri et al. 2021). Therefore, our study focused on evaluating the chemopreventive activity of terpenoid-rich extract from *C. odontophyllum* Miq. leaves on UVB-irradiated human keratinocytes (HaCaT).

MATERIALS AND METHODS

Plant sample and TRCO extraction

The previously described methods were followed in order to prepare TRCO (Abdul Aziz et al. 2022). A stock solution of TRCO was prepared by dissolving 100 mg of the extract in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). The liquid was then vortexed and filtered into a 1.5 mL microcentrifuge tube using a 0.22 µm syringe filter (Bioflow, Malaysia). The 100 mg/mL stock solution was used to prepare the 500 µg/ml (TRCO500) and 1000 µg/ml (TRCO1000) solutions. Export and research permits, with permit numbers SBC-2020-EP-58-MWH and SBC-2019-RDP-20-MWH, were granted by the Sarawak Biodiversity Centre (SBC). The leaves were also deposited to the Herbarium at UKM, where voucher ID028/2020 was used to confirm the species.

GC-MS analysis

GC-MS analysis was performed using an Agilent 7890A gas chromatograph (USA) coupled with an Agilent 5975C inert MSD mass spectrometer system equipped with a triple-axis detector, following the procedure outlined by Marina et al (2013). A 30 m × 0.25 mm I.D. × 0.25 mm Phenomenex ZB5 capillary column coated with 5% phenyl methylpolysiloxane was used in the GC. A splitless injector was used to automate the injection of the sample, and 1 µL was injected in split mode. The carrier gas was helium, flowing at a rate of 1.3 mL per minute. The temperature schedule for the oven was set up as follows: a one-minute starting hold at 50 °C, a seven-minute climb at 180 °C, a ten-minute ramp at 300 °C, and a final hold at 300 °C. The temperature of the quadrupole, source, and transfer line were set at 150 °C, 280 °C, and 300 °C, respectively, while the injector was maintained at 300 °C. The mass spectrometer was operated in full scan/selected ion monitoring (SIM) mode with electron impact ionization at 70 eV. Full scan spectra between 40 and 220 m/z were gathered. To increase sensitivity for quantitative analysis, SIM mode was used. The MSD Chemstation program was used to identify every peak in the raw GC chromatogram. A library search was conducted using the NIST/EPA/NIH version

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2.0 database, and the results were compiled into a table.

UVB-irradiation

A UVX digital radiometer (Analytikjena USA) was used to measure the strength of the UVB radiation, which was produced using UVB lamps (Analytikjena USA). Before measuring the UVB dose rate, which was set at 30 mJ/cm^2, the lamps were warmed up for at least 5 minutes (Tyagi et al. 2015).

UVB-irradiated HaCaT (in vitro skin cancer model)

HaCaT's in vitro skin cancer model was modified and optimised, based on prior investigation (Tyagi et al. 2015). After the cells achieved 80% confluency at passage 5 (P5), the medium was taken out of the 6-well plates. After giving the cells two PBS washes, 1 mL of brand-new media was given to each well. After that, the plates were incubated in an environment containing 5% CO_2 for 30 minutes at 37 °C. Following incubation, the UVB-induced group's HaCaT cells were subjected to 30 mJ/cm^2 of UVB radiation, whereas the non-UVB-induced group did not receive any UVB exposure. Following UVB treatment, the cells were cultured in fresh media at 37 °C with 5% CO_2 until confluent. This process was repeated through passages 6 to 10 (P6 to P10).

Preparation of lysates

All TRCO groups were subjected to the oncogenic transformation. In 1 mL of medium, TRCO500 and TRCO1000 were added before P5 to P10 were exposed to UVB rays. Each well containing HaCaT cells from all treatment groups (control, UVB-only, UVB + TRCO500, and UVB + TRCO1000) received 150 µL of ice-cold RIPA lysis solution at P11. The wells were then incubated on ice for 5 minutes. The cells were then extracted and lysed by gently scraping each well with a cell scraper. The resulting cell suspension was then transferred into cold microcentrifuge tubes. The cells were centrifuged for 10 minutes at 12000 rpm in 4 °C after being incubated on ice for 30 minutes with constant agitation. Carefully transferred to a fresh microcentrifuge tube, the supernatant—which did not include the pellet—was kept at -80°C until needed for protein quantification (using BSA; Elabscience, USA; Catalog No. E-BC-K318-M) and other studies.

Chemopreventive activity

Tumour protein p53 (TP53) assay

The human total TP53 ELISA kit (Catalog No. EH3898) from FineTest, China, was used to measure the TP53 levels in the experimental groups. Standards and samples (n=9) were all set in accordance with the kit's manufacturer's instructions. Subsequently, diluted samples (100 µL) were then dispensed into 96-well plates for the assay procedure, and an OD 450 nm microplate reader (Multiskan Go, Thermo Scientific, USA) was used to analyse the results. The standard curve that was previously constructed was used to determine the TP53 levels in the samples.

Marker of proliferation Ki-67 (KI67) assay

Utilising the human KI67 ELISA kit (Catalog No. EH0684) from FineTest, China, the amount of KI67 in the treatment groups was determined. Standard and samples (n=9) were all set in accordance with the kit's instructions. For the experiment, diluted samples (100 µL) were added to 96-well plates, and a microplate reader (Multiskan Go, Thermo Scientific, USA) was used to measure the absorbance at 450 nm. The produced standard curve was used to determine the KI67 levels in the tested samples.

Vascular endothelial growth factor (VEGF) assay

Jess Western analysis (ProteinSimple, USA) was conducted to assess the VEGF protein marker. Prepared lysate sample was utilised in this procedure. The lysate was diluted with a 0.1× sample buffer to get a concentration of 1.2 mg/mL. Subsequently, four parts of the diluted sample were mixed with one part of the 5× fluorescent master mixture containing sample buffer, fluorescent standard, and 40 mM of dithiothreitol, followed by heating to 95 °C for 5 minutes. Each sample was then loaded with 3 µL into the plate, utilising fluorescent separation

cartridges 12-230 kDa Jess (ProteinSimple, #SM-FL004). Primary antibodies were diluted in antibody buffers (ProteinSimple, #042-203), and secondary antibodies supplied by the company (ProteinSimple, #042-206/205) were used as provided. For each sample, 10 µL of primary and secondary antibodies were loaded. The plate underwent rotation for 5 minutes at 1000 × G to eliminate bubbles before being loaded into the Jess machine. The Jess system automatically performed protein separation, blocking, antibody incubation, and signal detection. Data analysis was conducted using Compass for SW software provided by the manufacturer. Normalisation reagents facilitated the detection of protein levels in the capillaries by binding to biomolecules' amine groups. With Jess technology, no loading control was necessary.

RESULTS

Terpenoids profiling from GC-MS

Terpenoids were detected in the extract by gas chromatography-mass spectrometry (GC-MS) analysis. Spathulenol and phytol were identified as the main terpenoids in the leaf extract (Figure 2). Detailed terpenoid profile is provided in Table 1, including retention time (RT), peak area (%), and quality assessment (Qual). Terpenoid identifications with a quality score above 80 are deemed reliable, while those between 70 to 79 are often accurate, and those between 60 to 69 are considered speculative, following guidelines established by Stein (1999).

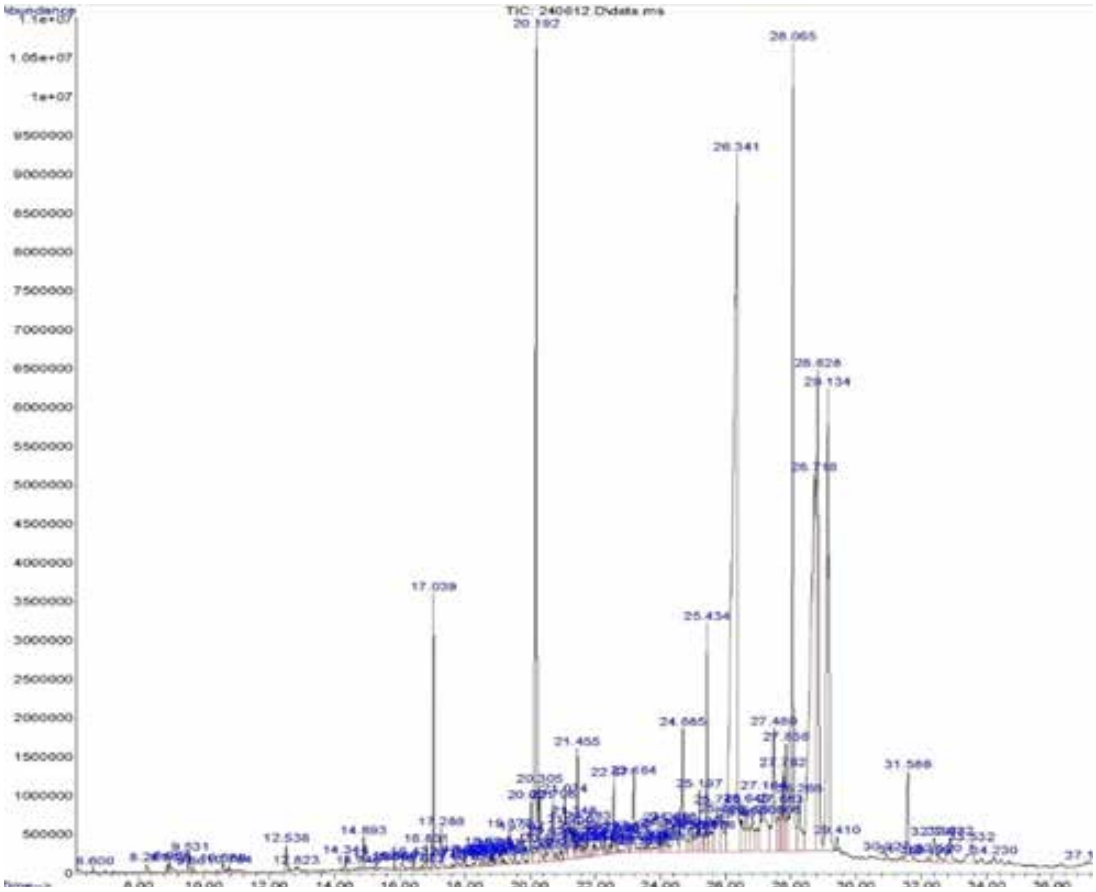

Table 1 Terpenoids profile in the *C. odontophyllum* Miq. leaf hexane extract

Figure 2 GC-MS chromatogram of *C. odontophyllum* Miq. leaf hexane extract

Retention Time	Area %	Library/ID	Quality
6.5977	0.0468	α-Pinene	97
8.8751	0.0614	p-Cymene	91
8.9698	0.1105	D-Limonene	96
12.5405	0.2248	Terpinen-4-ol	90
16.4329	0.0826	Copaene	98
17.2845	0.2879	Caryophyllene	99
17.6315	0.042	Alloaromadendrene	93
17.9533	0.1013	Humulene	94
17.7325	0.1043	β-Vatirenene	91
18.5652	0.0582	β-Humulene	93
18.6914	0.1885	Bicyclo[4.4.0]dec-1-ene,2-isopropyl-5-methyl-9-methylene-	94
18.7797	0.0712	β-Vatirenene	95
18.8365	0.0677	β-Bisabolene	94
19.1141	0.1277	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	38
19.379	0.3413	Dihydroactinidiolide	95
19.7575	0.3698	ɣ-Himachalene	47
20.0225	0.5846	Aromadendrene, dehydro-	51
20.1928	7.1602	Spathulenol	91
20.3064	0.6237	β-Selinene	64
20.4578	0.1376	α-Guaiene	93
20.7101	0.4709	Patchoulane	91
21.076	0.4624	Spathulenol	53
21.2527	0.6672	ɣ-Muurolene	64
21.4546	0.8797	α-Cadinol	83
21.7826	0.2393	trans-Z-α-Bisabolene epoxide	55
22.5712	0.5623	Patchoulane	60
24.2051	0.2585	2-Pentadecanone, 6,10,14-trimethyl-	49
28.066	8.743	Phytol	96
32.8795	0.6153	4,8,12,16-Tetramethylheptadecan-4-olide	93

Effects of TRCO on TP53 level

The results indicate that the HaCaT group exposed to UVB without TRCO significantly ($p<0.05$) reduced the levels of TP53 protein (21.60 ± 0.60 ng/mg prot) compared to the control group (31.21 ± 0.51 ng/mg prot). Pretreatment with TRCO500 (28.62 ± 0.15 / mg prot) and TRCO1000 (24.69 ± 0.69 ng/ mg prot) significantly ($p<0.05$) increased TP53 protein levels compared to the UVB-exposed group without TRCO (Figure 3).

Effects of TRCO on KI67 level

The pre-treatment groups with TRCO500 and TRCO1000 showed a significant reduction ($p<0.05$) in the levels of KI67 protein within the cells (91.54 ± 1.32 ng/mg prot and 83.75 ± 2.55 ng/mg prot, respectively) compared to the group affected by UVB without TRCO (105.31 ± 0.92 ng/mg prot) (Figure 4).

Effects of TRCO on VEGF protein expression

Vascular endothelial growth factor, or VEGF, is linked to tumour growth and metastasis and functions as a major regulator of angiogenesis. To examine VEGF protein expression, protein matching is performed to ensure consistent loading of samples in each well. Fluorescence matching measures protein trapped in capillaries, indicated by increased intensity of the band as shown in Figure 5 qualitatively. The UVB irradiation group without treatment shows high VEGF expression compared to the control group. The TRCO500 group displayed reduction in VEGF expression compared to the UVB irradiation group without treatment. Qualitatively, it is also possible that TRCO1000 also reduces VEGF expression.

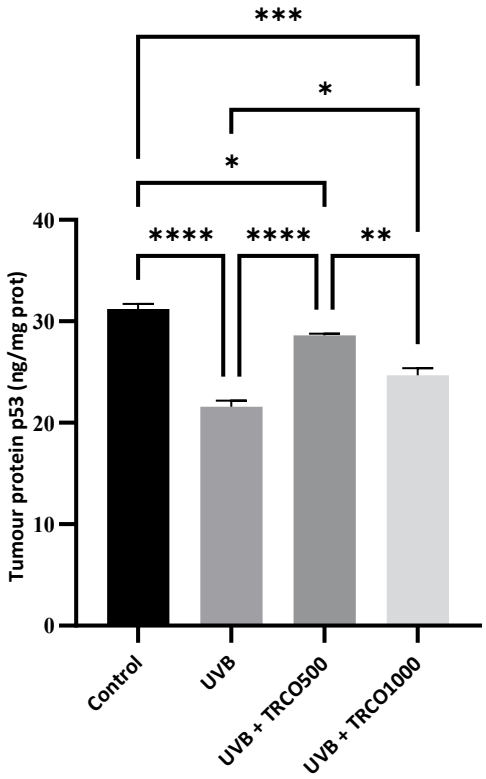

Figure 3 The TP53 level in HaCaT groups Values indicate mean ± SEM, n = 9, *, **, ***, **** p<0.05; one-way ANOVA was used for group comparisons, and a post-hoc test was performed subsequently

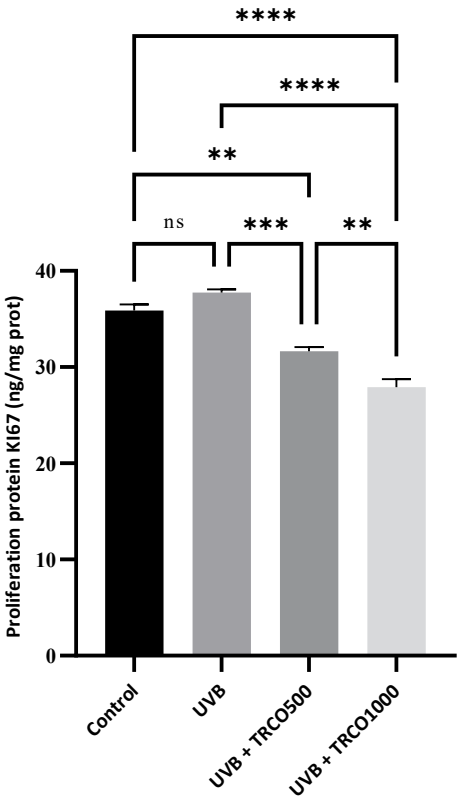

Figure 4 The KI67 level in HaCaT groups. Values indicate mean ± SEM, n = 9, *, **, ***, **** p<0.05; one-way ANOVA was used for group comparisons, and a post-hoc test was performed subsequently

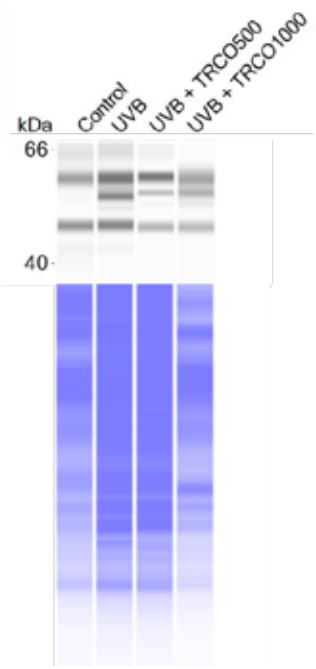

Figure 5 The VEGF protein expression in HaCaT groups

DISCUSSION

Terpenoids are prevalent in the hexane extract of *C. odontophyllum* Miq. leaves (TRCO), according to GC-MS analysis. TRCO was produced by extracting the leaves of *C. odontophyllum* Miq. using n-hexane, a non-polar solvent. This process successfully separated terpenoid compounds, which are primarily insoluble in water and highly non-polar. Two of the main terpenoids found in the hexane extract are phytol and spathulenol. According to earlier studies, spathulenol possesses anti-inflammatory, anti-proliferative, and antioxidant properties (do Nascimento et al. 2018). Diterpene alcohol phytol, which comes from chlorophyll, has several advantageous properties, including antibacterial, autophagic, apoptosis inducing, anti-nociceptive, anti-inflammatory, and immunomodulatory properties (de Alencar et al. 2019).

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Based on the findings, chronic UVB exposure significantly ($p<0.05$) reduced levels of the TP53 protein and increased levels of the KI67 protein and VEGF expression. The significant decrease ($p<0.05$) in TP53 protein levels due to UVB exposure in both models could indicate genetic damage to cells or cellular oxidative stress (Vostálová et al. 2009). As for the KI67 protein, the higher its levels, the more cells are in an active phase of cell proliferation. The levels of both TP53 and KI67 proteins can reflect uncontrolled cell proliferation activity, which can be associated with cancer carcinogenesis. Additionally, high expression of VEGF can enhance the body's ability to form new blood vessels, which often occur in response to tumour growth that requires blood to support its expansion (Xu et al. 2020). A combination of significantly decreased ($p<0.05$) levels of TP53 protein, along with significantly increased ($p<0.05$) levels of KI67 and VEGF proteins, may indicate a more aggressive biological state and potentially lead to increased invasiveness.

Pre-treatment with TRCO which is rich in terpenoids, especially spathulenol and phytol, has shown its effectiveness as a potential skin cancer chemopreventive agent. Bahadori et al. (2017) have reported that spathulenol, a tricyclic sesquiterpene terpenoid (Figure 6), exhibits a range of pharmacological actions, such as immunoinhibitory, anti-inflammatory, anticancer, and apoptosis-inducing properties. Additionally, spathulenol has been suggested as a possible option for treating cancer patients who are resistant to drugs (Hosseini et al. 2021). Previous studies have shown that spathulenol exhibits cytotoxic properties against melanoma, leukaemia, liver cancer, chronic myeloid leukaemia, and human promyelocytic leukaemia (Cianfaglione et al. 2017, Galvão et al. 2022). Moreover, it has been documented that spathulenol exhibits antioxidative, anti-inflammatory, and antiproliferative characteristics (El-Beltagi et al. 2020).

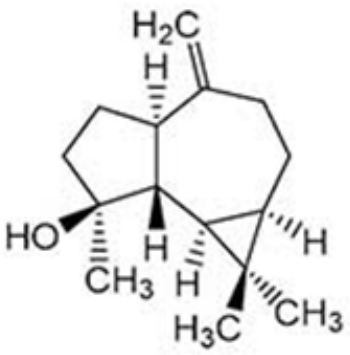

Figure 6 Chemical structure of spathulenol ($C_{15}H_{24}O$)

Phytol is a type of diterpene terpenoid alcohol (Figure 7) that is commonly found in TRCO, and found to have various biological activities, including anti-cancer, anti-nociceptive, antioxidant, and cancer chemopreventive properties (Santos et al. 2013, de Alencar et al. 2019 & Al-Saraireh et al. 2021). Phytol has been shown in cancer preventive and therapy studies to promote autophagy and suppress cell proliferation in AGS human gastric adenocarcinoma cells and MCF-7 breast cancer cells (Di Sotto et al. 2020). There are also studies reporting that phytol has cytotoxic properties against MCF-7 cells and shows antioxidant and anti-nociceptive effects (Al-Saraireh et al. 2021). These compounds demonstrate antioxidant properties, anti-proliferative abilities to inhibit cell growth, and anti-inflammatory properties that can alleviate inflammation processes related to carcinogenesis. Thus, pre-treatment using TRCO provides new insights into preventing the formation and carcinogenesis of UVB-induced skin cancer.

In the in vitro model conducted, TRCO administered as pre-treatment in the skin cancer model can act as a protector against UVB radiation and as an exogenous antioxidant that may neutralize free radicals. As a result of this protection, TRCO provided a cancer chemopreventive effect by significantly ($p<0.05$) increasing the levels of TP53 protein, acting as a tumour suppressor, and decreasing cell proliferation as indicated by a reduction in KI67 protein levels. Additionally, TRCO could decrease VEGF, which functions in angiogenesis and metastasis of cancer cells compared to the UVB-irradiated group without TRCO pre-treatment. Research by Xia et al. (2021) has demonstrated a correlation between the expression of VEGF, KI67 and TP53 and the recurrence of hepatocellular carcinoma, highlighting the significance of these biomarkers in predicting the course of the disease. The intricate interactions between VEGF, KI67 and

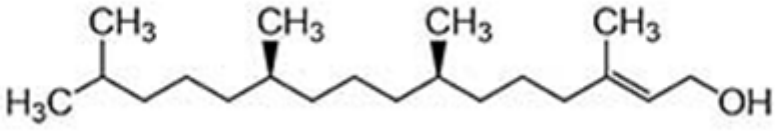

Figure 7 Chemical structure of phytol ($C_{20}H_{40}O$)

TP53 demonstrate the importance of these proteins in the development of skin cancer and in chemoprevention. The use of natural substances or dietary approaches to target these biomarkers holds potential for enhancing the prevention and therapeutic results of skin cancer. Hence, as a result of these activities, TRCO demonstrates a cancer chemopreventive effect in in vitro skin cancer models.

CONCLUSION

The findings of our study indicate that TRCO may be beneficial in the chemoprevention of UVB-induced skin cancer. TRCO exhibited chemopreventive actions by upregulating TP53, inhibiting VEGF production, and functioning as an anti-proliferative drug via the KI67 pathway. In summary, TRCO exhibits a promise for additional research through terpenoid isolation, offering a potential therapeutic intervention for the prevention of skin cancer. Our results may also lead to the development of *C. odontophyllum* Miq. which is native to Sarawak as a potential chemoprevention agent in the fight against and prevention of cancer and can even increase the commercial value of *C. odontophyllum* Miq. from Sarawak.

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