

The Protective Potential of Terpenoid-Rich *Canarium odontophyllum* Miq. Leaves against 7,12-Dimethylbenz[a]anthracene and Ultraviolet B-induced Two-stage Skin Carcinogenesis in BALB/c Mice

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Non-melanoma skin cancer remains among the most prevalent malignancies globally. This study investigated the protective potential of a terpenoid-rich extract from *Canarium odontophyllum* Miq. leaves (TRCO) in a 7,12-dimethylbenz[a]anthracene (DMBA) and ultraviolet B (UVB)-induced two-stage skin carcinogenesis (DMBA/UVB) model using BALB/c mice, focusing primarily on its action during the tumor promotion stage. Mice were topically treated with TRCO at 100 µg (TRCO100) and 200 µg (TRCO200) prior to DMBA/UVB exposure. The protective activities were evaluated based on macroscopic skin changes, histopathology, antioxidant enzyme activities, melanogenesis parameters, and expression levels of key tumor-associated markers (p53, Ki67, and VEGF). TRCO treatment significantly reduced DMBA/UVB-induced erythema, epidermal hyperplasia, and melanogenesis. TRCO modulated antioxidant enzyme activities (total SOD, CAT, GSH-Px and GST) and significantly lowered oxidative stress biomarkers. Expression of p53, Ki67, and VEGF was markedly modulated, with the TRCO100 group showing pronounced variations. These findings suggest that TRCO exerts promising protective potential against preneoplastic skin progression in a two-stage carcinogenesis model, most likely through integrated antioxidant, anti-inflammatory, and antiproliferative tissue mechanisms.

Key Words Chemoprevention, 7,12-dimethylbenz[a]anthracene, Ultraviolet rays, Skin neoplasms, Terpenoids

INTRODUCTION

Skin cancer, encompassing both melanoma and non-melanoma types such as basal cell carcinoma and squamous cell carcinoma, is a major public health concern globally, with incidence rates continuing to rise. Although melanoma is typically more aggressive, non-melanoma skin cancers are more common and are strongly linked to long-term exposure to ultraviolet B (UVB) radiation. Chronically, UVB has been well-documented as both a potent tumor initiator and a promoter of skin cancer development by generating oxidative stress, promoting inflammation and inducing reactive oxygen species (ROS), which can provoke DNA damage and inflam-

mation [1,2]. These effects collectively disrupt cellular homeostasis and drive the multistage process of skin carcinogenesis.

The multistage process of skin carcinogenesis involves a coordinated transition through initiation, promotion, and progression. Initiation is characterized by genetic alterations in cell cycle regulatory genes, with p53 mutations being particularly significant due to the gene's role in preserving genomic integrity and controlling programmed cell death. The promotion phase involves the proliferation of the mutated cells. Progression is marked by the evolution of these altered cells into a malignant phenotype [3,4]. p53 is frequently mutated in skin cancers and is a key regulator of the cell cycle and apoptosis.

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Ki67 indicates cellular proliferation, while VEGF drives angiogenesis necessary for tumor growth.

In UVB-driven carcinogenesis, excessive generation of ROS overwhelms endogenous antioxidant defenses and contributes to oxidative DNA damage, lipid peroxidation, and protein carbonylation. The persistent oxidative stress simultaneously activates inflammatory pathways, amplifying cytokine release and further enhancing cellular proliferation and angiogenesis. Additionally, activation of tyrosinase increases melanin synthesis as a photoprotective response; however, chronic stimulation of melanogenesis can exacerbate oxidative burden and contribute to dysplastic changes. These interconnected pathways of oxidative stress, inflammation, and dysregulated melanogenesis collectively accelerate the transition from initiation to malignant progression in UVB-induced skin carcinogenesis.

To experimentally recapitulate this complex process in a controlled and time-efficient manner, a two-stage skin carcinogenesis model is widely employed. This model utilizes 7,12-dimethylbenz[a]anthracene (DMBA) as a chemical initiator to induce defined genetic mutations, followed by repeated UVB exposure as a tumor promoter. Unlike the UVB alone model, which relies on cumulative radiation to drive both initiation and promotion, the DMBA/UVB paradigm accelerates carcinogenesis by separating these stages mechanistically, which allows for clearer interrogation of tumor promotion pathways and therapeutic interventions.

Canarium odontophyllum Miq., an indigenous plant from Sarawak, Malaysia, locally known as "dabai", possesses leaves (Fig. 1) rich in terpenoid compounds including spathulenol and phytol. While our previous work demonstrated that a terpenoid-rich extract of *C. odontophyllum* (TRCO) exhibits protective properties in monocultured human HaCaT keratinocytes in vitro [5], cell models cannot reproduce the intricate



Figure 1. *Canarium odontophyllum* Miq. Leaves.

microenvironment of live skin. Consequently, this study evaluates the in vivo protective potential of topically applied TRCO in a BALB/c mouse model subjected to DMBA/UVB-induced two-stage skin carcinogenesis [6]. By evaluating histopathological features, antioxidant responses, melanogenic activity, and oncogenic protein expression within intact skin tissue, this work aims to clarify the translational value of TRCO as a topical skin cancer chemopreventive agent.

MATERIALS AND METHODS

Preparation and characterization of TRCO

The leaves of *C. odontophyllum* were collected in Kuching, Sarawak, Malaysia, under appropriate institutional permits (Sarawak Biodiversity Centre export permit SBC-2020-EP-58-MWH; research permit SBC-2019-RDP-20-MWH) and authenticated at the Universiti Kebangsaan Malaysia (UKM) Herbarium (voucher number: ID028/2020). The terpenoid-rich extract was prepared as described by Abdul Aziz et al. [5]. Gas chromatography-mass spectrometry (GC-MS) characterization from this established preparation confirmed that the extract is composed of major bioactive terpenoids, notably spathulenol and phytol, alongside primary lipophilic constituents such as palmitic acid. A stock solution (100 mg/mL) was prepared in dimethyl sulfoxide (DMSO), filtered through a 0.22 μm syringe filter, and stored at -20°C . Working solutions of 500 $\mu\text{g/mL}$ (TRCO100) and 1,000 $\mu\text{g/mL}$ (TRCO200) were prepared using 70% acetone as the vehicle. Each mouse received 200 μL of the respective formulation by topical application, delivering 100 μg and 200 μg of TRCO, respectively.

Study design and experimental overview

A randomized controlled study was conducted to evaluate the protective action of TRCO during the promotion phase of a two-stage murine skin carcinogenesis (DMBA/UVB) model. A total of 24 healthy female BALB/c female mice (6 weeks old; body weight 20–30 g) were obtained from the Animal Resource Unit, UKM. Female mice were selected to minimize behavioural aggression and hormonal variability. The animals were housed in groups of six under standard laboratory conditions ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $55\% \pm 10\%$ relative humidity, 12-hour light/dark cycle) with ad libitum access to a standard rodent diet and water. The mice were randomly assigned using a computer-generated list into four groups ($n = 6$ independent biological replicates per group): (i) Control: Unexposed, vehicle-treated skin, (ii) DMBA/UVB + TRCO0: initiated with DMBA, promoted with UVB, treated with vehicle only, (iii) DMBA/UVB + TRCO100: initiated with DMBA, promoted with UVB, treated with 100 μg TRCO and (iv) DMBA/UVB + TRCO200: initiated with DMBA, promoted with UVB, treated with 200 μg TRCO.

Following anesthesia of mice with a ketamine-xylazine (KTX) mixture, the shaved dorsal skin surface was topically treated with 200 μL of acetone containing 200 nmol L^{-1}

7,12-dimethylbenz[a]anthracene) (DMBA) (0.01 mL of a 2 mM DMBA stock solution in 99.99 mL of 70% acetone). Then, TRCO was administered topically (200 μ L) 30 minutes prior to UVB exposure. UVB irradiation (180 mJ/cm²) was done three times per week using a calibrated UVB lamp (Analytik-jena) over a 24-week period (Fig. 2). At the end of the study, mice were anesthetized using KTX and euthanized by cervical dislocation. Dorsal skin samples were collected for further analysis. All procedures were conducted in accordance with institutional guidelines to minimize animal discomfort and distress.

The primary outcome of our study was the reduction of DMBA/UVB-induced skin damage, assessed by epidermal thickness and histopathological scoring. Secondary outcomes included antioxidant enzyme activities, oxidative stress markers (lipid peroxidation and protein carbonyl levels), melanogenesis parameters (tyrosinase activity and melanin content), and expression of carcinogenesis-related proteins (p53, Ki67, and VEGF). All histopathological and biochemical assessments were performed with investigators blinded to treatment allocation.

The study was conducted in accordance with ARRIVE 2.0 [7] and the NIH Guide for the Care and Use of Laboratory Animals [8], and all procedures were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (Approval No. FSK/2019/AHMAD ROHI/25-SEPT./1042-OCT.-2019-DEC.-2021). The inclusion criteria were healthy female BALB/c mice aged 6 weeks at the start of the study, while the exclusion criteria consisted of mice exhibiting abnormal skin lesions, weight loss greater than 10% post-acclimatization, or signs of illness. No mice met the exclusion criteria during the study.

Sample size justification

The sample size ($n = 6$ per group) was justified using two complementary statistical frameworks. First, standard power calculations were performed for the primary endpoint of

epidermal thickness. Assuming a statistical power of 80% to detect a 20% difference between group means at $\alpha = 0.05$ confirmed that six independent biological replicates per group provide adequate statistical sensitivity. Next, to further support this study design in the absence of extensive historical variance data for the TRCO extract, the resource equation approach [9] was applied. The resource equation defines the acceptable range for the error degrees of freedom (DF) in an ANOVA when the standard deviation and the effect size cannot be reliably estimated. According to the resource equation framework established by Arifin and Zahiruddin [9], the ethically and statistically acceptable range for the error degrees of freedom in a group comparison design is between 10 and 20. For this study, which involved four distinct experimental groups ($k = 4$), a sample size of six mice per group yielded an error DF of exactly 20 ($DF = k[n - 1] = 4[6 - 1] = 20$). This value lies precisely at the optimised upper boundary of the resource equation, ensuring robust exploratory power while adhering to animal welfare principles by avoiding unnecessary animal use.

Histopathological evaluation

Dorsal skin tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 3.5 μ m. Hematoxylin and eosin (H&E) staining was performed. Epidermal thickness and histological abnormalities were evaluated microscopically and quantified using Leica Application Suite v3.4.0.

Biochemical assays

Skin tissues were homogenized, and total protein was quantified using the BCA assay. Antioxidant enzyme activities such as total superoxide dismutase (T-SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione S-transferase (GST), as well as oxidative stress markers such as lipid peroxidation and protein carbonyl content were quantified using commercial kits (Elabscience), according to the man-

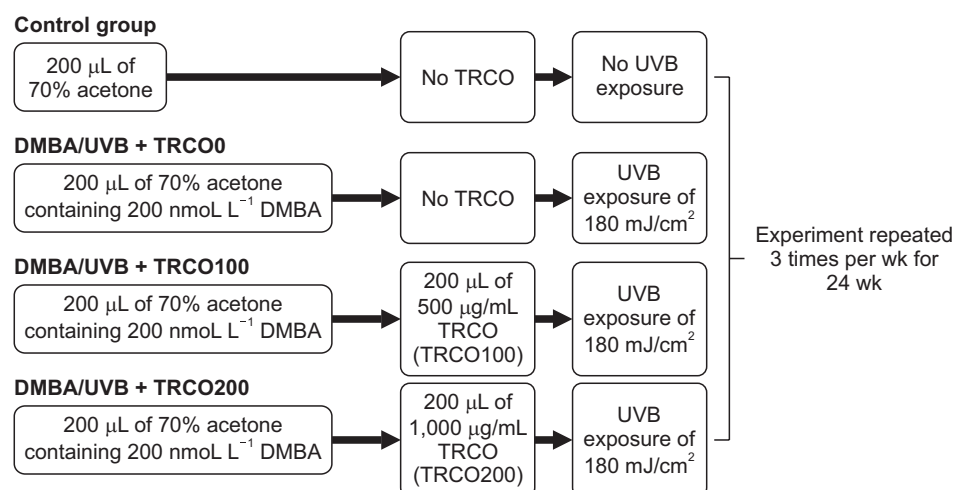


Figure 2. Experimental design of a DMBA/UVB-induced skin carcinogenesis model in BALB/c mice treated with TRCO ($n = 6$ for each group). TRCO was topically applied 30 minutes prior to each UVB irradiation, 3 times a week for 24 weeks. DMBA, 7,12-dimethylbenz[a]anthracene; UVB, ultraviolet B; TRCO, a terpenoid-rich extract from *Canarium odontophyllum* Miq. leaves.

ufacturer's instructions. All absorbance readings were taken using a microplate reader (Multiskan GO, Thermo Scientific). All individual mice were evaluated in technical triplicates.

Tyrosinase activity and melanin content

Tyrosinase activity was determined in terms of the rate of L-DOPA oxidation [10], and expressed as fold-change relative to the control. Melanin content was quantified as described by Arung et al. [11], using synthetic melanin as a standard based on absorbance at 475 nm. Data were normalized to protein content. All individual mice were evaluated in techni-

cal triplicates.

Protein expression analysis

p53 and Ki67 levels were measured using ELISA kits (Fine-Test). VEGF expression was evaluated using an automated capillary-based immunoassay system (Jess, ProteinSimple). Densitometric data generated by Jess were expressed as chemiluminescent peak areas (measured in "pixels"), which directly correspond to the absolute chemiluminescent signal intensity of the bound antibody. All chemiluminescent values were normalized against internal total protein loading runs

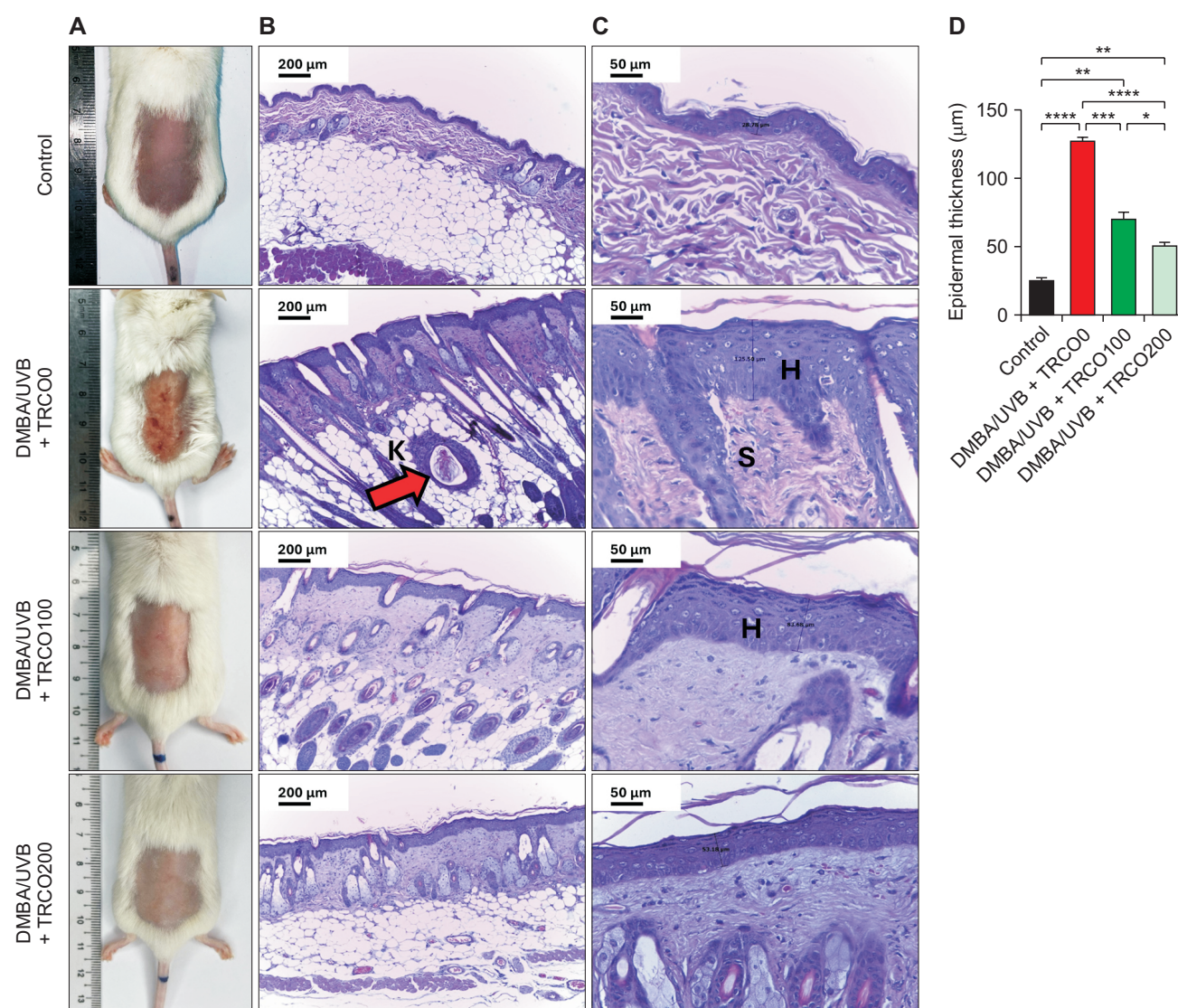


Figure 3. TRCO attenuates DMBA/UVB-induced skin carcinogenesis damage. (A) Effect of TRCO on skin morphology of BALB/c mice in a DMBA/UVB-induced skin carcinogenesis model after a 24-wk treatment. (B) Histopathology of dorsal skin of BALB/c mice at 100 × magnification using H&E staining. (C) Histopathology of dorsal skin of BALB/c mice at 400 × magnification using H&E staining. (D) The epidermal thickness of dorsal skin of BALB/c mice. The value represents the mean ± SEM of n = 6. All individual mice were evaluated in technical triplicates. The significant difference between groups were analyzed using one-way ANOVA and post-hoc Tukey (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). The thickness of the epidermis was measured using the Leica Application Suite 3.4.0 software. TRCO, a terpenoid-rich extract from *Canarium odontophyllum* Miq. leaves; DMBA, 7,12-dimethylbenz[*a*]anthracene; UVB, ultraviolet B; H&E, Hematoxylin and Eosin; SEM, standard error of the mean; K, keratin pearl; H, hyperkeratosis; S, solar elastosis.

using manufacturer-provided software.

Statistical analysis

Data analysis was conducted using GraphPad Prism version 9.3.1 (GraphPad Software). All datasets were presented as mean $\pm$ standard error of the mean (SEM). Statistical differences between groups were determined using a One-Way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Statistical significance was defined at a threshold of $P < 0.05$.

RESULTS

TRCO attenuates DMBA/UVB-induced morphological and histopathological damage

Macroscopic observation at week 24 as shown in Figure 3A revealed notable erythema and scaling in the DMBA/UVB + TRCO0 group (Fig. 3A). Both TRCO-treated groups (DMBA/UVB + TRCO100 and DMBA/UVB + TRCO200) exhibited visibly reduced erythema compared to the DMBA/UVB + TRCO0 group. Histological evaluation of mice in the DMBA/UVB + TRCO0 group demonstrated the keratin pearl formation (Fig. 3B), presence of hyperkeratosis, and solar elastosis (Fig. 3C), and increased epidermal thickness (Fig. 3D), signaling indicative of early preneoplastic squamous cell progression. Both TRCO-treated groups showed reduced histo-

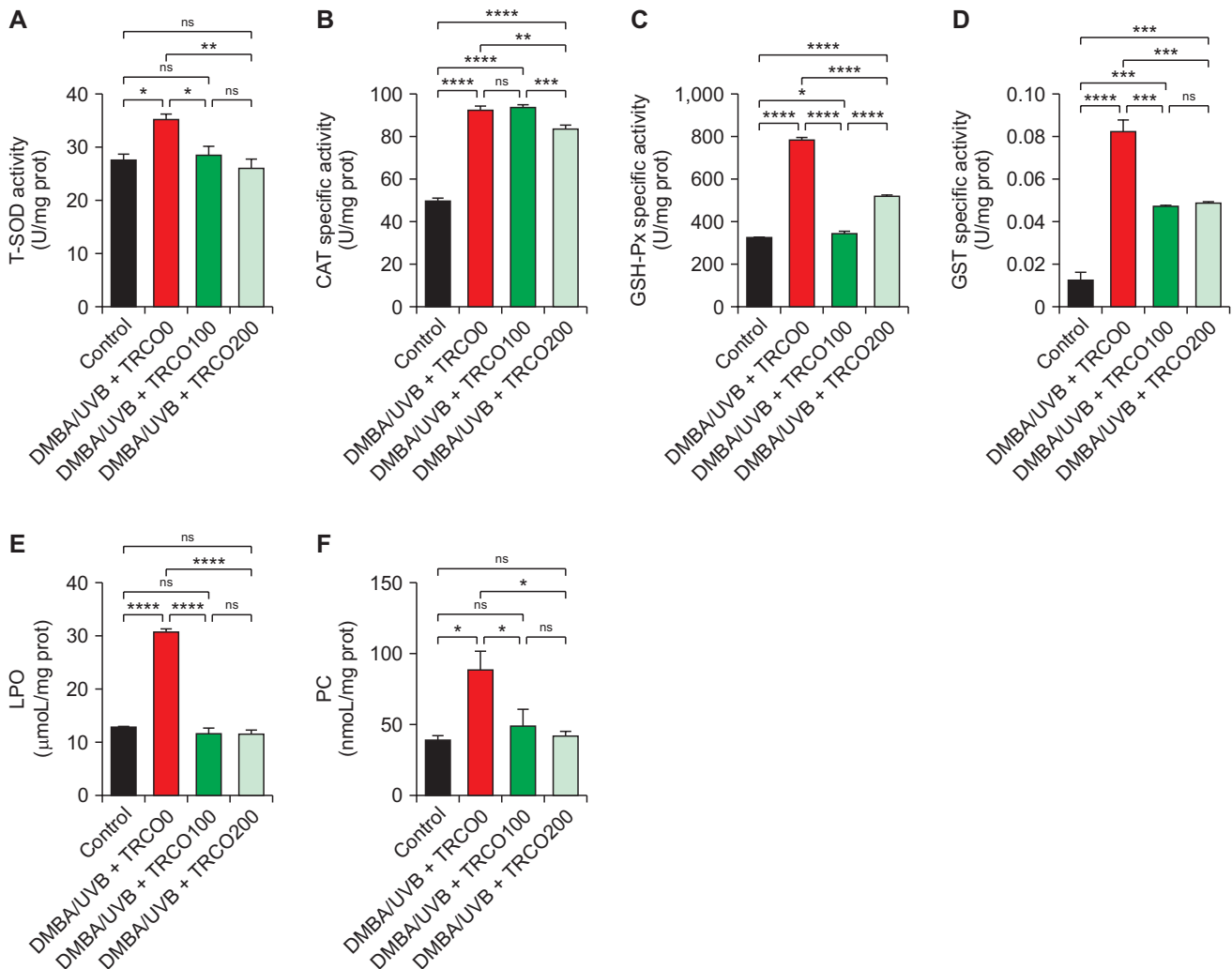


Figure 4. TRCO modulates antioxidant enzyme activities and reduces oxidative stress biomarkers in a DMBA/UVB-induced mouse carcinogenesis model. (A) T-SOD, (B) CAT, (C) GSH-Px, (D) GST, (E) LPO, (F) PC. The value represents the mean $\pm$ SEM of $n = 6$. All individual mice were evaluated in technical triplicates. The significant difference between groups were analyzed using one-way ANOVA and post-hoc Tukey ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$). TRCO, a terpenoid-rich extract from *Canarium odontophyllum* Miq. leaves; DMBA, 7,12-dimethylbenz[a]anthracene; UVB, ultraviolet B; T-SOD, total superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; LPO, lipid peroxide; PC, protein carbonyl; SEM, standard error of the mean; ns, not significant.

pathological features and significantly decreased epidermal thickness, with the DMBA/UVB + TRCO100 group showing pronounced improvement (Fig. 3).

TRCO modulates antioxidant enzyme activities and reduces oxidative stress biomarkers

The activities of representative antioxidant enzymes (T-SOD, CAT, GSH-Px, and GST) were elevated in the DMBA/UVB + TRCO0 group (Fig. 4A–4D). The result showed that chronic DMBA/UVB exposure induced a compensatory increase in antioxidant enzyme activity. As for TRCO treatment, these enzyme levels were normalized, indicating reduced oxidative burden.

Specifically, T-SOD activity was significantly elevated in the DMBA/UVB + TRCO0 group (35.39 ± 0.93 U/mg protein). In DMBA/UVB + TRCO100 and DMBA/UVB + TRCO200 groups, the T-SOD activity was significantly decreased to 28.72 ± 1.51 and 26.17 ± 1.68 U/mg protein, respectively. Likewise, CAT activity increased significantly in the DMBA/UVB + TRCO0 group (92.98 ± 1.43 U/mg protein vs. control 49.98 ± 0.80 U/mg protein). The DMBA/UVB + TRCO200 group had significantly lowered the CAT activity (84.03 ± 1.26 U/mg protein), while DMBA/UVB + TRCO100 showed no significant reduction. The GSH-Px activity was highest in the DMBA/UVB + TRCO0 group (788.37 ± 6.85 U/mg protein). DMBA/UVB + TRCO100 and DMBA/UVB + TRCO200 groups exhibited significant reductions to 347.60 ± 4.93 and 522.87 ± 3.16 , respectively. Finally, the GST activity was significantly increased in the DMBA/UVB + TRCO0 (0.083 ± 0.005 U/mg protein) group, while both DMBA/UVB + TRCO100 (0.047 ± 0.0001 U/mg protein), and DMBA/UVB + TRCO200 (0.049 ± 0.0003 U/mg protein) groups showed significant reductions.

As for oxidative stress, lipid peroxidation was significantly elevated in the DMBA/UVB + TRCO0 group (30.92 ± 0.35 μ mol/g protein vs. control 12.92 ± 0.05 μ mol/g protein) (Fig. 4E). In DMBA/UVB + TRCO100 and DMBA/UVB + TRCO200 groups, there were significantly reduced LPO levels (11.76 ± 0.88 and 11.57 ± 0.83 μ mol/g protein, respectively). Further, the protein carbonyl content, a marker of protein oxidation, increased markedly in DMBA/UVB + TRCO0 (88.97 ± 12.69 nmol/mg protein) compared to the control group (39.46 ± 2.77 nmol/mg protein). DMBA/UVB + TRCO100 and DMBA/UVB + TRCO200 treatments groups exhibited significantly reduced PC levels (49.48 ± 11.15 and 42.25 ± 2.93 nmol/mg protein, respectively) (Fig. 4F).

TRCO suppresses hyperactive tissue melanogenesis

DMBA/UVB exposure significantly increased tyrosinase activity (Fig. 5A) and melanin content (Fig. 5B). The DMBA/UVB + TRCO200 group showed a markedly suppressed tyrosinase activity (1.49 ± 0.01 -fold), compared to the control group (Fig. 5A). DMBA/UVB + TRCO100 showed a no statistically signif-

icant reduction (2.05 ± 0.01 -fold).

Melanin content was significantly elevated in the DMBA/UVB + TRCO0 group (20.08 ± 0.06 μ g/mg protein) compared to the control group (12.25 ± 0.31 μ g/mg protein). Both DMBA/UVB + TRCO100 and DMBA/UVB + TRCO200 groups significantly reduced melanin levels (17.99 ± 0.26 and 15.36 ± 0.38 μ g/mg protein, respectively), as shown in Figure 5B.

TRCO modulates expression of carcinogenesis-related proteins

p53 expression was significantly upregulated in the DMBA/UVB + TRCO0 group (17.37 ± 0.25 ng/mL vs. control 14.10 ± 0.36 ng/mL). p53 expression was significantly reduced to 13.59 ± 0.67 ng/mL in the DMBA/UVB + TRCO100 group, while the DMBA/UVB + TRCO200 group showed a non-significant decrease (16.31 ± 0.37 ng/mL), as shown in Figure 6A. The expression of Ki67, a proliferation marker, was significantly elevated in the DMBA/UVB + TRCO0 group (63.62 ± 1.11 ng/mL vs. control 49.13 ± 1.66 ng/mL). The DMBA/UVB + TRCO100 group showed significantly reduced Ki67 levels (45.44 ± 1.38 ng/mL), whereas the DMBA/UVB + TRCO200 group showed a non-significant reduction (59.03 ± 2.74 ng/mL), as shown in Figure 6B. Finally, VEGF expression was markedly elevated in the DMBA/UVB + TRCO0 group (871,611.4 pixels) in comparison to the control group (752,934.6 pixels). In the DMBA/UVB + TRCO100 group, the VEGF expression was reduced to 693,695.5 pixels, while

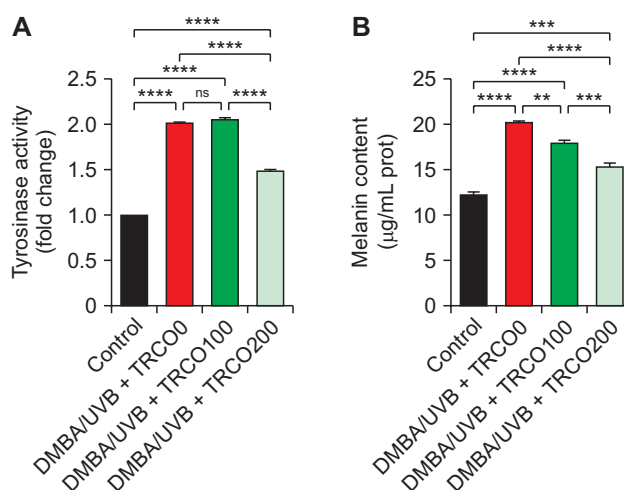


Figure 5. TRCO suppresses melanogenesis in a DMBA/UVB-induced murine carcinogenesis model. (A) Tyrosinase activity of dorsal skin of BALB/c mice model treated with TRCO. (B) Melanin content of dorsal skin of BALB/c mice. The value represents the mean $\pm$ SEM of $n = 6$. All individual mice were evaluated in technical triplicates. The significant difference between groups were analysed using one-way ANOVA and post-hoc Tukey ($**P < 0.01$, $***P < 0.001$, $****P < 0.0001$). TRCO, a terpenoid-rich extract from *Canarium odontophyllum* Miq. leaves; DMBA, 7,12-dimethylbenz[a]anthracene; UVB, ultraviolet B; SEM, standard error of the mean; ns, not significant.

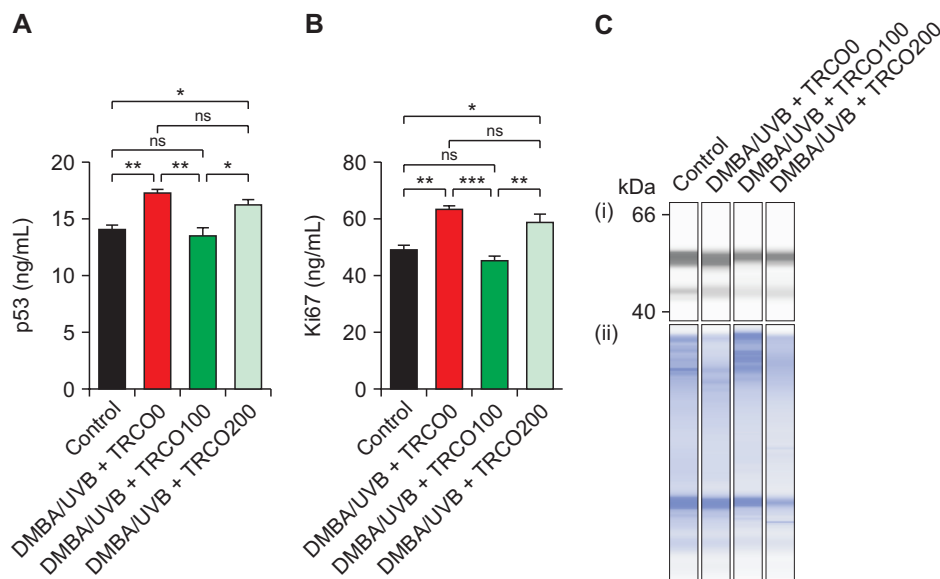


Figure 6. TRCO downregulates carcinogenesis-related protein expression in a DMBA/UVB-induced mouse skin carcinogenesis model. (A, B) The tumor suppressor protein p53 (A) and the proliferation marker Ki67 expression. The value represents the mean $\pm$ SEM of $n = 6$. All individual mice were evaluated in technical triplicates. The significant difference between groups were analyzed using one-way ANOVA and post-hoc Tukey ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (C) VEGF expression assessed using Jess Western's automatic capillary immunoassay test. (i) A densitometric image of VEGF expression across all groups. (ii) Total protein loading profiles were employed to confirm consistent protein loading and appropriate normalization across all lanes. TRCO, a terpenoid-rich extract from *Canarium odontophyllum* Miq. leaves; DMBA, 7,12-dimethylbenz[a]anthracene; UVB, ultraviolet B; p53, tumor protein p53; Ki67, marker of cellular proliferation Ki67; VEGF, vascular endothelial growth factor; SEM, standard error of the mean; kDa, kilodalton; ns, not significant.

the DMBA/UVB + TRCO200 group showed a lesser reduction (852,041.7 pixels) as compared to the DMBA/UVB + TRCO100 group. Normalization using internal controls confirmed these differences (Figure 6C and Table 1).

DISCUSSION

This study evaluated the protective action of TRCO, the leaf extract from *Canarium odontophyllum* Miq. in a DMBA-initiated and UV-promoted two-stage skin carcinogenesis model. A single topical dose of DMBA establishes stable genetic initiation, while chronic repetitive UVB irradiation acts as the driving force for tumor promotion. The present in vivo findings are complementary to our previous in vitro data [12] by providing critical translational evidence. Cell culture models fail to reflect the complex architectural context of skin, which includes the epidermis, dermis and other structural components. By tracking these parameters in intact tissue, we demonstrated that TRCO successfully traverses the epidermal barrier to prevent preneoplastic tissue changes, significantly suppressing epidermal hyperplasia, hyperkeratosis, the formation of keratin pearls and solar elastosis.

p53 is a key tumor suppressor protein. In a two-stage mouse skin carcinogenesis model, elevated total p53 expression in the DMBA/UVB + TRCO0 group typically represents a physiological, wild-type p53-mediated response to chronic DNA damage, or the selection and expansion of initiated

Table 1. Effects of TRCO on VEGF expression in dorsal skin of a DMBA/UVB-induced skin carcinogenesis in BALB/c mice

BALB/c mice treatment group	VEGF expression (pixel)
Control	752,934.6
UVB + TRCO0	871,611.4
UVB + TRCO100	693,695.5
UVB + TRCO200	852,041.7

"Pixels" refers to the chemiluminescent densitometric peak area obtained from the automated Jess capillary immunoassay system. TRCO, a terpenoid-rich extract from *Canarium odontophyllum* Miq. leaves; DMBA, 7,12-dimethylbenz[a]anthracene; UVB, ultraviolet B; VEGF, vascular endothelial growth factor.

clones accumulating mutant, non-functional p53 [13]. However, because total p53 protein was not differentiated into wild type and mutant configurations, this interpretation remains speculative and is an explicit limitation of this study. One possible explanation is that the lower p53 levels observed in the TRCO100 group could indicate that TRCO shielded the epidermal layer from upstream genotoxic insults, thereby reducing the requirement for a robust p53-mediated DNA damage response. There is also the alternative possibility that TRCO modified the clonal expression of mutant p53 populations. Hence, future studies using TRCO should further studies will be necessary to distinguish the TRCO effect between wild-type and mutant p53 configurations.

Our biochemical analyses regarding endogenous anti-

oxidant enzyme activities require a similarly nuanced interpretation. The sharp elevations in T-SOD, CAT, GSH-Px, and GST in the untreated DMBA/UVB cohort represent a well-documented compensatory tissue adaptation to cope with high levels of ROS. The lower enzyme levels observed in the TRCO-treated groups could mean suggest that TRCO may function as an efficient direct radical scavenger, reducing overall ROS generation and minimizing the need for compensatory host enzyme upregulation. Conversely, it is also possible that high topical doses of TRCO directly suppressed endogenous antioxidant enzyme expression. To resolve these contrasting explanations, we point to our macromolecular damage markers: both lipid peroxidation and protein carbonyl contents were significantly lowered by TRCO. This concurrent reduction in structural tissue damage supports the conclusion that TRCO maintains redox homeostasis by limiting overall oxidative stress at both the lipid and protein levels [12].

Melanin biosynthesis was also affected by chronic UVB exposure, as evidenced by increased tyrosinase activity and melanin content. The hyperpigmentation is typically a protective response to UV-induced damage [14]. TRCO, particularly at the higher dose, effectively downregulated tyrosinase activity and melanin synthesis, suggesting potential application in managing hyperpigmentation and photoaging, in addition to chemoprevention [15].

To provide deeper mechanistic substance beyond descriptive associations, terpenoids can be integrated into established molecular signaling pathways. Terpenoids have been shown to modulate the mitogen-activated protein kinase (MAPK) pathway, which controls downstream transcription factors driving cellular proliferation and tissue hyperplasia in cancer [16-18]. Furthermore, terpenoids frequently inhibit the activation and nuclear translocation of NF- κ B, a core transcription factor driving inflammatory cytokine release and angiogenesis via VEGF induction [19,20]. Concurrently, terpenoids interact with the Nrf2/Keap1 signaling network, promoting the nuclear accumulation of Nrf2 to maintain baseline redox balance and prevent the accumulation of severe lipid and protein oxidation products [21]. Although terpenoids have been widely studied for their modulation of molecular signaling pathways, the specific effects of TRCO remain uncharacterized, highlighting the need for further studies.

An interesting aspect of our data is the non-linear dose response relationship, where the lower dose (TRCO100) frequently displayed superior protective efficacy compared to the higher dose (TRCO200), particularly in the modulation of Ki67 and VEGF. As markers of angiogenesis such as CD31 staining or microvessel density were not quantified, our findings are limited to the modulation of VEGF expression rather than providing definitive evidence of an anti-angiogenic effect. On the other hand, while the superiority of TRCO100 can be speculated as a hormesis in which intermediate phytochemical concentrations prompt optimal cellular adaptation, alterna-

tive physiological mechanisms should be considered. Higher topical concentrations of TRCO (200 μ g) may exceed the skin's uptake capacity, leading to preferential accumulation within the stratum corneum without a proportional increase in penetration into the deeper proliferative basal layer [22]. To discern which explanation best fits the TRCO's lower dose superior protective efficacy, further studies are needed.

Finally, the methodological boundaries of this study must be clearly acknowledged. The investigation was confined to the 24-week preneoplastic phase, enabling the evaluation of early microarchitectural and biochemical adaptations but not extending to long-term macro-tumor outcomes such as tumor incidence, multiplicity, or volume. As such, interpretation of efficacy should be made with caution: TRCO demonstrates promising protective potential against early-stage preneoplastic skin progression in a two-stage chemical and photocarcinogenesis (DMBA/UVB) model, rather than definitive therapeutic effects on growth and progression of established tumors. Despite these limitations, the present findings collectively suggest TRCO as a compelling natural candidate for the chemoprevention of DMBA/UVB-induced skin carcinogenesis, acting through mechanisms that include attenuation of oxidative stress, regulation of pigmentation pathways, and modulation of key molecular modulators of carcinogenesis. Future studies should incorporate extended longitudinal designs to capture macro-tumor kinetics, distinguish between wild-type and mutant p53 profiles, assess phosphorylation states in the MAPK and NF- κ B signaling pathways, as well as quantify microvessel density using endothelial markers like CD31 to confirm an angiogenic effect and further substantiate these mechanistic insights. Additionally, further work is also warranted to isolate and characterize the active terpenoid constituents of TRCO, define their precise molecular targets, and validate efficacy in advanced preclinical and clinical settings.

In conclusion, topically applied TRCO exhibits promising protective potential against early preneoplastic tissue progression in a DMBA/UVB-induced two-stage murine skin carcinogenesis model, operating primarily during the tumor promotion phase. TRCO application effectively preserves skin tissue architecture, attenuates oxidative stress, regulates pigmentation pathways, and modulates some key molecular modulators of carcinogenesis such as p53, Ki67, and VEGF. These results highlight the potential development of TRCO as a natural, topically applied photoprotective agent. Future research must prioritize long-term macro-tumor kinetic trials, dermal penetration studies across variable vehicles, and targeted upstream signaling pathway analyses to validate its safety and translational utility in dermatological formulations.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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