

Chemical profiling and cytotoxicity screening of agarwood essential oil (*Aquilaria sinensis*) in brine shrimp nauplii and cancer cell lines

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Abstract

Agarwood essential oil (AEO) has gained attention from healthcare industries due to its numerous pharmacological properties. However, a comprehensive understanding of the chemical composition and its cytotoxic property is lacking. The objective of this study was to investigate the chemical profile as well as the cytotoxic concentration range of AEO derived from *Aquilaria sinensis* wood. Gas chromatography-mass spectrometry (GC-MS) was employed to identify the AEO components. Results showed that sesquiterpenes and sesquiterpenoids constitute 95.85% of the AEO. Among the major compounds identified are *allo*-aromadendrene (13.04%), dihydro-eudesmol (8.81%), α -eudesmol (8.48%), bulnesol (7.63%), τ -cadinol (4.95%), dehydrofukinone (3.83%), valerenol (3.54%), cis-nerolidol (2.75%), agarospirol (2.72%), dehydrojinkoh-eremol (2.53%), selina-3,11-dien-9-al (2.36%), guaialol (2.12%) and caryophyllene oxide (2.0%). The presence of volatile quality marker compounds such as 10-epi-Y-eudesmol, aromadendrane, β -agarofuran, α -agarofuran, γ -eudesmol, agarospirol and guaialol, indicates that the extracted AEO is of premium grade. Interestingly, the AEO displayed moderate to high toxicity in brine shrimp lethality test (BLST). All studied tumor cell lines (MDA-MB-231, HepG2, B16F10) exhibited varying degrees of sensitivity to AEO, which resulted in time and dose-dependent reduction of cell proliferation. Our findings showed that AEO contains bioactive components that may be exploited in future studies for the development of anti-cancer therapeutics.

Introduction

Cancer is a pathological condition caused by uncontrolled cell proliferation, which is a hallmark of oncogenesis and progression¹. According to the latest issue of the International Agency for Research on Cancer (IARC) in 2020, breast cancer in women has now become the most frequently diagnosed cancer, surpassing lung cancer². Approximately 2.3 million new cases, accounting for 11.7% of diagnoses, were reported, followed by lung (11.4%), colorectal (10.0%), prostate (7.3%) and stomach cancer (5.6%). Despite this shift, lung cancer remains the leading cause of cancer-related deaths, with an estimated 1.8 million fatalities (18%). Colorectal cancer stands at 9.4%, followed by liver (8.3%), stomach (7.7%) and breast cancer (6.9%)². Among men, lung cancer is the leading cause of mortality³. The primary cause of cancer-related death in women is breast cancer³. On the other hand, acute lymphoblastic leukemia is the top hematological cancer in children and adolescents, representing 20% of all cancers diagnosed in persons aged < 20 years⁴.

Depending on the type of cancer and how advanced it is, the standard treatments are surgery, chemotherapy and radiation. However, resistance to radiotherapy and chemotherapy may result in treatment failure or even cancer recurrence⁵. Thus, the development of new therapeutic agents to overcome these pressing issues is necessary. Chemotherapy is an important component of modern cancer therapy, which has evolved over centuries. While the historical use of plants in medicine dates back to the dawn of civilization, the World Health Organization (WHO) reports that people in Asia and some parts of Africa widely rely on herbal remedies for disease management⁶. At present, a significant

amount of modern pharmaceutical drugs, particularly those used in cancer therapy, are derived from natural ingredients. Phytochemicals like vincristine, camptothecin, and paclitaxel have emerged as important as well as reliable chemotherapeutic agents in cancer treatment⁷. This long-standing reliance on natural sources highlights the ongoing pursuit of efficacious therapeutic agents sourced from plants.

Agarwood, also known as eaglewood, oud, aloeswood, and gaharu is a highly valuable fragrant heartwood used for a wide range of non-timber purposes in perfume, cosmetics industry, medicinal preparations as well as for religious ritual purposes⁸. The genus *Aquilaria* or simply known as agarwood from the family Thymelaeaceae, has different species including *crassna*, *malaccensis*, and *sinensis*. Agarwood is well known for its resinous secretion against injuries and this tree is commonly found in Southeast Asian countries and Southern China. Agarwood is rich in bioactive compounds, including sesquiterpenes, chromones, flavonoids, benzophenones, diterpenoids, triterpenoids and lignans, which show myriad pharmacological effects^{9,10,11}. A number of studies reported that these chemicals extracted from *Aquilaria* wood exhibit acetylcholinesterase inhibition, anti-fungal, anti-bacterial, anti-inflammatory, anti-cancer and analgesic actions^{12,13,14}.

Previous study indicates that plant-derived essential oil possesses anti-tumor potential against leukemia as well as cancers of the mouth, breast, lung, prostate, liver, and colon¹⁵. Study revealed that specific components of essential oils might increase the cytotoxic action of chemotherapy drugs, such as docetaxel, paclitaxel, and 5-fluorouracil, on different cancer cell lines. This suggests that the dosage of these drugs may be reduced while still having the same effect¹⁶. Till date, the cytotoxic and chemical profile of AEO derived from *A. sinensis* is understudied, which hampered its feasibility to be used for potential medication or therapeutic agents. Hence, the present study was conducted to characterize the chemical constituents of AEO via GC-MS and to evaluate its cytotoxic profile using brine shrimp (*Artemia salina*) and cancer cell lines (MDA-MB-231, B16F10, HepG2).

Materials and methods

Plants material

The agarwood from *A. sinensis* was harvested from a cultivated agarwood plantation in Tangkak, Johor (Fig. 1a) with permission from DAdvance Agarwood Solutions Sdn. Bhd. The agarwood plantation farm is registered under Malaysian Timber Industry Board (MTIB) and it is located at a longitude of 2.2951° N and latitude of 102.5756° E. Taxonomic identification was performed by Forest Research Institute of Malaysia (FRIM). All plant experiments were conducted in accordance with relevant institutional, national, and international guidelines and legislation.

Chemicals and reagents

Dulbecco's Modified Eagle's medium (DMEM), trypsin, phosphate buffer saline (PBS) and fetal bovine serum (FBS) were purchased from Gibco, UK. Standard drug doxorubicin (Dox) and 3-(4,5-

dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma–Aldrich, U.S.A.

Extraction of agarwood essential oil

The agarwood was separated from the white wood, crushed into smaller pieces, and air-dried under the sun for 1–2 days until completely dry. Subsequently, the dried agarwood dust was ground into powder (Fig. 1b) and stored in a bag at room temperature for further extraction. Before the extraction process, 20 kg of agarwood powder underwent a 14-day soaking period in filtered water to facilitate the release of AEO. The soaked powder and 200 L of filtered water were then introduced into a 250 L stainless steel tank of a novel designed distiller (Lian Sin Enterprise, Selangor, Malaysia) (Fig. 1c) at a sample:water ratio of 1:10. The mixture was heated to a boil at 100°C, generating steam containing both water and AEO. The steam was condensed and collected in an essential oil separation funnel. The excess condensed water was recycled back to the distillation tank during the extraction process. After 120 h of hydro-distillation, the water will be removed, followed by the retrieval of the floating AEO through the bottom of the separation funnel, as illustrated in Fig. 1d. The AEO was obtained as a pale yellow liquid (Fig. 1e).

Chemical profiling by GC-MS

The volatile constituents of the sample were analyzed using a gas chromatography system, the Agilent 7890B (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with an Agilent 5977A quadrupole mass spectrometer and Agilent DB-1ms capillary column (30 m × 0.25 mm × 0.25 µm film thickness) based on the method adopted from Eissa et al.¹⁷ with modifications. GC was performed using the following conditions: autosampler injection (1 µL); injection temperature (230°C), carrier gas (helium) flow rate (1 mL/min); programmed oven temperature, 70°C held for 3 min, ramped from 70 to 230°C at a rate of 3°C/min, held for 3 min. The detector temperature was 240°C. The detected peaks from the total ion chromatography (TIC) and mass chromatograms were identified based on the National Institute of Standards and Technology (NIST) 2020 mass spectral library.

Brine shrimp lethality test (BSLT)

The brine shrimp lethality test was performed according to the protocol of Asaduzzaman et al¹⁸. The method estimates *in vivo* lethality in a simple zoological organism, using nauplii of the brine shrimp *Artemia salina*. Artificial seawater (ASW) was prepared by mixing 7.5 g of a commercially available salt mixture with 500 mL of distilled water according to the instructions. *A. salina* eggs were added to a small commercial nauplius hatching tank and incubated in an ASW under a halogen lamp providing direct light and heat. The hatched shrimp was allowed to mature for 24 h as nauplii. Hatched shrimp were attracted to light, and brine shrimp eggless nauplii were collected from illuminated sections of the aquarium. Ten nauplii were counted visually in the stem of a graduated Pasteur pipette against an illuminated background and transferred to a test tube. A stock solution (50 mg/mL) is prepared by dissolving the AEO in saline containing 15% propylene glycol and 1% Tween 80. This can be used to serially dilute it to produce a range of concentrations 31.25, 62.5, 125, 250, 500, 1000 µg/mL. Solvent alone was used as

negative control. Testing was performed with three replicates for each dosage and 10 nauplii per replicate. Each test tube was illuminated for 24, 48 and 72 h. Survivors were counted visually by two independent enumerators. The LC₅₀ values were determined with probit analysis using the Statistical Package for the Social Sciences (SPSS) application.

Cell culture

Human breast adenocarcinoma (MDA-MB-231), hepatocellular (HepG2) and murine melanoma (B16F10) cell lines were purchased from American Type Culture Collection (ATCC). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin and incubated at 37°C, 5% CO₂ as described previously¹⁹.

Cell viability assay

MTT assay was performed to measure cell viability, according to previous protocol²⁰. The cells were seeded in 100 µL of complete culture media, at a density of 5×10^4 per mL in 96 well plates. The seeded cells were incubated for 12 h at 37°C and 5% CO₂. The cells were then treated with AEO at different concentrations including 0.975, 1.95, 3.91, 7.81, 15.625, 31.25, 62.5, 125, 250, 500 µg/mL. A total of 20 µL of MTT solution was added to each well after the cells were treated with AEO for the indicated time point. After 3 h of incubation, the media was discarded and 100 µL of DMSO was added to each well. The absorption of produced formazan was measured at 570 nm using a BioTek EPOCH 2 microplate reader. The cell viability was calculated as following:

Cell viability (%) = (OD Sample / OD Control) × 100

Statistical analysis

All the experiments were conducted on three replicates and data was expressed as mean ± standard deviation. The statistical analysis was performed using IBM SPSS Statistical Software version 25 (IBM Corp., Armonk, N.Y., USA). One-way analysis of variance (ANOVA) was performed to determine the significant differences between means at a 95% confidence interval ($p < 0.05$).

Results and discussion

Yield, smells, and appearance of AEO

The agarwood used in this study originated from *A. sinensis* cultivated in Tangkak, Johor (Fig. 1a). By adopting the ITS2, matK, and rbcL barcode sequences, the agarwood sample was identified as *A. sinensis*. The AEO was extracted from agarwood (*A. sinensis*) powder using hydro-distillation (HD) (Fig. 1c). HD is the most conventional method employed by industries for the extraction of essential oil from agarwood using water, making it a relatively safe and environmentally-friendly process^{21,22}. The AEO obtained had a clear, yellowish brown colour with a strong woody scent (Fig. 1e).

The average yield of AEO through HD for 120 h at 100°C was reported as 1 g/kg of dried agarwood powder, equivalent to 0.1% (v/w). The yield of AEO in this experiment was similar to that of resinous heartwood and agarwood leaves reported in the previous literature. Sulaiman et al.²³ reported that the maximum extraction yield of AEO from *A. malaccensis* was 0.18% using the HD method for 72 h at 100°C. Other research done by Wu et al.²⁴ investigated the extraction of leaf aromatic oils from *A. sinensis* through HD, with a reported yield of 0.1% at 100°C for 5 h. Despite its practicability and safety, HD remains an time-consuming and low efficiency extraction method. In this work, soaking agarwood powder as a pre-treatment was performed to enhance the swelling of the cell wall of agarwood, thereby releasing more AEO²⁵.

Several new extraction methods, including solvent extraction (SE), enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and supercritical fluid extraction (SFE) have been explored for extracting AEO^{26,27,28}. In Radzi et al.²⁹ study, agarwood that underwent microwave pre-treatment demonstrated a threefold increase in yield compared to conventional HD. On the other hand, SFE exhibited a significant increase in AEO yield, rising from 0.061% (obtained by HD) to 0.317%³⁰. Nevertheless, these extraction methods are still not widely utilized in the agarwood industry due to the safety concerns (SE), high costs (EAE, SFE), and upscaling issues (MAE, UAE)²².

Chemical composition analysis

Profiling of chemical constituents in plant species is an important step for the investigation of their medical effects. The chemical constituents identified from the medicinal plants are the potential sources for drug development. As AEO is valued for its distinctive and pleasant fragrance, GC-MS is extensively employed to identify its compounds and assess its quality³¹. In this study, the AEO was eluted and analyzed by GC-MS using DB-1ms capillary column. The identification of the individual components of the AEO was based on the matching of their mass spectra with the NIST 2020 library. Figure 2 displays the GC-MS spectra of AEO, revealing the presence of 59 chemical compounds. The compounds were numbered in accordance with corresponding peaks. Visually, small peaks could be observed in large quantities, corresponding to the compounds that could not be determined (Fig. 2). Chemical compounds showed up before retention time (RT) of 25.3 min were mainly sesquiterpenes and sesquiterpenoids. Most of the sesquiterpenoids and other compounds were detected between 25.3 and 40.0 min.

The chemical compounds found in AEO are listed in Table 1 according to their sequence of elution on a DB-1ms capillary column. A summary is provided in Table 1 for the distribution of chemical classes, retention time, % contents of the constituent components, as well as CAS number. Overall, the total percentage of sesquiterpenoids was the highest at 77.36%, followed by sesquiterpenes (18.49%) and other compounds (1.41%). This finding is in good agreement with previous studies which found sesquiterpenoids as the major component in AEO^{32,33,34}. However, the amount of sesquiterpenoids varied depending on the agarwood species, origin, and extraction methods. Other studies found varying amounts of sesquiterpenoids, ranging from 63.87% (India, *A. malaccensis*) to 67.40% (Malaysia, *A.*

malaccensis) and 49.91% (Thailand, *A. crassna*)³⁵. The findings in Tian et al.³⁶ showed that sesquiterpenoids were present in AEO (*A. sinensis*) at 68.68% through steam distillation, whereas the supercritical extraction method yielded sesquiterpenoids at 23.78%

The most abundant compound identified in AEO was allo-aromadendrene (13.04%), followed by dihydro-eudesmol (8.81%), α -eudesmol (8.48%), bulnesol (7.63%), τ -cadinol (4.95%), dehydrofukinone (3.83%), valerenol (3.54%), 10-*epi*- γ -eudesmol (3.07%), cis-nerolidol (2.75%), agarospirol (2.72%), dehydrojinkoh-eremol (2.53%), selina-3,11-dien-9-al (2.36%), guaialol (2.12%), caryophyllene oxide (2.00%), cyperotundone (1.87%), *nor*-Ketoagarofuran (1.75%), selina-3,11-dien-9-ol (1.66%), γ -eudesmol (1.42%), hinesol (1.41%), selina-3,11-dien-9-one (1.24%), jinkoh-eremol (1.21%), β -eudesmol (1.14%), kusunol (1.07%), and *epi*- α -Bisabolol (1.06%), all of which except allo-aromadendrene (sesquiterpenes) belong to sesquiterpenoids according to their structural characteristics (Table 1). While the sesquiterpenes and other compounds were present as minor components, accounting for less than 1.00% individually (Table 1). Major compounds detected in this study were consistent with those found in previously published works, wherein the AEO was extracted from *Aquilaria* spp. using HD^{13,31,37}. Further, most of these compounds are responsible for the superior quality of agarwood and exhibit various pharmacological activities³⁷. Other common compounds that were detected belong to aromatic compounds. According to literature, allo-aromadendrene contributes to the characteristic woody odor of AEO, while agarospirol gives out the smell of spicy, peppery, and woody^{38,39,40}.

Different studies have highlighted different chemical constituents as the marker compounds for classifying the AEO. Ismail et al.⁴¹ concluded 10-*epi*- γ -eudesmol, aromadendrane, β -agarofuran, α -agarofuran and γ -eudesmol as the marker compounds in high quality agarwood oils derived from *A. malaccensis*. The presence of four characteristic compounds (benzylacetone, agarospirol, aristolene, and guaialol) has been used as quality indicators for AEO (*A. sinensis*) in a study conducted by Tian et al.³⁷. The majority of these compounds, except benzylacetone and aristolene, were present in substantial quantities in the AEO, further confirming the studied sample as a high value fragrant material.

In addition, 2-(2-phenylethyl)chromone is the major aromatic constituent in agarwood, which has been extensively reported^{31,42}. However, no chromone compounds, as well as their derivatives, were detected in the present study. They are rarely identified by GC-MS but are more commonly detected via LC-MS/MS^{31,42}.

Table 1
Chemical compounds identified in AEO obtained from *A. sinensis*. (NA - Not available)

No	Compounds	Molecular formula	Retention time (min)	Peak area (%)	Phytochemical groups	CAS no
1	4-Phenyl-2-butanone	C ₁₀ H ₁₂ O	12.841	0.53	Other compounds	2550-26-7
2	α-Gurjunene	C ₁₅ H ₂₄	20.132	0.10	Sesquiterpene	489-40-7
3	Aromadendrene	C ₁₅ H ₂₄	21.667	0.10	Sesquiterpene	489-39-4
4	<i>allo</i> -Aromadendrene	C ₁₅ H ₂₄	22.585	13.04	Sesquiterpene	25246-27-9
5	α-Curcumene	C ₁₅ H ₂₂	22.79	0.44	Sesquiterpene	644-30-4
6	γ-Selinene	C ₁₅ H ₂₄	23.032	0.28	Sesquiterpene	515-17-3
7	β-Agarofuran	C ₁₅ H ₂₄ O	23.146	0.35	Sesquiterpenoid	NA
8	β-Vetispiene	C ₁₅ H ₂₂	23.259	0.52	Sesquiterpene	28908-27-2
9	Valencene	C ₁₅ H ₂₄	23.436	0.56	Sesquiterpene	4630-07-3
10	α-Selinene	C ₁₅ H ₂₄	23.574	0.55	Sesquiterpene	473-13-2
11	Dihydro-β-agarofuran	C ₁₅ H ₂₆ O	23.642	0.46	Sesquiterpenoid	5956-09-2
12	δ-Guaiene	C ₁₅ H ₂₄	23.874	0.54	Sesquiterpene	3691-11-0
13	γ-Cadinene	C ₁₅ H ₂₄	24.08	0.26	Sesquiterpene	39029-41-9
14	α-Elemene	C ₁₅ H ₂₄	24.35	0.28	Sesquiterpene	5951-67-7
15	δ-Cadinene	C ₁₅ H ₂₄	24.461	0.12	Sesquiterpene	483-76-1
16	Kessane	C ₁₅ H ₂₆ O	24.598	0.17	Sesquiterpenoid	3321-66-2
17	Selina-3.7(11)-diene	C ₁₅ H ₂₄	24.925	0.34	Sesquiterpene	6813-21-4
18	α-Calacorene	C ₁₅ H ₂₀	25.051	0.30	Sesquiterpene	21391-99-1

No	Compounds	Molecular formula	Retention time (min)	Peak area (%)	Phytochemical groups	CAS no
19	Dehydro-aromadendrene	C ₁₅ H ₂₂	25.151	0.29	Sesquiterpene	NA
20	α-Agarofuran	C ₁₅ H ₂₄ O	25.339	0.60	Sesquiterpenoid	5956-12-7
21	<i>cis</i> -Nerolidol	C ₁₅ H ₂₆ O	25.779	2.75	Sesquiterpenoid	142-50-7
22	<i>nor</i> -Ketoagarofuran	C ₁₄ H ₂₂ O ₂	26.264	1.75	Sesquiterpenoid	5986-25-4
23	β-Vetivenene	C ₁₅ H ₂₂	26.668	0.77	Sesquiterpene	27840-40-0
24	Caryophyllene oxide	C ₁₅ H ₂₄ O	27.115	2.00	Sesquiterpenoid	1139-30-6
25	Tetradecanal	C ₁₄ H ₂₈ O	27.521	0.54	Other compounds	124-25-4
26	Guaiol	C ₁₅ H ₂₆ O	27.697	2.12	Sesquiterpenoid	489-86-1
27	10- <i>epi</i> -γ-Eudesmol	C ₁₅ H ₂₆ O	27.823	3.07	Sesquiterpenoid	15051-81-7
28	γ-Eudesmol	C ₁₅ H ₂₆ O	28.208	1.42	Sesquiterpenoid	1209-71-8
29	Agarospinol	C ₁₅ H ₂₆ O	28.379	2.72	Sesquiterpenoid	1460-73-7
30	Hinesol	C ₁₅ H ₂₆ O	28.475	1.41	Sesquiterpenoid	23811-08-7
31	τ-Cadinol	C ₁₅ H ₂₆ O	28.676	4.95	Sesquiterpenoid	5937-11-1
32	β-Eudesmol	C ₁₅ H ₂₆ O	28.801	1.14	Sesquiterpenoid	473-15-4
33	α-Eudesmol	C ₁₅ H ₂₆ O	28.971	8.48	Sesquiterpenoid	473-16-5
34	Jinkoh-eremol	C ₁₅ H ₂₆ O	29.174	1.21	Sesquiterpenoid	94201-17-9
35	Dihydro-eudesmol	C ₁₅ H ₂₈ O	29.58	8.81	Sesquiterpenoid	6770-16-7
36	Kusunol	C ₁₅ H ₂₆ O	29.716	1.07	Sesquiterpenoid	20489-45-6

No	Compounds	Molecular formula	Retention time (min)	Peak area (%)	Phytochemical groups	CAS no
37	Bulnesol	C ₁₅ H ₂₆ O	30.044	7.63	Sesquiterpenoid	22451-73-6
38	Dehydrojinkoh- eremol	C ₁₅ H ₂₄ O	30.297	2.53	Sesquiterpenoid	NA
39	Cyperotundone	C ₁₅ H ₂₂ O	30.503	1.87	Sesquiterpenoid	3466-15-7
40	<i>epi</i> - α -Bisabolol	C ₁₅ H ₂₆ O	30.808	1.06	Sesquiterpenoid	78148-59-1
41	α -Bisabolol	C ₁₅ H ₂₆ O	30.942	0.50	Sesquiterpenoid	515-69-5
42	Selina-3,11-dien-9-one	C ₁₅ H ₂₂ O	31.273	1.24	Sesquiterpenoid	NA
43	Rotundone	C ₁₅ H ₂₂ O	31.419	0.88	Sesquiterpenoid	18374-76-0
44	Valerenol	C ₁₅ H ₂₄ O	31.63	3.54	Sesquiterpenoid	101628-22-2
45	Selina-3,11-dien-9-ol	C ₁₅ H ₂₄ O	31.87	1.66	Sesquiterpenoid	NA
46	Selina-4,11-dien-14-oic acid	C ₁₅ H ₂₂ O ₂	32.082	0.51	Sesquiterpenoid	NA
47	α -Costol	C ₁₅ H ₂₂ O	32.259	0.73	Sesquiterpenoid	65018-15-7
48	Selina-3,11-dien-9-al	C ₁₅ H ₂₂ O	32.603	2.36	Sesquiterpenoid	NA
49	9,11-Eremophiladien-8-one	C ₁₅ H ₂₂ O	32.857	0.72	Sesquiterpenoid	NA
50	Aristolone	C ₁₅ H ₂₂ O	33.023	0.70	Sesquiterpenoid	6831-17-0
51	Guaia-1(10),11-dien-9-one	C ₁₅ H ₂₂ O	33.113	0.21	Sesquiterpenoid	NA
52	Dehydrofukinone	C ₁₅ H ₂₂ O	33.693	3.83	Sesquiterpenoid	19598-45-9
53	Sinenofuranol	C ₁₄ H ₂₄ O ₂	34.196	0.86	Sesquiterpenoid	NA
54	Dihydrokaranone	C ₁₅ H ₂₂ O	34.481	0.24	Sesquiterpenoid	NA
55	Guaia-1(10),11-dien-15-oic acid	C ₁₅ H ₂₂ O ₂	35.278	0.46	Sesquiterpenoid	NA

No	Compounds	Molecular formula	Retention time (min)	Peak area (%)	Phytochemical groups	CAS no
56	oxo-Agarospirol	C ₁₅ H ₂₄ O ₂	35.995	0.83	Sesquiterpenoid	NA
57	Methyl palmitate	C ₁₇ H ₃₄ O ₂	38.016	0.18	Other compounds	112-39-0
58	Dihydrocolumellarin	C ₁₅ H ₂₂ O ₂	38.262	0.52	Sesquiterpenoid	66873-38-9
59	1,5-Diphenyl-3-pentanone	C ₁₇ H ₁₈ O	40.03	0.16	Other compounds	5396-91-8

Brine shrimp lethality test (BSLT)

The BSLT method has been widely used by researchers to assess the cytotoxic concentration range and toxicity of essential oils. The LC₅₀ values obtained from brine shrimp lethality bioassay for AEO were 117.16 ± 8.21 µg/mL, 65.02 ± 2.70 µg/mL and 51.70 ± 3.32 µg/mL, for 24, 48 and 72 h, respectively (Table 2). The LC₅₀ values were assessed at 95% confidence using probit analysis. The valorized essential oils should be considered toxic or medium to highly toxic by comparison to Meyer's and Clarkson's toxicity index as summarized in Table 2. The solvent group showed little toxicity towards brine shrimp with a survival rate of more than 90%. In contrast, AEO exhibited > 90% mortality in the concentration range of 250–1000 µg/mL (Fig. 3).

Table 2
LC₅₀ value of AEO on brine shrimp lethality test (BSLT).

Time-point	LC ₅₀ (µg/mL)	95% confidence interval	Toxicity class (Meyer/Clarkson)
24 h	117.16 ± 8.21	101.15–121.54	Toxic/Medium Toxic
48 h	65.02 ± 2.70	53.77–87.42	Toxic/ Highly Toxic
72 h	51.70 ± 3.32	45.60–66.49	Toxic/ Highly Toxic

Elumba et al.⁴³, Del Socorro et al.⁴⁴ suggested that some of the tested extracts with LC₅₀ below 100 µg/mL which are categorized as toxic, does not always indicate its danger or out-right toxicity toward humans, but may also suggest a potential anti-tumor or anti-cancer activity⁴⁵. To the best of our knowledge, this is the first report on the toxicity effect of AEO in brine shrimp. We hypothesized that the lethality of brine shrimp larvae may be linked to the presence of bioactive compounds in the AEO, which can interfere with biochemical and physiological processes⁴⁶. The toxic compounds can enter the digestive tracts of brine shrimp larvae, where the substances absorbed have the ability to interact with targets, such as lipids, enzymes, cell membranes, and nucleic acids, affecting the body's processes and ultimately resulting in death^{47,48}.

Cell line viability test (MTT)

Table 3 and Fig. 4a-c show the growth inhibitory values determined from the dose-response curves of AEO and anti-cancer drugs used in cell lines MDA-MB-231, B16F10 and HepG2. The strongest anti-proliferative activity of AEO was observed against B16F10 cell line (IC_{50} 48.9 ± 3.1 $\mu\text{g/mL}$), followed by HepG2 cells (IC_{50} 56.2 ± 3.9 $\mu\text{g/mL}$) and MDA-MB-231 cell line (IC_{50} 61.3 ± 3.2 $\mu\text{g/mL}$) after 24 h treatment. As shown in Fig. 5, morphological analysis revealed that AEO-induced cell rounding, loss of cell-cell contact and cell detachment, an appearance normally seen in cells undergoing apoptosis. Nevertheless, AEO treatment at 48 and 72 h also showed growth inhibitory potential on all tested cancer cell-lines. These results suggest that AEO induced a time and dose-dependent inhibition of cell proliferation, although each cell-line showed different sensitivity, in accordance with the determined IC_{50} . In addition, the anti-proliferative effect of the referent chemo-drug, doxorubicin is stated in Table 3.

At present, there is no study on AEO extracted from agarwood used against melanoma and hepatic cancer. Previous study reported that *A. crassna* essential oil mixture showed significant anti-proliferation activity against human cancer cell lines such as colorectal HCT 116 (IC_{50} 28 $\mu\text{g/mL}$), pancreatic PANC-1 (IC_{50} 32 $\mu\text{g/mL}$), prostate PC3 (IC_{50} 79 $\mu\text{g/mL}$) and breast MCF-7 (IC_{50} 110 $\mu\text{g/mL}$)⁴⁹. The same study proved that the growth inhibitory effect of AEO is attributed to the presence of sesquiterpene such as β -caryophyllene. This compound has been shown to inhibit colorectal cancer cell proliferation, clonality, migration, invasion, and spheroid formation⁴⁹. Further investigation revealed that the cell death mechanism induced by β -caryophyllene is related to the apoptotic properties such as DNA fragmentation and mitochondrial pathways. Although quantitative analysis using GC-MS showed β -caryophyllene is one of the major compounds (8.1%) in *A. crassna* essential oil, this compound is not detected in *A. sinensis* AEO used in the present study, indicating the uniqueness of AEO chemical composition derived from different agarwood species. Of note, our GC-MS analysis detected the presence of caryophyllene oxide, a sesquiterpene that results from the oxidation of β -caryophyllene.

The same group of researchers also reported that *A. crassna* essential oil exhibited potent cytotoxic activity against human pancreatic cancer MIA PaCa-2 cells with an IC_{50} (11 ± 2.18 $\mu\text{g/mL}$)⁵⁰. They showed that cell migration and the colony formation ability of MIA PaCa-2 was effectively inhibited at 5 and 10 $\mu\text{g/mL}$, respectively. Furthermore, they discovered that AEO exhibited significant inhibition of HCT 116 colorectal cancer cell-induced subcutaneous tumors in nude mice⁵⁰. It is interesting to note that these studies reported previously were using AEO derived from *A. crassna*. Another scientific investigation found that AEO possesses anti-proliferative activity towards MCF-7 breast cancer cells with IC_{50} observed at 44 $\mu\text{g/mL}$ ⁵¹. However, the AEO used in this study was purchased directly from a Malaysian market, and it was unclear which type of agarwood they were using to produce AEO.

Besides sesquiterpenes, chromones, such as 2-(2-hydroxy-2-phenylethyl)chromone, demonstrated the ability to inhibit tumor development at non-cytotoxic concentrations, displaying half-inhibitory values ranging from 25 to 38 μM against different tumor cell lines⁵². Two novel chromone derivatives, namely 7-hydroxy-2-[2-(3'-methoxy-4'-hydroxyphenyl)-ethyl]chromone and 6,7-dimethoxy-2-[2-(3'-hydroxyphenyl)-

ethyl]chromone, isolated from agarwood ethanol extract, exhibited weak cytotoxic activities (IC₅₀: 18.82–37.95 mg/mL) against SMMC-7721, MGC-803, and OV-90 cell lines⁵³.

Table 3
IC₅₀ values of AEO and doxorubicin (Dox) on B16F10, MDA-MB-231 and HepG2 cell lines at different treatment time-points.

Cell line	AEO IC ₅₀ (µg/mL)			Dox IC ₅₀ (µg/mL)		
	24 h	48 h	72 h	24 h	48 h	72 h
B16F10	48.9 ± 3.1	38.7 ± 5.0	30.5 ± 2.6	< 10	< 10	< 5
MDA-MB-231	61.3 ± 3.2	54.4 ± 4.8	39.4 ± 3.7	< 10	< 10	< 5
HepG2	56.2 ± 3.9	41.6 ± 2.4	36.8 ± 1.1	< 10	< 10	< 5

Conclusion

The AEO was successfully extracted from agarwood (*A. sinensis*) powder using HD. The presence of key quality marker compounds suggest that the extracted AEO is of premium grade. This study demonstrated for the first time the cytotoxic effects of AEO on brine shrimps as well as melanoma (B16F10), hepatocarcinoma (HepG2) and breast adenocarcinoma (MDA-MB-231) cancer cell lines. We showed that the anti-proliferative activity of the AEO is time and dose-dependent. Furthermore, our results indicated that the cytotoxic or growth inhibitory effect may be attributable to the presence of sesquiterpene and sesquiterpenoid (constitutes 95.85%), which are the major components in AEO. Nevertheless, we also observed a positive correlation between BSLT and *in vitro* testing of cancer cell lines, which is consistent with previous reported studies on essential oil derived from other sources. Future studies should be directed to elucidate the cytotoxic agent in AEO that has the potential anti-cancer properties.

Declarations

Author Contributions

C.Y.L., W.F.W. and S.W.C designed the experiments. S.W.C., M.V.L, X.W., M.L.T. and K.M.L. performed the experiments. S.W.C. and X.W. performed the AEO extraction. M.V.L. and K.M.L. performed the cell culture. C.Y.L. and W.F.W performed data analysis. C.Y.L., W.F.W., S.W.C.and M.L.T. support the research items. C.Y.L., W.F.W., S.W.C. and M.L.T. wrote the paper. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data that support the findings of this study are available from the corresponding author, (C.Y.L), upon reasonable request.

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Figures

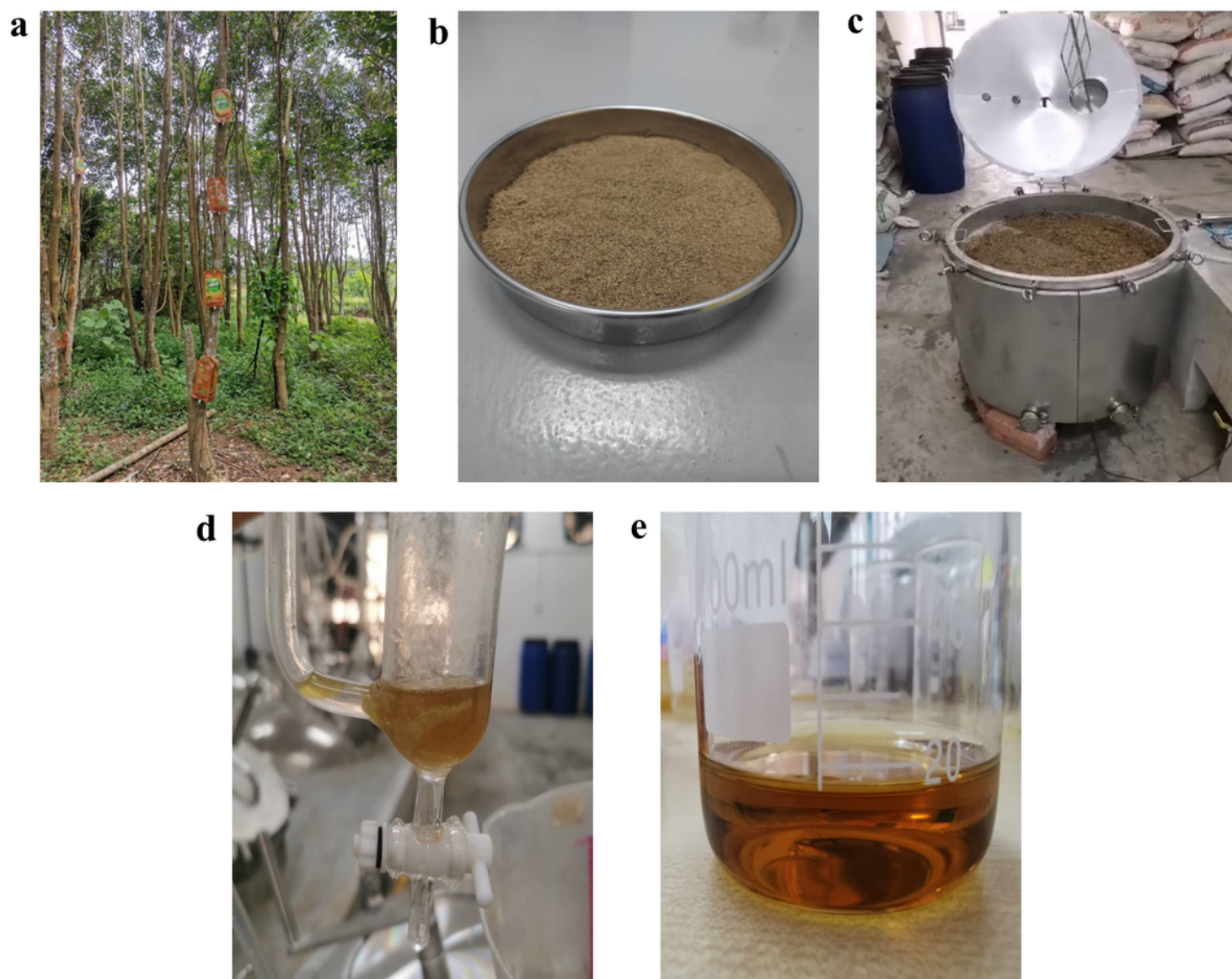


Figure 1

Illustrations of (a) Cultivated agarwood (*A. sinensis*) plantation. (b) Agarwood powder. (c) Agarwood powder in distillation tank. (d) AEO collected at the bottom of the separation funnel. (e) Final AEO.

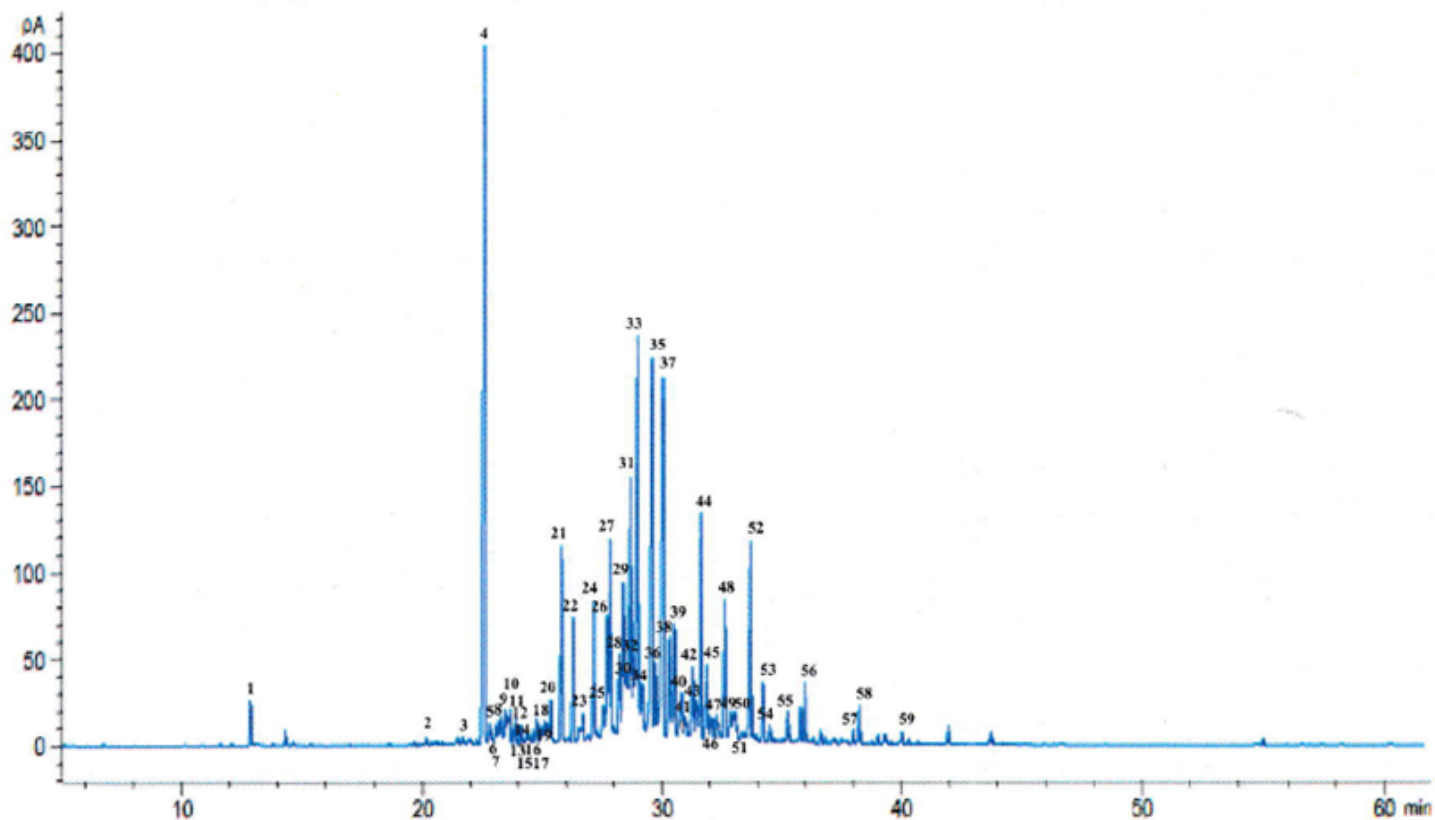


Figure 2

Chromatogram of the analysis by GC for AEO obtained from *A. sinensis*.

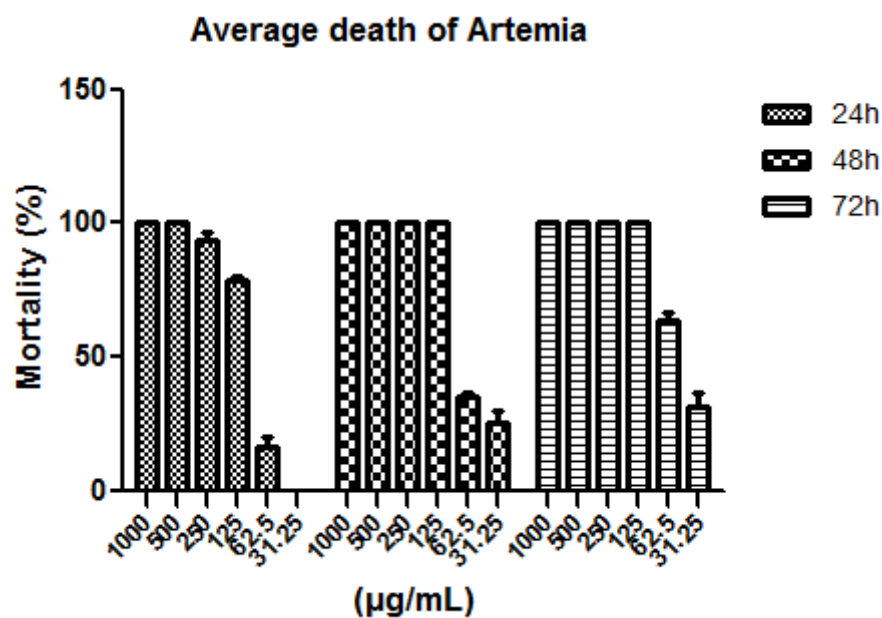


Figure 3

Evaluation of acute toxicity effect of AEO on brine shrimp at different treatment time-points.

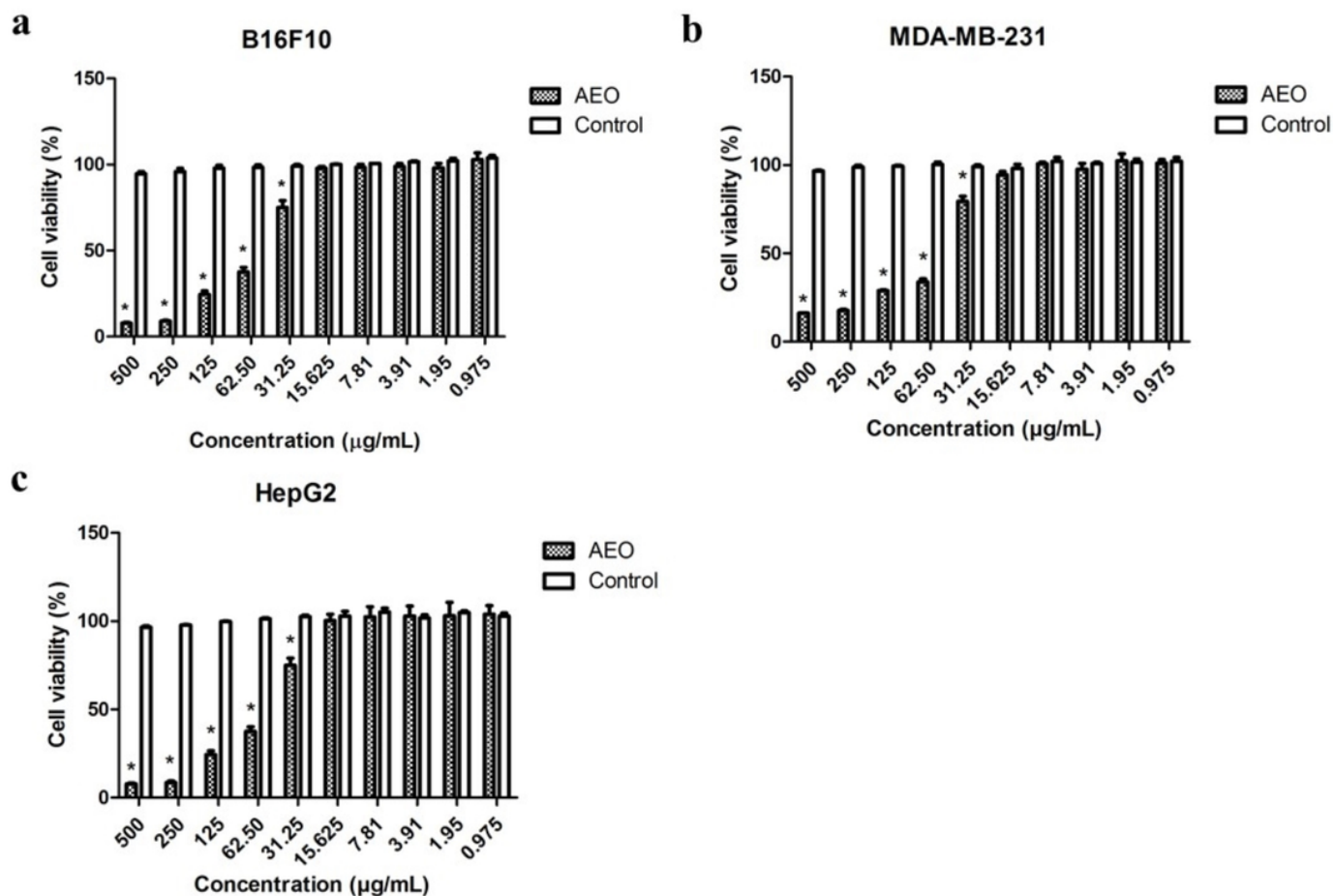


Figure 4

Evaluation of effect of AEO on cancer cell lines by MTT assay. Percentages of viable cells of (a) B16F10 (b) MDA-MB-231 (c) HepG2 at 24 h time-point after AEO treatments. Each experiment was repeated for a minimum of 3 times and plotted as bar graphs with error bars. One-way ANOVA was conducted and the p value was calculated between control and AEO treated samples, where, *: p value < 0.05.

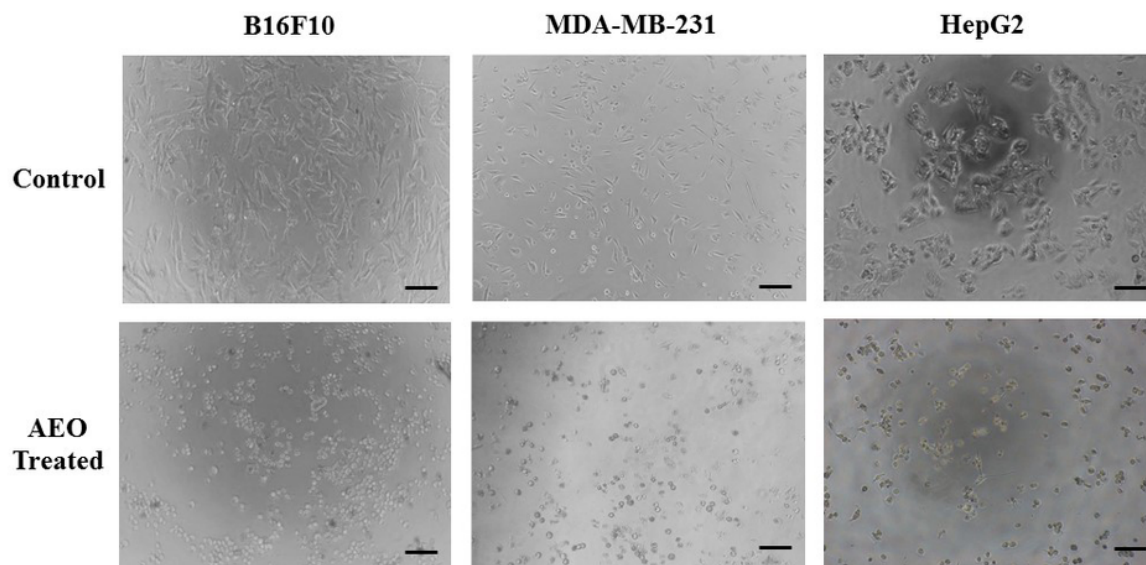


Figure 5

Morphology changes of B16F10, MDA-MB-231, HepG2 cells after AEO treatment (61.5 $\mu\text{g/mL}$) for 24 h. Treated cells were rounded up and cell to cell adhesion was lost. Images showing 40 x magnification. Black bar in each image represents 100 μm .