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Biological Activities and GCMS Analysis of the Methanolic Extract of *Christia vespertilionis* (L.F.) Bakh. F. Leaves

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Abstract

Christia vespertilionis (L.F.) Bakh. F. (red butterfly wing) is commonly used as a herbal medicine to treat symptoms of many diseases. The present study aimed to determine the *in vitro* antimalarial and cytotoxic activities as well as to investigate the chemical compositions of the methanolic extract of *C. vespertilionis* leaves (CVME). The antimalarial activity of CVME against a chloroquine-sensitive (3D7) strain of *Plasmodium falciparum* was assessed by using a malarial SYBR Green I fluorescence-based (MSF) assay. The cytotoxic effect of CVME on cancerous (MDA-MB-231 and MCF-7) and noncancerous (NIH/3T3) cell lines were evaluated by using methyl tetrazolium (MTT) assay. Chemical constituents of CVME were analysed by using a gas chromatography/mass spectrometry (GCMS). CVME exhibited a moderate antimalarial activity with an IC₅₀ value of 43.87 ± 2.04 µg/mL. CVME is considered as weak cytotoxic and significantly inhibited proliferation of MDA-MB-231 cells with an IC₅₀ value of 37.45 ± 1.05 µg/mL (*p*<0.05) as compared to standard MCF-7 (IC₅₀>100 µg/mL) and NIH/3T3 cells (IC₅₀>100 µg/mL). This effect is selectively cytotoxic towards certain cancerous cells only. GCMS analysis suggests that CVME potentially contains several pharmacologically bioactive compounds such as tetrahydro-2-methyl-thiophene (61.77%), phytol (8.59%), 10-undecenoic acid (5.00%), 6-methyl heptyl-2-propanoate (4.96%) and 2-(2-benzothiazolylthio)-1-(3,5-dimethylpyrazolyl)-ethanone (2.70%) that might possess antimalarial and cytotoxic activities. It implies that CVME has selective cytotoxic activity against the breast cancer cell line (MDA-MB-231) and possesses an antimalarial activity against the 3D7 malaria parasite, suggesting the presence of bioactive compounds in *C. vespertilionis* leaves that could be a potential source of phytochemicals with high medicinal value to be used in cancer and malaria treatment.

Keywords: *Christia vespertilionis*, antimalarial activity, anticancer activity, cytotoxic activity, bioactive compounds.

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Introduction

Malaria is a disease caused by *Plasmodium* parasites that are transmitted to humans by infected female *Anopheles* mosquitoes. In 2017, more than 210 million people worldwide suffered from the infection and up to 435 000 of these died [1]. Of the five human malaria parasites, *P. falciparum* is the most virulent species causing severe malaria and high mortality [1,2]. The use of chloroquine to treat *P. falciparum* has led to the widespread appearance of chloroquine-resistant strains throughout the affected regions and extended to other antimalarial drugs [3,4]. The recent use of artemisinin-based combination therapies (ACTs) to treat uncomplicated and severe *P. falciparum* has made good progress in the treatment and control of malaria [1]. However, the emergence of parasites partially resistant to artemisinin (ART) in multiple locations in the Greater Mekong and other Southeast Asia has been reported [5,6]. This reduced sensitivity of *P. falciparum* to ART could greatly impede the malaria control and elimination efforts. The discovery and development of new potential antimalarial drugs with unique structures and mechanisms of action are urgently needed. One possible source lies in the use of herbal medicines that contain bioactive compounds with antimalarial properties.

Cancer is the second leading cause of death worldwide and caused 9.6 million deaths in 2018 [7]. In Malaysia, the most common type of cancer is breast cancer followed by colorectal and respiratory tract cancer [8]. Breast cancer is also a common malignancy among women in the United States and was estimated to affect more than 200 000 individuals and cause more than 40 000 deaths in 2018 [9]. Tamoxifen is a common anticancer drug used for the treatment of estrogen receptor-positive breast cancer [10]. Despite its efficacy in preventing recurrence and treating breast cancer, tamoxifen has been associated with several side effects such as the risk of developing uterine cancer, endometrial cancer, blood clots, cataracts and stroke [11]. Therefore, the search for new cancer therapies that do not have adverse side effects is in huge demand.

Phytochemicals or secondary metabolites produced from medicinal plants have been utilised for decades as a source of drug candidates for various ailments including malaria and cancer. Quinine and artemisinin are plant-based antimalarial drugs isolated from a cinchona bark [12] and a Chinese herb, *Artemisia annua*, respectively [13]. Both antimalarial drugs are also displayed in anticancer activities [10,14]. For cancer treatment, 48 out of 65 new anticancer drugs registered from 1981-2002 were derived from medicinal plants [14]. For instance, vincristine (Oncovin®) and paclitaxel (Taxol®) were from *Catharanthus roseus* and *Taxus brevifolia* plants, respectively. *Christia vespertilionis*, an ornamental plant belongs to the family Faboideae [15] has been reported to have antitumor [16] and anticancer activities [17], and also been used by native people to treat intermittent fever caused by malaria [18]. The medicinal plants that have an *in vitro* antimalarial activity must be further investigated for their cytotoxicity for prediction of the safety of the plant. Therefore, the present study aims to determine the *in vitro* antimalarial and cytotoxic activities as well as the phytochemical composition of the *C. vespertilionis* methanol leaf extract.

Materials and Methods

1.1 Plant material

The *C. vespertilionis* plant (CV) was purchased from the Rural Transformation Centre Kelantan, Malaysia. The plant was identified and authenticated by a botanist at Kuliyah of Pharmacy, International Islamic University Malaysia (IIUM) (voucher specimen no. PIUM 0273-1).

1.2 Plant extraction

The CV leaves were separated, washed, dried and grounded into a powder. The CV powder (50 g) was successively macerated in 500 mL of petroleum ether, ethyl acetate, methanol and water, respectively (1:10 powder/solvent) (Figure 1). After 24 hours of extraction, the mixtures were filtered using 70 mm filter papers to remove any residues. The filtrates were concentrated under reduced pressure until organic solvents were eliminated and subsequently freeze-dried until the water was removed to produce CV petroleum ether extract (CVPEE), CV ethyl acetate extract (CVEAE), CV methanol extract (CVME) and CV aqueous extract (CVAE). All extracts were stored at 4°C until further use. Due to the low yield of CVPEE, CVEAE and CVAE obtained, only CVME was used in the subsequent antimalarial and cytotoxic activity studies.

1.3 Antimalarial activity study

1.3.1 Ethics statement

Blood collection from informed consent healthy donors for the *in vitro* malaria parasite culture was performed in accordance with the ethical approval obtained from Human Research Ethics Committee Universiti Sains Malaysia (HREC USM) (USM/JEPeM/18050263).

1.3.2 *P. falciparum* *in vitro* culture

Parasites (3D7 strain) were cultured in culture flasks containing fresh human type O⁺ erythrocytes at 2% haematocrit and RPMI 1640 medium (Gibco, 72400-047) supplemented with a final concentration of 2 mg/mL glucose (Sigma, G-8270), 0.025 mg/mL gentamicin (Duopharma, 42491-M), 0.05 mg/mL hypoxanthine and 5 mg/mL albumax (Gibco, 11021-029) [19]. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. The parasite growth and development were monitored by using Giemsa-stained thin blood smears. The cultures predominantly at ring stages were synchronised by using 5% sorbitol treatment and used in the subsequent antimalarial activity assay after 2-hour post-synchronisation [20].

1.3.3 Malarial SYBR Green I fluorescence-based (MSF) assay

This assay was used to determine the antimalarial activity of CVME. The CVME stock solution (30 mg/mL in 100% DMSO) was diluted with the culture medium at 10 concentrations of two-fold serial dilutions in 96-well microtiter plates [19,21]. The DMSO final concentration in each well did not exceed 1%. The CVME solutions (20 µL) of different concentrations ranging from 11.7-3000 µg/mL were transferred into another 96-well microtiter

plates containing parasite suspensions (180 μ L) (2% parasitaemia, 2% haematocrit). The wells with artemisinin, a standard antimalarial drug were used as positive controls, and with 1% DMSO and without CVME were used as negative controls, respectively. Following 48 hours of treatment at 37°C, parasite suspensions (20 μ L) were transferred into microcentrifuge tubes and added with 20 $\times$ SYBR Green I solution (20 μ L) (Invitrogen). After 1 hour of incubation at room temperature, the fluorescence signal was measured with a microplate reader at excitation and emission wavelengths of 490 and 530 nm, respectively. A graph plotted as the logarithm of CVME concentration versus the percentage of parasite inhibition was generated and the half-maximal inhibitory concentration (IC₅₀) of CVME was determined by using probit regression analysis with GraphPad Prism software (Version 6). All tests were conducted in triplicate on three different occasions.

1.4 Cytotoxic activity study

1.4.1 Cell culture

Breast cancer cell lines, MCF-7 (ATCC® HTB-22™) and MDA-MB-231 (ATCC® HTB-26™), and normal mouse fibroblast cell line, NIH/3T3 (ATCC® CRL-1658™) were cultured in DMEM supplemented with 10% foetal calf serum and 1% penicillin-streptomycin solution. The cells were grown at 37°C with 5% CO₂. The cells were fed until confluent and subcultured by trypsinisation.

1.4.2 Methyl tetrazolium (MTT) assay

This assay was conducted to determine the cytotoxicity of CVME [22]. Exponentially growing cancerous and noncancerous cells were seeded into 96-well microtiter plates at a density of 5×10^4 cells per well. After 24 hours of incubation, the old media was discarded and fresh media (200 μ L) was added. After 72 hours, the CVME solutions (2 μ L) of different concentrations ranging from 0.40-100 μ g/mL were added into respective wells. The wells received tamoxifen, a standard anticancer drug was used as positive controls, and 1% DMSO as negative controls. After 72 hours of treatment, the old media was removed and MTT reagent (50 μ L) (2 mg/mL in DMEM) was added. After 4 hours of incubation at 37°C, the MTT solution was aspirated out and the purple formazan crystals formed at the bottom of the wells were dissolved with DMSO (200 μ L). The absorbance at 570 nm was read on a microplate reader. The half-maximal inhibitory concentration (IC₅₀) was calculated from a graph plotted as the logarithm of CVME concentration versus the percentage of cell viability curve by using GraphPad Prism software (Version 6). All tests were conducted in triplicate on three different occasions.

1.5 Gas chromatography-mass spectrometry (GC-MS)

This analysis was performed on a GC-MS (Perkin Elmer Clarus™ SQ8) fitted with an Elite-5MS capillary column (30 mL $\times$ 0.25 μ m film thickness). Purified helium (99.99%) was used as a carrier gas at a constant flow of 1 mL/min. All data were obtained by collecting the full-scan mass spectra within the scan range of 40-500 m/z. The sample was prepared in methanol and the injected sample volume was 1 μ L with a split ratio of 10:1. The oven

temperature program was 110°C and accelerated to 200°C at a rate of 10°C/min up to 280°C at a rate of 5°C/min. Phytochemical identification was performed by diluting 10 mg of CVME in 1 mL of methanol. The unknown compounds were identified by comparing the spectra obtained with National Institute Standard and Technology (NIST) Mass Spectral Library.

1.6 Statistical analysis

Statistical analysis was performed by using Microsoft Excel and GraphPad Prism software (Version 6). Each experiment was carried out in triplicate on three different occasions. Results were presented as mean $\pm$ standard deviation (SD). Statistical significance of the data was calculated by using student's t-test and one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests. Value of $p < 0.05$ was considered as statistically significant.

Result

1. The extraction yield of CV extracts

The highest yield of the extract was obtained from water (4.40%) followed by methanol (2.82%), petroleum ether (0.96%) and ethyl acetate (0.82%). Based on the extraction yield and better solvent of organic molecules, CV methanol extract (CVME) was used in the subsequent antimalarial and anticancer activity assays.

2. The antimalarial activity of CVME

According to WHO guidelines and previous data [24], the antimalarial activity of a plant extract can be classified as follows: promising (IC₅₀ < 15 μ g/mL), moderate (15 < IC₅₀ < 50 μ g/mL) and inactive activity (IC₅₀ > 50 μ g/mL). CVME has a moderate antimalarial activity (IC₅₀ = 43.87 $\pm$ 2.04 μ g/mL) (Figure 2). Artemisinin, as a standard antimalarial drug, was potent in inhibited the parasite growth with an IC₅₀ value of 4.0 $\pm$ 0.22 ng/mL.

3. The cytotoxic activity of CVME

Inhibition of cell proliferation following treatment with CVME was assessed on cancerous (MCF-7 and MDA-MB-231) and noncancerous (NIH/3T3) cells by using MTT assay. CVME caused a reduction in percentage of viability of MDA-MB-231 (IC₅₀ = 37.45 $\pm$ 1.05 μ g/mL), but not MCF-7 (IC₅₀ > 100 μ g/mL) (Figure 3). CVME was also found to be non-toxic on normal cell NIH/3T3 (IC₅₀ > 100 μ g/mL). Although tamoxifen, a standard anticancer drug, exerted a significant antiproliferative effect on both MDA-MB-231 (IC₅₀ = 4.90 $\pm$ 1.06 μ g/mL) and MCF-7 (IC₅₀ = 1.04 $\pm$ 1.03 μ g/mL) ($p < 0.05$), this drug was found to be toxic on NIH/3T3 (IC₅₀ = 3.64 $\pm$ 1.10 μ g/mL).

4. The screening of chemical compounds of CVME

GC-MS analysis was compared with the NIST mass spectra library to identify the compounds in CVME (Figure 4). The analysis resulted in the identification of 26 compounds representing 100% of the total extract (see Supplementary Material 1). These compounds were

resolved at concentrations ranging from 61.77 to 0.01% and the retention time ranging from 1.40 to 34.72 minutes. Five most major phytochemicals identified are summarised in Table 1.

Discussion

Based on the previous study, methanolic extract of *C. vespertilionis* has been shown to possess antioxidant and antimicrobial effects [25,26]. The methanolic leaf extract has also been proven to yield antimalarial activity [29] and abundant with polar compounds [27]. Therefore, CVME was selected for this current investigation.

Despite the widely use of CV by indigenous communities to treat malaria-related fever [29,30], no study of the plant against a chloroquine-sensitive (3D7) strain of *P. falciparum* has been done thus far. Based on the current study, CVME is a good candidate for use as an improved traditional drug (ITD) for the treatment of malaria although it is classified as a moderate antimalarial activity. For instance, aqueous-methanolic extract of CV stems showed 87.8% suppression of parasitemia in *P. berghei*-infected mice [29]. The results obtained here as well as from the previous studies [18,29] is a primary confirmation of the traditional effect reported. There are a total of 15 compounds that have been detected from the plant by a previous study [29] i.e. D:C-friedoolean-8-en-29 α -ol, ursolic-acid-methyl-ester, oleanolic acid methyl ester, erythrodiol, 2'-hydroxy-genestin, orobol, 2,3-dihydro-2'-hydroxy-genestin, quercetin-3-O-glucoside, catechin-3-O- β -D-glucopyranoside, stigmasterol, β -sitosterol acetate, β -sitosterol, monoterpene geraniol, 2'-hydroxydecanylpentadec-5, 8, 10, 12-tetraenoate (christanoate) and 7-isopropylidene-1-methyl-1, 2, 6, 7, 8, 9-hexahydronaphthalene (christene). Among them, christene was found to have an active antimalarial activity with an IC₅₀ of 9.0 μ g/mL, implying that this compound could be potentially investigated in future. The other compounds in the extract present in CVME could also be responsible for the additive effect of antimalarial activity. Also, according to Hofer et al. [16], pheophorbide-a, a chlorophyll catabolite extracted in the *C. Vespertilionis* also display an antiproliferative activity, showing that the compound can be considered as a potential anticancer agent.

In this study, CVME had a moderate effect on a breast cancer cell, MDA-MB-231 and the cytotoxicity was significantly higher than that obtained on a nonmalignant cell line, NIH/3T3. Although it has an effect on a normal cell line tested, the findings proved that *C. vespertilionis* might be selective in term of its target; it has a more potent effect on cancer cells. This kind of selectivity is a good characteristic of a potential anticancer candidate as a drug that can affect both noncancerous and cancerous cells can cause adverse side effects [31]. MCF-7 was found to be less sensitive to CVME likely due to the presence of oestrogen receptor on the cell that facilitates the cell growth and hampers the induction of cell death [32]. The standard drug, tamoxifen also affects the normal fibroblast cells, NIH/3T3, in contrast with its ability as a selective anticancer drug against breast cancer cells [43]. This could be associated with the cell types used in which tamoxifen has been shown to affect both normal- and tumour-fibroblast cell functions by downregulating cytokines release [43,44]. This is consistent with previous *in vitro*

reports of delayed proliferation rate fibroblast cells [43,45]. According to the U.S. National Cancer Institute (NCI) guidelines, only crude extracts with IC₅₀ values <30 μ g/mL after 72 hours of exposure are acknowledged as promising agents for anticancer candidates [33]. Although the IC₅₀ value of CVME against MDA-MB-231 was higher than the NCI reference value, the value was still under 100 μ g/mL, suggesting the cytotoxic capability of the extract [34]. To the best of our knowledge, this is the first study investigating the cytotoxic activity of CV against breast cancer cell lines. Previous studies only reported the inhibition of the proliferation of cervix carcinoma, medullary thyroid carcinoma, and small intestinal neuroendocrine tumour following treatment with different CV extracts [16,18]. In light of all the above reports, it is conceivable that CV leaves possess a cytotoxic effect against several cancer cell lines.

Generally, the moderate effects of CVME against both *P. falciparum* and breast cancer cell line might due to the extraction process in which not all main component in leaves extract were extracted out. The heterogeneity of bioactive compounds presents in CVME as compared with isolated compound usually used in clinical settings might interfere and interact with each other, therefore might dampen the biological activities of the extract.

In this study, CVME subjected to GC-MS analysis revealed the presence of 26 compounds. Of the 26 compounds identified, the most prevailing compounds were tetrahydro-2-methyl- thiophene (61.77%), phytol (8.59%), 10-undecenoic acid (5.00%), 6-methylheptyl-2-propenoate (4.96%) and 2-(2-benzothiazolylthio)-1-(3,5-dimethylpyrazolyl)-ethanone (2.70%). The compounds in CVME that were reported to have an anticancer activity are phytol, 10-undecenoic acid and 2-propenoic acid, 6-methylheptyl ester. Phytol is a product of chlorophyll metabolism in plants [35]. This compound was reported to block the teratogenic effect of retinol [36] and exhibit antioxidant activity *in vitro* and antinociceptive *in vivo* [37]. 10-undecenoic acid and 2-propenoic acid, 6- methylheptyl ester were also suggested to exert antioxidant, anticarcinogenic and chemopreventive activities [38,39].

Thiophene, tetrahydro-2-methyl- was the major compound identified in CVME. No specific data have recorded the anticancer activity of any plant extracts that contain this compound. However, thiophene and its derivatives were reported to have an antimicrobial, antiinflammatory, antifungal, antioxidant and antitumor activities [40]. Thiophene also has antiallergic, antidepressant and antidiabetic activities [41]. Ethanone, 2-(2-benzothiazolylthio)-1-(3,5-dimethylpyrazolyl)- was also reported to have antimicrobial activity as suggested by Velayutham and Karthi [42]. These compounds, thiophene and ethanone could be responsible for the antimalarial activity of CVME against 3D7 parasites.

Conclusion

It implies that CVME has a moderate cytotoxic activity against the breast cancer cell line (MDA-MB-231) and possesses a moderate antimalarial activity against the 3D7 malaria parasite, suggesting the presence of bioactive compounds in *C. vespertilionis* leaves that could be a potential source of phytochemicals with high medicinal value to be used in cancer and malaria treatment. Further

investigations, such as bio-guided fractionation, need to be carried out first to isolate and then to elucidate the structure of the active compounds in CV.

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Dried powder of *C. vespertilionis* leaves

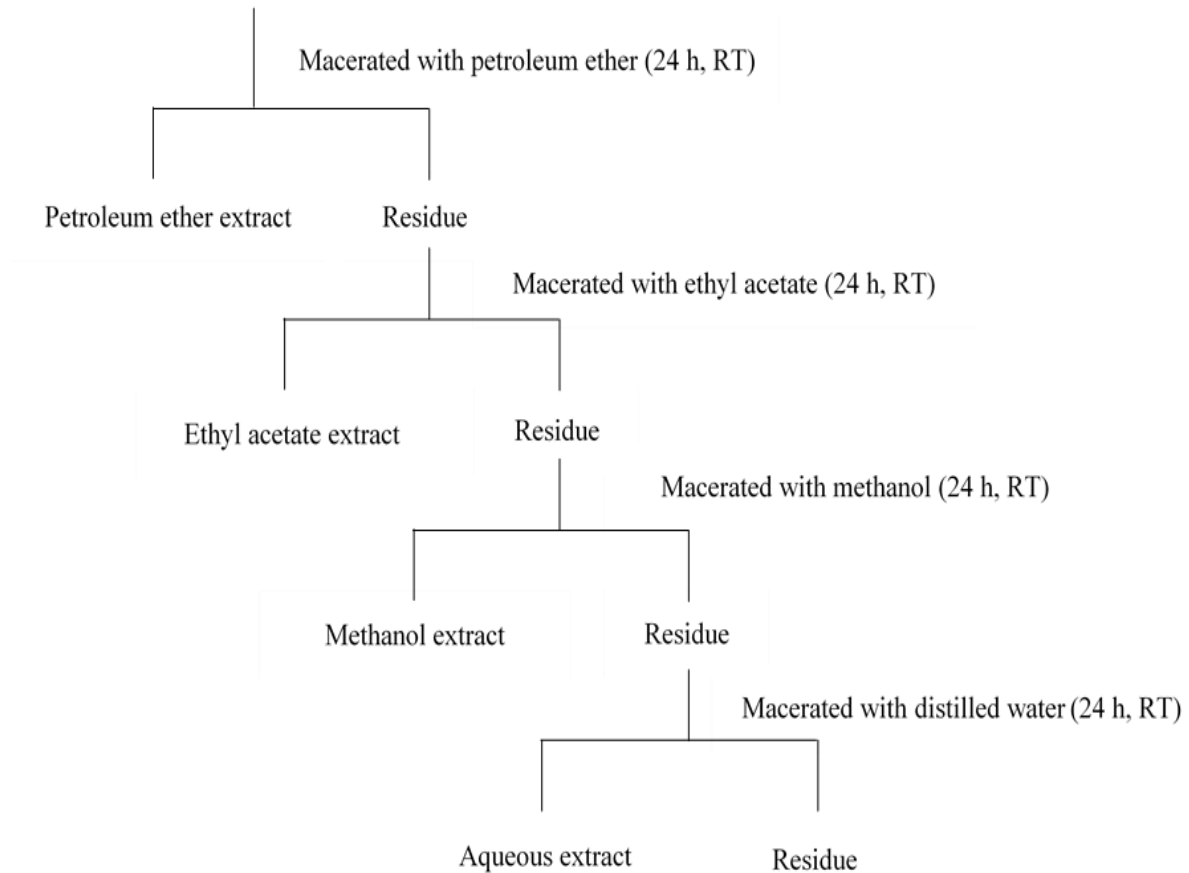


Figure 1. Successive extraction of *C. vespertilionis* leaves

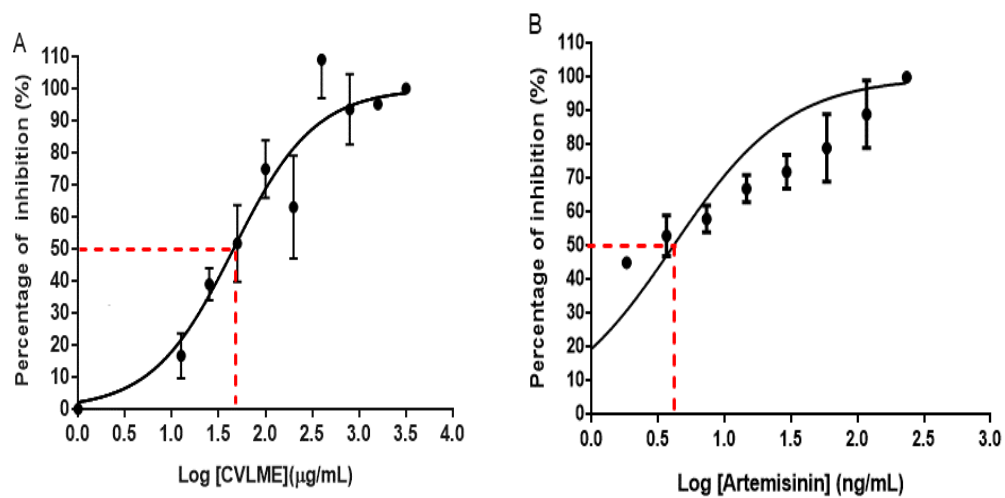


Figure 2. *In vitro* susceptibility of *P. falciparum* to (A) CVME and (B) artemisinin. Log extract or drug concentration-response curve was plotted to determine the IC₅₀ value ± SD. Data are from a representative of three experiments.

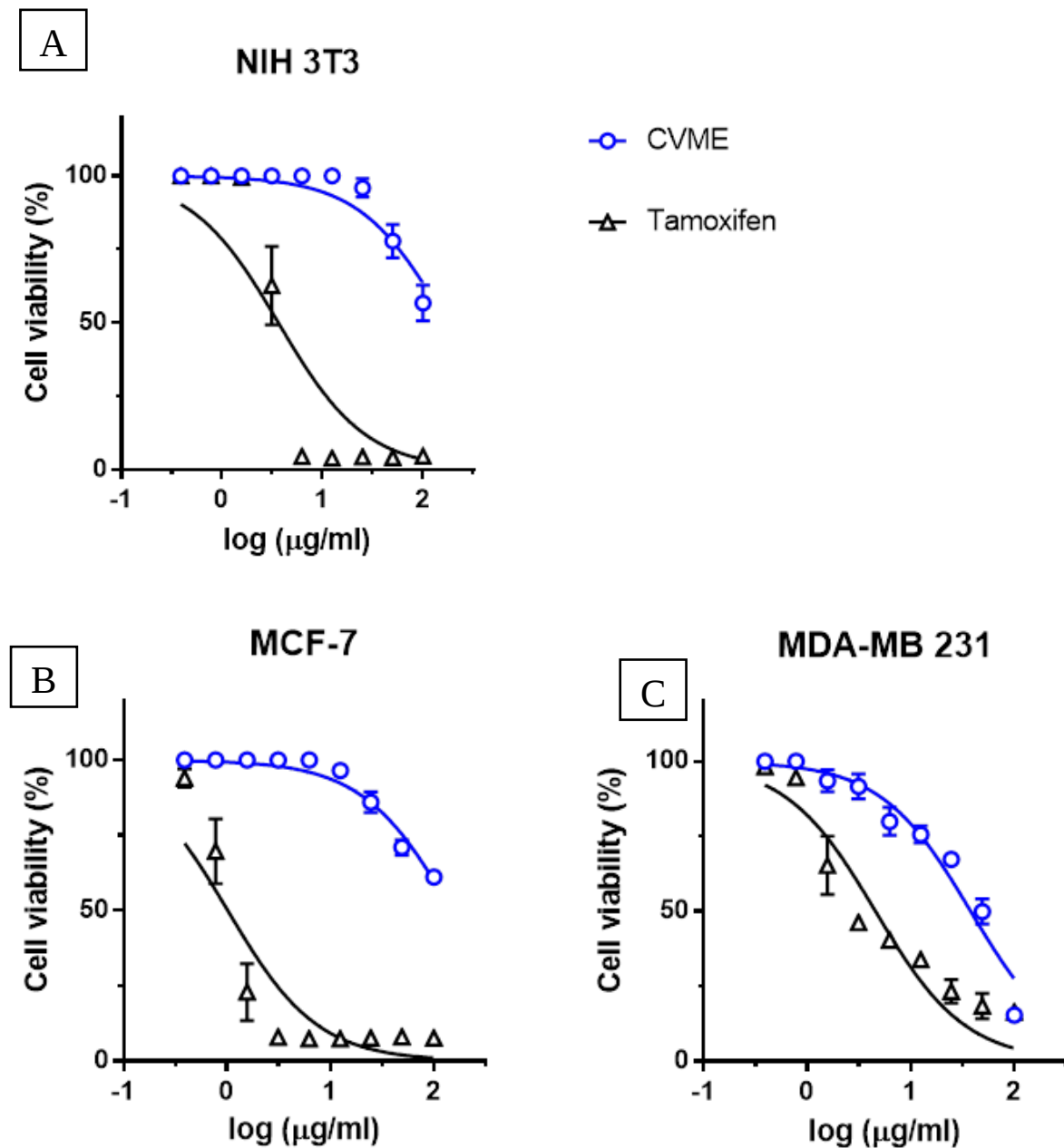


Figure 3. Cytotoxic activity of CVME and tamoxifen on (A) NIH/3T3, (B) MCF-7 and (C) MDA-MB-231. Each point shows the percentage of viable cells compared to the negative control, DMSO. Values are expressed as the mean $\pm$ SD from three independent experiments.

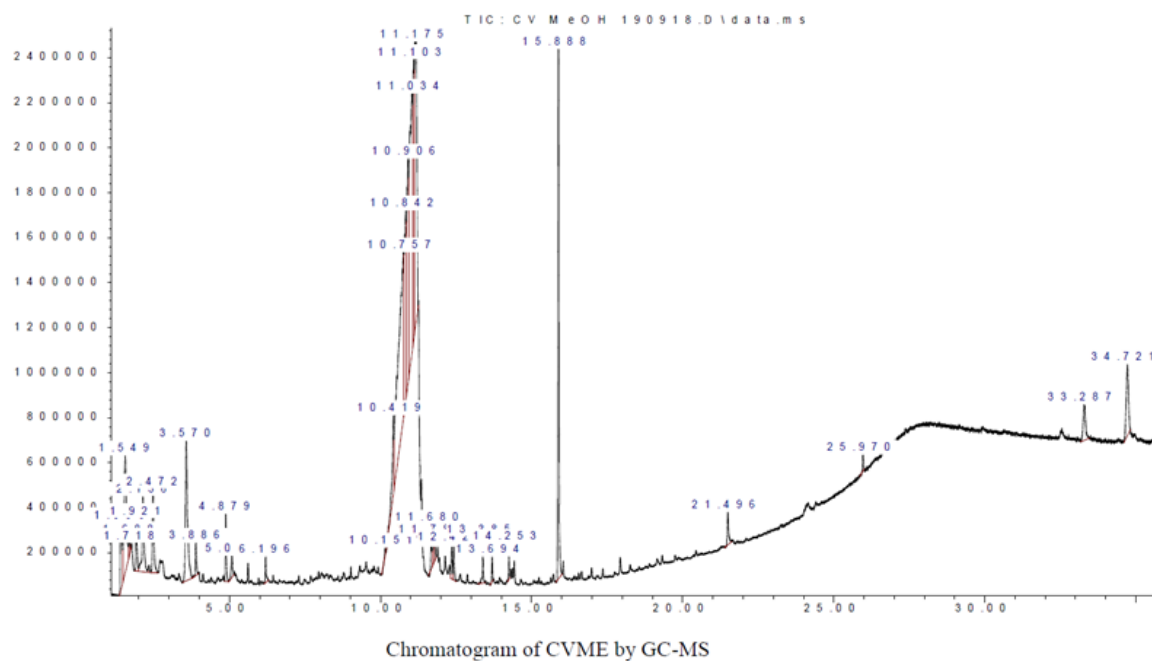


Figure 4. Chromatogram of CVME by GC-MS

Table

Table 1. GC-MS analysis of the five most abundant compounds in CVME.

Abundance	Compound	Nature of compound	% of the total
#1	Tetrahydro-2-methyl-thiophene	Heterocyclic	61.77
#2	Phytol	Diterpene	8.59
#3	10-Undecenoic acid	Fatty acid	5.00
#4	6-methylheptylester-2-propenoic acid,	Ester	4.96
#5	2-(2-benzothiazolylthio)-1-(3,5-dimethylpyrazolyl)-ethanone	Aromatic	2.70