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# FATTY ACIDS AND TERPENOIDS FROM *Canarium odontophyllum* MIQ. LEAF AND THEIR ANTIOXIDANT AND CYTOTOXIC EFFECTS ON UVB-INDUCED IMMORTALIZED HUMAN KERATINOCYTES CELLS (HACAT)

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## ABSTRACT

The study evaluated the antioxidant capacity of hexane extract of *Canarium odontophyllum* Miq. leaf; its fatty acids and terpenoids content; and cytotoxic effects on UVB-induced human keratinocytes (HaCaT). FRAP assay was used to determine antioxidant capacity. GC-MS analysis to identify the fatty acids and terpenoids' in the hexane extract of *Canarium odontophyllum* Miq. leaf. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to measure the cytotoxic effects of the extract on UVB-induced human keratinocytes. Serial doses of up to 1000 µg/mL extract were administered before UVB irradiation of the cells. FRAP assay showed the extract was found to exhibit antioxidant activity but no significant difference in ascorbic acid equivalent antioxidant capacity (AEAC) between dose 500 µg/mL (5.00 ± 0.35 AEAC) and 1000 µg/mL (5.70 ± 0.29 AEAC) extract. GC-MS analysis showed the extract contained 88.93% of fatty acids and terpenoids, especially n-hexadecanoic acid, spathulenol, and phytol. MTT assay showed no $IC_{50}$ value for the tested extract dose on UVB-induced HaCaT. Thus, the results suggest the potential application of hexane extract of *C. odontophyllum* Miq. leaf in terpenoids' studies. In-depth research and isolation of compounds of interest should be done to develop it as a viable medical phytotherapeutic agent.

**Key words:** Terpenoids, fatty acids, *Canarium odontophyllum*, human keratinocytes, antioxidant

## INTRODUCTION

UVB is a well-known environmental carcinogen that causes epidermal DNA damage, making it the primary cause of skin cancer (D'Orazio *et al*., 2013; Laikova *et al*., 2019). It generates free radicals in the skin, which play a critical part in skin carcinogenesis, and antioxidants play a crucial role in protecting the human body from free radical damage (Nimse & Pal, 2015).

Fatty acids have been known to possess antioxidant and radical scavenging activities (Nengroo & Rauf, 2019), while antiviral, antibacterial, antimalarial, anti-inflammatory, cholesterol synthesis inhibition, and anticancer activities are among the many medicinal properties of the terpenoids group (Yang *et al*., 2020). The use of natural compounds is seen as safer and a promising alternative to replace the widely used synthetic antioxidants (Pokorný, 2007).

In previous research, the plants in the genus Canarium have shown antioxidant, antibacterial, antifungal, anti-tumor, anti-inflammatory, hepatoprotective, analgesic, and anti-diabetic properties (Mogana & Wiart, 2011; Basri & Nor, 2014). Up to now, minimal investigations have been conducted to investigate the biological properties of *Canarium odontophyllum*. The native seasonal fruit of Sarawak, Malaysia, also known locally as 'dabai' is consumed as a snack food by the locals (Latiff *et al*., 2000). Several studies have been conducted on *C. odontophyllum* Miq leaf (Basri *et al*., 2015; Basri *et al*., 2017; Budin *et al*., 2018), and the active compounds, especially the fatty acids and terpenoids, which can be extracted using a nonpolar organic solvent such as hexane, might be accountable for their biological properties. Our recently published article summarized *C. odontophyllum* leaf phytoextracts and their medicinal properties (Abdul Aziz *et al*., 2022). Therefore, in this study, we employed the ferric reducing/antioxidant power assay (FRAP) to

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determine the antioxidant capacity of hexane extract of *C. odontophyllum* Miq. leaf (HCOL). Then, GC-MS analysis was done to determine the fatty acids and terpenoid content in HCOL. Finally, we employed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on UVB-induced human keratinocytes cells (HaCaT) to mimic the condition where human skin to measure the effects of the extract on cell viability and cytotoxicity.

## MATERIALS AND METHODS

### Plant sample

Fresh leaves of *C. odontophyllum* Miq. (Figure 1) were collected from Kuching, Sarawak, Malaysia, in December 2019. The permit for export and the permit for research and development were obtained from Sarawak Biodiversity Centre with permit numbers SBC-2020-EP-58-MWH and SBC-2019-RDP-20-MWH, respectively. The leaf was deposited in UKM Herbarium with voucher number ID028/2020.

### Extraction procedure

The leaves were air-dried for about 3 days at room temperature. A commercial grinder was used to grind the dried leaves. Extraction was done using n-hexane (non-polar) to obtain fatty acids and terpenoids. The extract was then evaporated using Rotavapor (Buchi Switzerland) to dry with the method described previously (Basri & Nor, 2014).

### Stock preparation

Crude extract of 100 mg was weighed and mixed with 1 mL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich USA). The mixture was then vortexed and filtered using a 0.22 µm syringe filter (Bioflow Malaysia) into a 1.5 mL microcentrifuge. The HCOL stock (100 mg/mL) was kept at -20 °C until further use.

### Antioxidant capacity

The antioxidant capacity of HCOL was measured using the FRAP assay method for assessing antioxidant power by reduction of Ferric ($Fe^{3+}$) to Ferrous ions ($Fe^{2+}$) at low pH, which causes the formation of colored ferrous-tripyridyltriazine complex, as described previously (Benzie & Strain, 1996).

*Reagents for FRAP assay*

a) Acetate buffer 300 mM pH 3.6: 16 mL glacial acetic acid was added into 3.1 g sodium acetate trihydrate and diluted with distilled water into 1 L.

b) TPTZ (2, 4, 6-tripyridyl-s-triazine) (Sigma-Aldrich USA) solution 10 mM, 10 mL: 0.031 g TPTZ powder dissolved in 10 mL 40 mM HCl (freshly prepared and stored in dark).

c) Iron (III) chloride ($FeCl_{3)}$ (Chemiz Malaysia) solution 20 mM, 10 mL: 0.054 g iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) (Chemiz Malaysia) powder dissolved in 10 mL distilled water (freshly prepared).

*FRAP assay procedure*

FRAP solution (a + b + c in ratio of 10:1:1) was incubated in 37 °C water bath. 50 µL of iron (II) sulfate ($FeSO_4$) (Chemiz Malaysia), ascorbic acid (Chemiz Malaysia) (positive control), and 50 µL sample were added to a 96-well plate (triplicate). 175 µL of warmed FRAP solution was added to the wells and incubated for 5 min at 37 °C. Then the absorbance was read at 595 nm. For the standard curve, ten concentrations of iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) (Chemiz Malaysia) (100 - 1000 µM) were used, and the

**Fig. 1.** Leaves of *Canarium odontophyllum* Miq. (dabai).

absorbance values were measured. The FRAP value of the samples was first expressed as µM $FeSO_4.7H_2O$. Then, serial dilution of ascorbic acid (AA) (50, 25, 12.5, 6.25, 3.125, 0 µg/mL) was used as a positive control to express the FRAP values as ascorbic acid equivalent antioxidant capacity (AEAC) in the unit µg AA/g HCOL.

**GC-MS Analysis for Fatty Acids and Terpenoids**

GC-MS was carried out on an Agilent 7890A gas chromatograph (USA) directly coupled to the mass spectrometer system (MS) of an Agilent 5975C inert MSD with a triple-axis detector as described previously (Marina *et al*., 2013). A Phenomenex ZB5 capillary column (30 m 0.25 mm I.D. 0.25 mm) coated with 5% phenyl methylpolysiloxane was used in the GC. A splitless injector was used to automate injection. In split mode, 1 µL of the sample was injected. With a flow rate of 1.3 mL/min, helium was used as the carrier gas. The oven was set to the following temperature: 50 °C for 1 min, then 7 °C/min from 50 °C to 180 °C, then 50ºC/min from 180 °C to 300ºC, and then 10 min at 300 °C. The temperature of the injector was kept at 300 °C. Temperatures were set at 280 °C for the transfer line, 300 °C for the source, and 150 °C for the quadrupole. With an electron impact ionization of 70 eV, the mass spectrometer was operated in full scan/selected ion monitoring (SIM) mode. From 40 to 220 m/z, full scan spectra were acquired. Quantitative analysis was carried out in the Selected Ion Monitoring (SIM) mode to improve sensitivity. All peaks in the raw GC chromatogram were found using the MSD Chemstation, and a library search was conducted using the NIST/EPA/NIH version 2.0, and the results were combined in a table.

**Cell viability**

*Immortalized Human Keratinocytes Cell Line (HaCaT)*

A vial of HaCaT (human epidermal keratinocyte) cell line, catalog number EP-CL-0090, was obtained from Elabscience (USA).

*HaCaT cell revival*

HaCaT cell stock was obtained from a -80 °C freezer and immediately transferred to a 37 °C water bath to dethaw rapidly. Upon dethawing, the HaCaT cell stock was transferred to a 15 mL centrifuge tube containing 3 mL enriched Dulbecco's modified Eagle medium (DMEM) (Capricorn Germany) added with 15% fetal bovine serum (FBS) (Capricorn Germany) and 1% Penicillin/Streptomycin (Capricorn, Germany) media and centrifuged at 1600 rpm for 3 min. Then, the supernatant was carefully discarded without touching the cell pellet. Then, the cell pellet was transferred to a T-25 flask containing 7 mL of DMEM. Next, the cells were observed under an inverted microscope for cell distribution before being incubated at 37 °C in 5% $CO_2$. After being incubated for 24 h, the cells were observed for contamination and confluency. Then, fresh 7 mL of enriched DMEM media was added after removing the old media from the T-25 flask.

*HaCaT cell subculture*

HaCat cells were subcultured upon reaching 80% confluency. Firstly, media was discarded from the T-25 flask with a serological pipette. Then, the cells were washed twice with a 5 mL phosphate buffer solution (PBS) (Oxoid USA). Next, 3 mL of trypsin-EDTA (Capricorn Germany) solution was added to the T-25 flask and incubated for 15 min at 37 °C in a 5% $CO_2$ incubator to detach the cells. After incubation, the cells were observed under an inverted microscope for detachment. Next, 7 mL of enriched DMEM media was added to the T-25 flask to inactivate trypsin-EDTA. Then, the contents of the T-25 flask were transferred to a 15 mL centrifuge tube and centrifuged at 1600 rpm for 3 min. Next, the supernatant was discarded, and 1 mL of DMEM was added to the cell pellet to create cell suspension. Then, 13 mL of fresh enriched DMEM was added to a new T-75 flask. Upon cell counting, the appropriate volume from the cell suspension was added to 1 mL of fresh enriched DMEM. The 1 mL DMEM cell suspension should contain $1\times10^6$ cells/mL and be added to the T-75 flask containing 13 mL enriched DMEM. The subculture flask was labeled and incubated at 37 °C, 5% $CO_2$ for 48 h. The flask was checked every day for contamination and confluency.

*HaCaT cell calculation*

Firstly, old media was discarded from the cell culture flask. Then, cells were washed twice with 5 mL PBS. Next, 3 mL of pre-warmed trypsin-EDTA was added to the cell culture and incubated for 15 min at 37 ºC, a 5% $CO_2$ incubator to detach the cells. After incubation, the cells are observed under an inverted microscope for detachment. Next, 7 mL of DMEM media was added to the cell culture flask. Then, the flask contents were transferred to a 15 mL centrifuge tube and centrifuged at 1600 rpm for 3 min. Next, the supernatant was carefully discarded without disturbing the cell pellet. 10 µL from the cell pellet was pipetted out and mixed with 190 µL of Trypan blue. Then, 10 µL of cell and Trypan blue suspension was added to the hemocytometer for cell counting. Under the inverted microscope, the number of viable cells was counted from the 4 corner squares. The number of cells per mL was determined using Equation 1.

Equation 1:

$$\left(\frac{\text{Total number live cells in 4 major corners}}{4}\right)\times \text{dilution factor} \times 10^4$$

Then, the volume of cell suspension seeded into a new T-75 flask was determined through Equation 2.

Equation 2:

$$M1V1 = M2V2$$

Where,
*M*1 = concentration total number of cells (cells/mL)
*V*1= volume of cell suspension
*M*2 = preferred concentration of cells for seeding (e.g $1 \times 10^6$)
*V*2= final volume of cells required for seeding

*MTT assay*

HCOL extract was evaluated for cell viability using the Mosmann MTT assay (Mossman, 1983) with some modifications to determine UVB-induced HaCaT viability and cytotoxicity. The test was carried out in triplicate. Briefly, $1 \times 10^5$ cells/mL with DMEM were seeded in a 96-well microplate except for blanks. The microplates were then incubated at $CO_2$ 5%, 37 ºC overnight to allow the cells to attach to the wells. After that, the medium in each well was replaced with 100 µL of DMEM with or without menadione (Sigma-Aldrich USA) (0, 0.0625, 0.125, 0.25, 0.5, 1 mM) or different concentrations of HCOL (0, 62.5, 125, 250, 500, 1000 µg/mL). The microplate was incubated for another 30 min before UVB irradiation (30 mJ/cm$^2$) using a UVB lamp (Analytikjena USA). Then the wells were washed with PBS, and 25 µL of 5 mg/mL MTT solution was added to each well, then incubated for another 4 h in the incubator. Afterward, 100 µL DMSO was loaded into each well, and the microplate was gently agitated for 10 min to dissolve the formed purple formazan crystals. A microplate reader (Multiskan Go, Thermo Scientific USA) was used to determine the absorbance at 570 nm. The percentage of viable cells was determined by using Equation 3:

$$CV=\left(\frac{\text{OD Treatment}}{\text{OD Control}}\right)\times 100\%$$

The concentrations that kill 50% of HaCaT cells ($IC_{50}$) were calculated using dose-response curves.

## RESULTS AND DISCUSSION

### Antioxidant capacity (FRAP assay)

From a previous study, screening of *C. odontophyllum* Miq. leaf extracts (aqueous, methanol, and acetone) showed antioxidant capabilities (Basri *et al*., 2014). In our study, as shown in Figure 2, the HCOL was found to exhibit antioxidant activity with no significant difference in ascorbic acid equivalent antioxidant capacity (AEAC) between dose 500 µg/mL (5.00 ± 0.35 AEAC) and 1000 µg/mL (5.70 ± 0.29 AEAC) HCOL.

### GC-MS analysis

Based on the GS-MS chromatogram (Figure 3), 88.93% of the extract contains fatty acids and terpenoids. Fatty acids and terpenoids identified are listed in Table 1.

The GC-MS study revealed that the leaf hexane extract contained 20 fatty acids and 26 terpenoids. However, identifications (qual) above 80 are considered credible, 70 to 79 are frequently correct, and 60 to 69 are highly speculative (Stein, 1999). We identified spathulenol and phytol as major terpenoids and n-hexadecanoic acid (palmitic acid) as primary fatty acids in the leaf extract. Spathulenol has been proven to have antioxidant, anti-inflammatory, and anti-proliferative activities (do Nascimento *et al*., 2018). Phytol, chlorophyll-derived diterpene alcohol, has been shown to have antioxidant, autophagy, apoptosis-inducing, antinociceptive, anti-inflammatory, immune-modulating, and antibacterial properties (Islam *et al*., 2018). n-Hexadecanoic acid is known to have anti-inflammatory (Aparna *et al*., 2012) and anti-tumor properties (Harada *et al*., 2002).

Based on the compounds found, the *C. odontophyllum* Miq. leaf might offer promising therapeutic effects as claimed in a previous article by Abdul Aziz *et al*. (2022). Hence, for any study

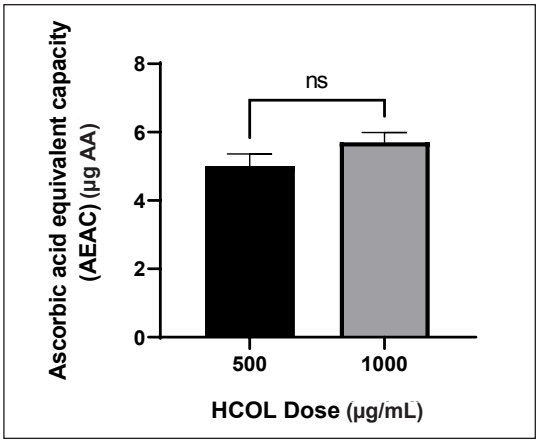


**Fig. 2.** Antioxidant capacity of hexane extract of *C. odontophyllum* Miq. leaf (HCOL) using FRAP assay. HCOL was found to exhibit dose-dependent antioxidant activity but no significant difference in ascorbic acid equivalent antioxidant capacity (AEAC) between dose 500 µg/mL (5.00 ± 0.35 AEAC) and 1000 µg/mL (5.70 ± 0.29 AEAC) HCOL.

regarding plant terpenoids, the hexane leaf extract is proper to be used to investigate the pharmacological and medicinal activities of its terpenoids. Further isolation and purification from the leaf extract could also predict its important bioactive chemical structure and formula. Their structural elucidation by screening for their biological activities will also benefit further drug development.

**Cytotoxic effects of HCOL on UVB-induced HaCaT**

Figure 4 depicts the cytotoxic effects (percentage of viability) of various concentrations of menadione and HCOL on HaCaT cells after being treated for 30 min before 30 mJ/cm$^2$ UVB irradiation. A higher percentage of cell viability can be seen in HCOL treated group compared to the menadione treated group (control).

The findings from the MTT assay indicate menadione is highly toxic to the cells, and the level of mortality is concentration-dependent. Treatment with HCOL showed a decrease in cell viability. The highest dose of 1000 µg/mL HCOL showed 53.16 ± 4.72% viable HaCaT. When antioxidants, especially in herbal extracts, directly react with MTT or formazan, unwanted reactions can give rise to high background absorbance values. However, this occurrence was minimal in this assay because the blanks (media and extract without cells) did not give any background reactions. $IC_{50}$ values were obtained and listed in Table 2 based on the dose-response curves plotted. However, the HCOL extract showed unobtainable $IC_{50}$ in the tested range of concentrations.

Our findings showed that HCOL is safe to use

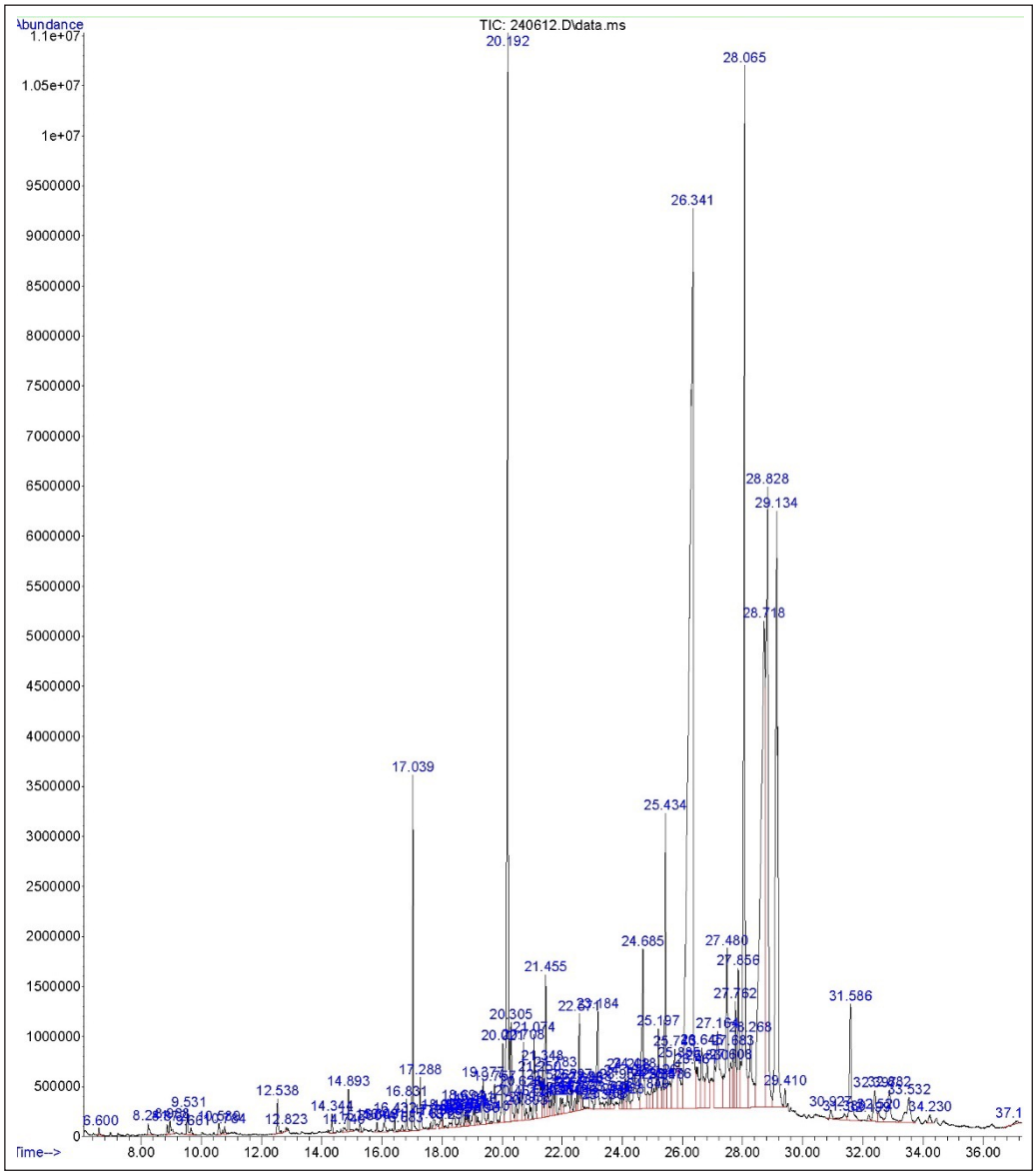


**Fig. 3.** GC-MS Chromatogram of hexane extract of *C. odontophyllum* Miq. leaf (HCOL).

**Table 1.** Fatty acids & terpenoids profile in the hexane extract of *C. odontophyllum* Miq. leaf (HCOL)

| RT | Area Pct | Library/ID | Qual |
|---|---|---|---|
| 6.5977 | 0.0468 | alpha-Pinene* | 97 |
| 8.8751 | 0.0614 | p-Cymene* | 91 |
| 8.9698 | 0.1105 | D-Limonene* | 96 |
| 10.7867 | 0.0487 | Nonanal | 64 |
| 12.5405 | 0.2248 | Terpinen-4-ol* | 90 |
| 14.7422 | 0.0582 | Nonanoic acid | 60 |
| 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 | beta-Vatirenene* | 91 |
| 18.5652 | 0.0582 | beta-Humulene* | 93 |
| 18.6914 | 0.1885 | Bicyclo[4.4.0]dec-1-ene,2-isopropyl-5-methyl-9-methylene-* | 94 |
| 18.7797 | 0.0712 | beta-Vatirenene* | 95 |
| 18.8365 | 0.0677 | beta-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 | gamma-Himachalene* | 47 |
| 20.0225 | 0.5846 | Aromadendrene, dehydro-* | 51 |
| 20.1928 | 7.1602 | Spathulenol* | 91 |
| 20.3064 | 0.6237 | beta-Selinene* | 64 |
| 20.4578 | 0.1376 | alpha-Guaiene* | 93 |
| 20.7101 | 0.4709 | Patchoulane* | 91 |
| 21.076 | 0.4624 | Spathulenol* | 53 |
| 21.2527 | 0.6672 | gamma-Muurolene* | 64 |
| 21.4546 | 0.8797 | alpha-Cadinol* | 83 |
| 21.7826 | 0.2393 | trans-Z-alpha-Bisabolene epoxide* | 55 |
| 21.9466 | 0.1271 | 9-Octadecenoic acid | 86 |
| 22.5712 | 0.5623 | Patchoulane* | 60 |
| 23.1831 | 0.8778 | Tetradecanoic acid | 99 |
| 23.3598 | 0.1024 | 9-Octadecenoic acid | 53 |
| 23.467 | 0.0895 | cis-Vaccenic acid | 97 |
| 23.6437 | 0.128 | Oleic acid | 94 |
| 23.9528 | 0.1726 | Pentadecanoic acid, methyl ester | 96 |
| 24.0285 | 0.0864 | Oleic acid | 96 |
| 24.2051 | 0.2585 | 2-Pentadecanone, 6,10,14-trimethyl-* | 49 |
| 24.6846 | 1.6695 | Pentadecanoic acid | 99 |
| 24.8486 | 0.2302 | 9-Octadecenoic acid | 95 |
| 24.9811 | 0.2564 | cis-Vaccenic acid | 98 |
| 25.1262 | 0.3997 | cis-Vaccenic acid | 94 |
| 25.347 | 0.2433 | Oleic acid | 97 |
| 25.4353 | 1.5211 | Hexadecanoic acid, methyl ester | 99 |
| 25.5741 | 0.4848 | Octadec-9-enoic acid | 95 |
| 25.7444 | 1.2274 | Oleic acid | 92 |
| 25.8832 | 0.7433 | Oleic acid | 70 |
| 26.3438 | 20.218 | n-Hexadecanoic acid | 99 |
| 26.6466 | 0.7244 | trans-13-Octadecenoic acid | 90 |
| 26.8295 | 0.7557 | Oleic acid | 96 |
| 27.1639 | 1.8785 | Oleic acid | 98 |
| 27.4793 | 1.8534 | Heptadecanoic acid | 91 |
| 27.6055 | 0.4811 | cis-Vaccenic acid | 99 |
| 27.6812 | 0.5251 | Octadec-9-enoic acid | 91 |
| 27.7632 | 0.7623 | 9,12-Octadecadienoic acid, methyl ester | 99 |
| 27.8578 | 1.0925 | 9-Octadecenoic acid (Z)-, methyl ester | 92 |
| 28.066 | 8.743 | Phytol* | 96 |
| 28.2679 | 1.1205 | Methyl stearate | 97 |
| 28.7158 | 11.3511 | 9,12-Octadecadienoic acid (Z,Z)- | 99 |
| 28.8294 | 7.7961 | 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- | 96 |
| 29.1322 | 7.5373 | Octadecanoic acid | 91 |
| 30.9238 | 0.0823 | Tetracosanoic acid | 93 |
| 32.8795 | 0.6153 | 4,8,12,16-Tetramethylheptadecan-4-olide* | 93 |
| 33.5293 | 0.5918 | Eicosanoic acid | 89 |

*: Terpenoids
RT: Retention time
Area Pct: % of the total measured area
Qual: Quality of identification, >80% regarded as identified

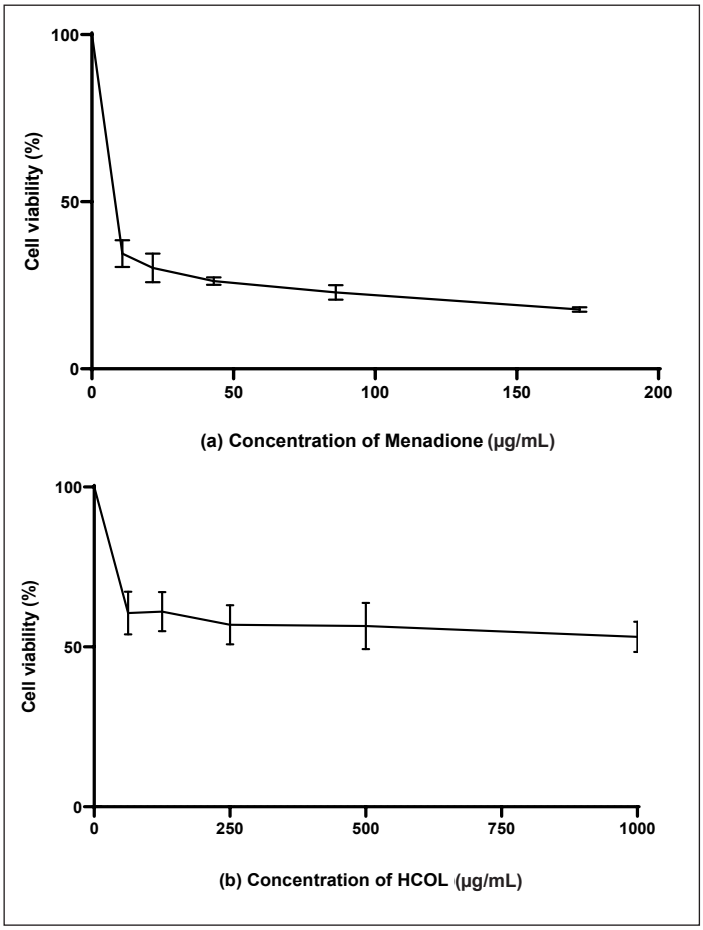


**Fig. 4.** Cytotoxic effects of (a) menadione and (b) HCOL on UVB-induced HaCaT using MTT assay. (mean ± SEM) (*n*=9)

**Table 2.** $IC_{50}$ values for HaCaT cell viability assay

| Sample | $IC_{50}$ ± SEM (µg/mL) |
|---|---|
| Menadione | 12.91 ± 7.92 |
| HCOL | Cannot be obtained |

on UVB-induced HaCaT cells. The previous study by Ghazali *et al*., (2017) also demonstrated the safe use of *C. odontophyllum* Miq. leaf where it was found that the acetone extract was not mutagenic. However, our study was limited to only crude hexane extract of *C. odontophyllum* Miq. leaf. Further investigations should be carried out with active compounds isolated from the extract.

## CONCLUSION

Hexane extract of *C. odontophyllum* Miq. leaf (HCOL) contains an abundance of fatty acids and terpenoids that possess several biological properties, especially antioxidants, to scavenge free radicals. The findings will be beneficial for further research and drug development. Nevertheless, terpenoids' isolation and biological activity will yield rewarding findings, thus opening new research areas into compounds and their medicinal potential.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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