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Antioxidant and cytotoxicity to liver cancer HepG2 cells *in vitro* of Korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen) seed extracts

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ABSTRACT

This study examined the total phenolic contents, *in vitro* antioxidant activity and anti-cancer effect of petroleum ether and aqueous: methanol (20:80, v/v) seed extracts of *Aframomum corrorima* (Ethiopian cardamom). The aqueous methanol extract contained higher total phenolic content (25 ± 5 mg gallic acid equivalent/100 g of dried extract and total flavonoid content (19 ± 0.4 mg quercetin equivalent/100 g of dried extract). The extract showed stronger antioxidant activity than the petroleum ether extract, $EC_{50} = 97 \pm 4$ μ g/mL in DPPH radical scavenging assay, and $EC_{50} = 258 \pm 15$ μ g/mL in ferric reducing antioxidant power assay. In the antiproliferative assay, the petroleum ether extract showed stronger ($EC_{50} = 105 \pm 7$ μ g/mL) effect than the aqueous: methanol (20:80, v/v) extract ($EC_{50} = 282 \pm 43$ μ g/mL), on hepatocellular carcinoma (HepG2) cells when incubated for 24 h. This is the initial report to demonstrate antioxidant activity and anti-cancer *in vitro* effect of seed extracts of *A. corrorima*.

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Introduction

Aframomum corrorima, locally known as Korarima or Ethiopia cardamom, is a monocotyledonous flowering plant belonging to the family Zingiberaceae.^[1] It is widely cultivated in the south and southwestern part of Ethiopia. The spice obtained from the plant's seeds (usually dried), is extensively used in Ethiopian cuisine and as herbal medicine to flavor food and beverages. The seeds (usually dried, sometimes fresh) ground with other spices are used to flavor all kinds of traditional sauces in the country. They have been used in the preparation of spiced hot red pepper,^[2] paste of chili pepper^[3,4] and to flavor butter, coffee, tea, and bread.^[1] Different parts of *A. corrorima* plant (leaf, seed, and pod) have been used in traditional medicine by the people of southern Ethiopia. The leaf is used to treat animal body swelling and skin wounds.^[5] A decoction of the rhizomes has been used against parasitic nematodes in ruminant animals.^[6] The seed is effective against tonic convulsions, carminative, purgative, headache, stomachache, skin wounds, and sore throat when taken orally (Abegaz, 1994).^[7] Seed extract and steam distilled essential oil of theseed of this plant have shown antimicrobial activities.^[8]

The seeds of *A. corrorima* contain different types of essential oil components with a typical odor. According to Baser and Kürkcüoglu.^[9] and Hymete *et al.*^[10] the main constituents found in the seed oil were 1, 8-Cineole and sabinene, whereas sesquiterpenestructures such as (*E*)-nerolidol, β -caryophyllene and caryophyllene oxide, dominated the husk oil of the sample collected from the

local market. According to Eyob *et al.* [10] studies conducted on fresh materials, the major constituents of the oil were found to be γ -terpinene in pods and 1, 8-Cineole in seeds. Also Eyob *et al.* [11] reported the essential oil component of leaf and rhizome. The volatile oil of the leaf was dominated by β -caryophyllene and γ -terpinene and β -pinene was the major monoterpene in rhizome. Recently, Hailu *et al.* [12] reported the existence of noticeable variability with regards to composition and characteristics of the essential oil of fruit and seed samples of *A. corrorima* collected from different parts of Ethiopia.

Although *A. corrorima* is widely used as a food flavoring and traditional medicine, in Ethiopia, scarce information is available on total phenolic content and antioxidant activity. [11] To our knowledge, there is no report on anti-cancer effects of the seed extracts. Therefore, the objective of the present study was to estimate the total phenolic and flavonoid contents, *in vitro* antioxidant activity (using various assays) and cytotoxicity effect on hepatocellular carcinoma (HepG2) cell of petroleum ether and aqueous methanol (20:80), v/v extracts of the dried seed.

Materials and methods

Chemicals

Cell titer 96 aqueous and one solution cell proliferation (MTS) assay (Promega, Madison, WI, USA) kits and sterile dimethyl sulfoxide (DMSO) (ATCC, Rockville, USA) were purchased for the experiment. Gallic acid, butylated hydroxytoluene (BHT), Folin–Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, and L-ascorbic acid were purchased from Sigma-Aldrich. Sorafenib was purchased from Signal chem (Richmond, BC, Canada) and dissolved in DMSO (cell-culture grade) (ATCC). The other chemicals and solvents used in this experiment were of analytical grade.

Sample preparation and extraction

Fresh fruit of *A. corrorima* was collected from farm, 6 km North-East of Yirgalem Town, Sidama Regional State, Southern Ethiopia. The fruits were air-dried for 10 days and then the seeds were separated from the husk and ground to fine powder using an electric grinder (FM100 model, China). The petroleum ether and aqueous: methanol (20:80, v/v) extracts were prepared by dissolving 3 g of the seeds fine powder separately in 30 mL of each solvent. The mixture was then subjected to sonication (model 750D, VWR Intl. Ltd., Montreal, QC, Canada) for 15 min x 3 times, with 10 min intervals in between sonication cycles to keep the temperature below 30°C during the extraction. The crude extract was centrifuged (model Durafuge 300, Precision Scientific, Richmond, VA, USA) at 3000 g for 15 min. Then the supernatant was filtered using Whatman number 1 filter paper. The aqueous: methanol (20:80, v/v) and petroleum ether extracts were evaporated to dryness under N₂ and the remaining water was freeze-dried for 8 h in a freeze dryer (model 2085C0000, Kinetics Thermal Systems, Stone Ridge, NY, USA). Samples of each treatment were extracted and analyzed in triplicate and immediately stored in amber vials at –20°C until used for the analysis.

Determination of total phenolic contents (TPC)

Total phenolic content was estimated by Folin–Ciocalteu method as described in Jundi *et al.* [13] To 0.1 mL of the extract (1 mg/mL), 1 mL Folin–Ciocalteu reagent (diluted ten times) was added and the mixture was left for 5 min and then 1 mL (75 g/L) of sodium carbonate was added. The absorbance of the resulting blue color was measured at 765 nm with a double beam UV- visible spectrophotometer (JENWAY, 96500, UK) after incubation for 90 min at room temperature. The total phenolic content was estimated from gallic acid (1–100 μ g/mL) calibration curve ($y = 0.015x + 0.023$, $R^2 = 0.99$) and results were expressed as (mgGAE/100 g of dried extract).

Determination of total flavonoids (TFC)

The TFC was determined as described in Engeda *et al.* [14] with minor modifications. The analysis was based on the formation of the yellow color of flavonoid-aluminum complex. Aluminum chloride (2 mL, 2%) was mixed with the same volume of the seed extract (1 mg/mL). Then absorbance at 415 nm was taken after 1 h of incubation at room temperature against a blank sample. The TFC was determined using a standard curve of quercetin at (1–40 µg/mL) and values were calculated as (mgQE/100 g of dried extract) using the equation, $y = 0.024x + 0.112$, $R^2 = 0.99$.

Determination of antioxidant activity

DPPH Radical Scavenging Method

The DPPH radical scavenging activity of the extracts from *A. corrorima* seed was determined as described by Engeda *et al.* [15] Different concentrations (50 to 100 µg/mL) of the extracts were taken in different test tubes. Freshly prepared DPPH solution (2 mL, 0.006%, w/v) in methanol was added in each of the test tubes containing 1 mL of the extract. The reaction mixture and the reference standards (ascorbic acid and BHT) were vortex mixed and left to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was then taken at 717 nm. Methanol was used as blank. The ability to scavenge the DPPH radical was calculated as DPPH scavenging (%) = $[(Ac-As)/Ac] \times 100$, where Ac is the absorbance of the control and As is the absorbance in presence of the sample extracts. The antioxidant activity of the extract was expressed as EC₅₀. The EC₅₀ value was defined as the concentration (in µg/mL) of extracts that scavenges the DPPH radical by 50%.

Ferric reducing antioxidant power

The ferric reducing power was determined according to the method of Yishaket *et al.* [16] Plant extract (1 mL) solution (final concentration 50–1000 µg/mL) was mixed with 2.5 mL potassium phosphate buffer (0.1 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. Then at 50°C the mixture was incubated for 20 min. Trichloroacetic acid (2.5 mL, 10%) was added to the mixture, which was then centrifuged at 3000 rpm (Centurion, 1000 series, UK) for 5 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL FeCl₃ (0.1%) and absorbance was measured at 700 nm. All tests were performed in triplicate. An increase in absorbance of the reaction indicated a higher reducing power. EC₅₀ values (µg/mL) were calculated and indicate the effective concentration at which the absorbance was 0.5 for reducing power.

Cell lines and culture conditions

Human hepatocellular carcinoma cells (HepG2) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as recommended by the ATCC. HepG2 cells were grown in Eagle's modified minimum essential media (EMEM) supplemented with 10% FBS (FBS; ATCC, Rockville, MD, USA) and 1% penicillin-streptomycin as described before. Briefly cells were maintained at 37°C in an incubator under 5% CO₂/95% air atmosphere at above 85% relative humidity constantly. The cells were cultured (about 80% of confluence) once a week in T-75 flasks. Cells were counted using a hemocytometer (Bright-Line Hemacytometer, Sigma-Aldrich, Mississauga, ON, Canada) and were plated in 96-well format for 24 h prior to the addition of test compounds. All the test samples were solubilized in sterile-filtered DMSO (<0.5% in the culture medium) prior to addition to the culture media. Control cells were also run in parallel and subjected to the same changes in media with <0.5% DMSO.

Cytotoxicity assay

Cytotoxicity to HepG2 cells

Cytotoxicity to HepG2 was determined using the Cell Titer 96™ Aqueous one solution cell proliferation (MTS) assay kit (Promega Madison, WI, USA) according to the manufacturer's instructions.^[17] HepG2 cells (1×10^4 cells/100 μ L/well) were seeded in a sterile flat bottom 96-well plate (BD Biosciences, Mississauga, ON, Canada) and incubated at 37°C, 5% CO₂ for overnight. The aqueous: methanol (20:80, v/v) and petroleum ether extracts of *A. corrorimaseeds* or sorafenib were prepared in media and 100 μ L of each treatment was added to each well, each treatment in three replications. Thereby, cells were exposed to various concentrations (10, 50, 100, 200, 300, and 500 μ g/mL) of each treatment. Controls consist of cells with media containing DMSO (< 0.5%) and blank wells contained media with no cells. After 24 and 48 h of incubation, 20 μ L of the MTS reagent in combination with phenazinemethosulfate (the electron coupling agent) was added to the wells, and cells were incubated in a humidified CO₂ incubator for 3 h. Absorbance at 490 nm (OD490) was monitored with a plate reader (FLUO star Optima, BMG Labtech, Durham, NC, USA) to obtain the number of viable cells relative to the control population. Percentage of viability in the test compound treated cells was expressed as a percentage compared to control (<0.5% DMSO), using the following formula: % cell viability = (OD490 of treated cells/OD490 of control cells) \times 100. Data were expressed as mean values \pm SD and obtained from three different experiments against each cell line ($n = 3$ per plate per time point).

Morphological observation under inverted phase-contrast microscope

HepG2 cells were equally seeded in 96-well flat-bottom tissue culture-treated plates (BD Biosciences) and then treated with various concentrations of the extracts, sorafenib, or DMSO (< 0.5%) control. After 24 h of treatment, the morphology of HepG2 cells was observed under an inverted phase-contrast microscope (Nikon Eclipse E 100, Nikon, ON, Canada) and images were captured at 100x magnification using Infinity digital microscopy camera (Lumenera corporation, ON, Canada).

Statistical analysis

Statistical analysis was performed using Statistical Analysis System (SPSS, Version 20). The data were subjected to analysis of variance (ANOVA). Duncan's multiple range test was used for mean comparisons at $p < .05$. Linear regression analysis was used to calculate EC₅₀ values.

Results and discussions

Total phenolic and flavonoid contents

The total phenolic and flavonoid contents of aqueous: methanol (20:80, v/v) extract were higher ($p < 0.05$) than that of petroleum ether extract (Table 1). According to the study conducted by Eyob *et al.*^[11] the total phenolic content in seeds (3.98 mg/g dried weight) was significantly higher than in pods (1.32 mg/g dried weight). These values were lower than that of the present study.

Table 1. Total phenolic and flavonoid contents of petroleum ether and aqueous: methanol (20:80, v/v) extracts from seed of *A. corrorima*.

Extract	TPC (mgGAE/100 g)* \pm SD	TFC (mgQE/100 g)** \pm SD
Petroleum ether	14 \pm 2	0.1 \pm 0.02
Aqueous: methanol (20: 80, v/v)	25 \pm 5	19 \pm 0.4

Where * and ** are total phenolic and total flavonoids contents, expressed as gallic acid and quercetin equivalents per hundred grams of dried extract, respectively. Values are expressed as mean \pm SD ($n = 3$) from triplicate experiments.

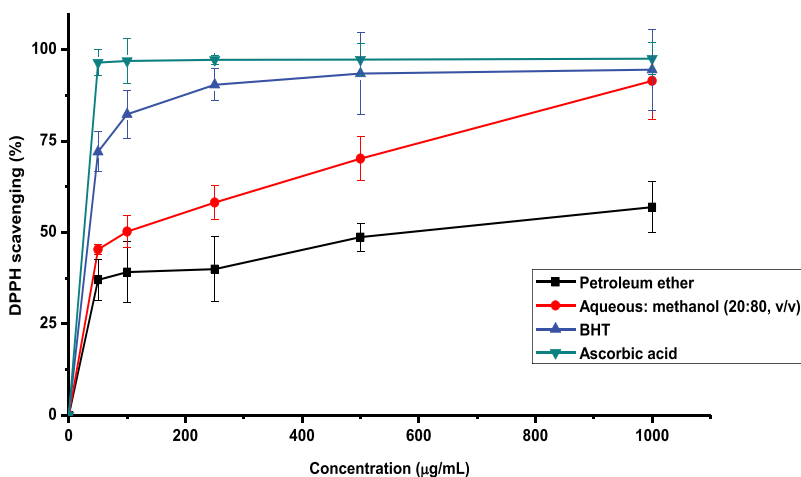


Figure 1. DPPH radical scavenging activity (%) of petroleum ether and aqueous: methanol (20:80, v/v) extracts from the dried seed of *A. corrorima* and controls (L-ascorbic acid and BHT). Values are the average of triplicate measurements (mean \pm SD).

Antioxidant activity

DPPH radical scavenging activity

The DPPH radical scavenging effects of *A. corrorima* seed extracts were shown in Figure 1. DPPH radical is scavenged by antioxidants through the donation of hydrogen forming the reduced DPPH. The color changes from purple 2, 2-diphenyl-1-picrylhydrazyl radical to reduced yellow diamagnetic 2, 2-diphenyl-1-picrylhydrazine molecule, which can be quantified by its absorbance reduction at wavelength 520 nm.^[18] At the concentration of 1 mg/mL, the DPPH radical scavenging effects were decreased in the order of L-ascorbic acid ($98 \pm 4\%$) > BHT ($95 \pm 11\%$) > aqueous: methanol (20:80, v/v) extract ($91 \pm 10\%$) > petroleum ether extract ($57 \pm 7\%$). As the concentration of the sample increased, the percent inhibition of DPPH radical also increased.^[4] This suggested that dried seed extracts of *A. corrorima* contain compounds that can donate electron/hydrogen easily and stabilizes free radicals.

The IC_{50} values were calculated from plotted graph of percentage scavenging activity against the concentration of the extracts (Table 2). The lower the EC_{50} value, the higher is the scavenging potential. Stronger scavenging activity (lower EC_{50} values) was recorded for aqueous: methanol (20:80, v/v) extract which appeared more than seven times stronger than the scavenging activity of petroleum ether. This might be in accordance with the total phenolic or flavonoid contents of *A. corrorima*.^[19] The DPPH scavenging activities of petroleum ether and aqueous: methanol (20:80, v/v) extracts were found to be significantly weaker ($p < 0.05$) than that of BHT and L-ascorbic acid.

Table 2. The EC_{50} ($\mu\text{g/mL}$) values of DPPH radical scavenging and ferric reducing antioxidant power activities in petroleum ether and aqueous: methanol (20:80, v/v) extracts from seed of *A. corrorima*.

Extract	DPPH scavenging ($\mu\text{g/mL}$) \pm SD	Ferric reducing ($\mu\text{g/mL}$) \pm SD
Petroleum ether	675 ± 11^c	818 ± 22^c
Aqueous: methanol (20:80, v/v)	97 ± 4^b	258 ± 15^b
BHT	9 ± 0.3^a	38 ± 1.2^a
L-ascorbic acid	6 ± 0.1^a	-

Values are expressed as mean \pm SD ($n = 3$) from triplicate experiments. Means with different letters in a column were significantly different at the level of $p < 0.05$.

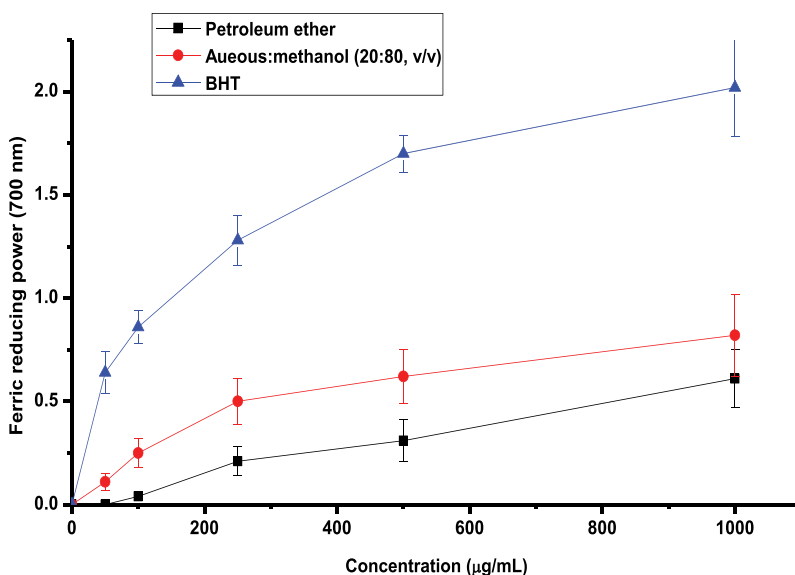


Figure 2. Ferric ion reducing antioxidant power of petroleum ether and aqueous: methanol (20:80, v/v) extracts from dried seed of *A. corrorima* at different concentrations ($\mu\text{g/mL}$) and the reference (BHT). Values are average of triplicate measurements (mean \pm SD).

Ferric reducing power

The antioxidant potential of the *A. corrorima* was estimated for its ability to reduce Fe (III) to Fe (II). Similar to DPPH scavenging as the concentration of the extracts increased, the ferric reducing power increased (Figure 2). In this study, the aqueous methanol extract showed stronger ferric reducing power. Significant differences ($p < 0.05$) were found between the two extracts (Table 2), the aqueous methanol extract showed three times stronger (smaller EC_{50}) than the ferric reducing power of petroleum ether extract, however this extract showed weaker reducing power than that of butylated hydroxytoluene (BHT).

Microscopic evaluation of morphological changes in HepG2 cells

To examine the effect of the petroleum ether and aqueous: methanol (20:80, v/v) extracts on cell morphology, HepG2 cells were treated with 10, 50, 100, 200, 300, and 500 $\mu\text{g/mL}$ of the extracts, sorafenib (positive control) and DMSO ($< 0.5\%$) for 24 h and morphological changes were observed by phase-contrast microscopy. The images (Figure 3) showed that the petroleum ether extract induced severe morphological changes of cell death, including rounding and shrinkage of cells in a dose-dependent manner. The pattern of cell death was similar to liver cancer drug sorafenib at a concentration of 300 $\mu\text{g/mL}$ and above. The control cells were well adhered to, displaying the normal morphology of HepG2 cells. In contrast, the majority of HepG2 cells treated with petroleum ether extract at the concentration of 300 $\mu\text{g/mL}$ or more became round and shrunken and could not be affixed to the walls and floating in the medium ($\times 100$ magnification).

Inhibition of HepG2 cell proliferation

HepG2 cells were treated with increasing concentrations (10, 50, 100, 200, 300, and 500 $\mu\text{g/mL}$) of aqueous methanol (20:80, v/v) extract, petroleum ether extract or sorafenib and cell viability were assayed at 24 and 48 h after treatment (Figure 4). Cell viability decreased in a dose-dependent manner and decreased with the increasing concentration of the extract. A strong negative correlation between the percentage of cell viability and concentration of the extract was observed (Figure 4). Petroleum

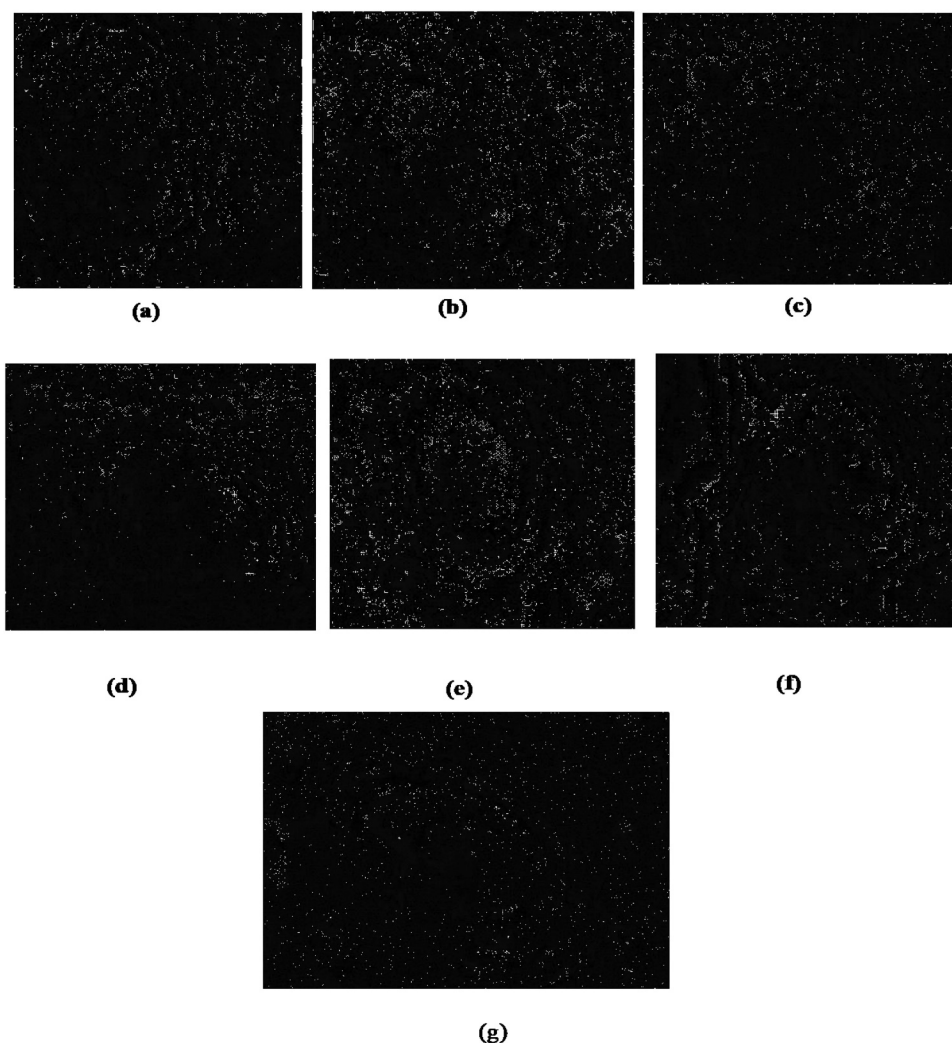


Figure 3. Inverted phase contrast microscopy showing petroleum ether extract of *A. corrorima* seed or sorafenib (positive control) induced changes of HepG2 cells at 24 h. (a) 10 $\mu\text{g}/\text{mL}$ petroleum ether extract; (b) 50 $\mu\text{g}/\text{mL}$ petroleum ether extract; (c) 100 $\mu\text{g}/\text{mL}$ petroleum ether extract; (d) 300 $\mu\text{g}/\text{mL}$ petroleum ether; (e) 500 $\mu\text{g}/\text{mL}$ petroleum ether; (f) 100 $\mu\text{g}/\text{mL}$ sorafenib (positive control); (g) negative control (DMSO).

ether extract inhibited the proliferation of HepG2 cells more than aqueous methanol (20:80, v/v) extract, with increasing inhibitory activity as the concentrations of extracts increased. After 24 and 48 h, petroleum ether extract showed $11 \pm 2\%$ and $0.5 \pm 0.0\%$ cell viability of HepG2 cells, respectively, at a concentration of 300 $\mu\text{g}/\text{mL}$, while at the same concentration, aqueous: methanol (80:20, v/v) extract showed $40 \pm 11\%$ and $34 \pm 15\%$ cell viability, respectively. There is a significant difference of EC_{50} value between petroleum ether ($\text{EC}_{50} = 105 \pm 7 \mu\text{g}/\text{mL}$) and aqueous methanol (20:80, v/v) ($\text{EC}_{50} = 282 \pm 43 \mu\text{g}/\text{mL}$) extracts treated for 24 h. Also, the EC_{50} values of petroleum ether and aqueous methanol (20:80, v/v) extracts were significantly higher ($p < 0.05$) than the EC_{50} value of sorafenib (positive control) at any time (Table 3). The relatively stronger cytotoxicity activity of the petroleum ether extract, containing low phenolic composition, suggests that the nature of compounds is determinant for these activities rather than their amounts. Studies showed that essential oils^[20] are playing a dominant role in anti-cancer activity rather than the polar phenolics. Under this study, the stronger cytotoxicity activity of petroleum ether extract may be because of bioactive monoterpene, 1,

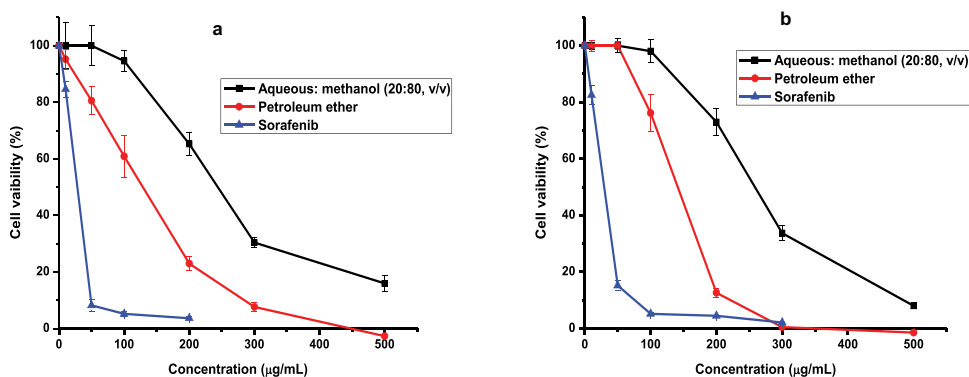


Figure 4. Percentage of cell viability of petroleum ether and aqueous methanol (20:80, v/v) extracts of *A. corrorima* on HepG2 cells after 24 h (A) and 48 h (B) of exposure, sorafenib as a reference. The MTS assay was used to measure the percentage of cell viability. Data are presented as mean \pm SD of three replicates from three independent experiments.

Table 3. EC₅₀ ($\mu\text{g/mL}$) values of petroleum ether and aqueous: methanol (20:80, v/v) extracts of *A. corrorima* and sorafenib (liver cancer drug) on HepG2 cells after 24 h and 48 h of exposure.

Test extracts	24 h	48 h
Petroleum ether	105 \pm 7 ^b	111 \pm 6 ^b
Aqueous: methanol (20:80, v/v)	282 \pm 43 ^c	308 \pm 22 ^c
Sorafenib (liver cancer drug)	25 \pm 2 ^a	13 \pm 2 ^a

EC₅₀ value of cell viability was measured by MTS assay. Data are presented as mean \pm SD of three replicates from three independent experiments. Values within a column with different subscript letters are significantly different at $p < 0.05$, compared to Sorafenib (positive control).

8-Cineole is the major component of the essential oil (44.3%) of seed of *A. corrorima*.^[11] A number of scientific reports have shown the antiproliferative activity of 1, 8-Cineole against different cell lines. According to Rodenaket *al.*^[21] 1, 8-Cineole, the main compound present in the *N. binaloudensis* oil has been reported to inhibit the growth of liver-derived (HepG2) cell lines. The recent study^[22] on 1, 8-Cineole-containing n-hexane extract of *Callistemon citrinus*, also showed cytotoxicity effect against the cancer cells. Therefore, the antiproliferative efficacy of petroleum ether extract may be related to the presence of 1, 8-Cineole and other phytochemical components in seed extract of *A. corrorima*.

Conclusion

The results obtained showed the *in-vitro* studies performed using the hepatocellular carcinoma (HepG2) cell reveals that the petroleum ether seed extract of *A. corrorima* showed a moderate anti-cancer activity; however, the aqueous methanol (20:80, v/v) extract exhibited weaker activity. Even though there was an increase in the cell growth inhibition when the concentration of the sample was increased, the EC₅₀ values were more than 100 $\mu\text{g/mL}$ for the cell lines studied as shown by the MTS assay method. In this study, however, the findings did not show any relationship between antiproliferative activity and phenolic content, and antioxidant activity. Petroleum ether extract, which contained a lower phenolic composition and weaker antioxidant activity, exhibited a stronger antiproliferative activity. The overall results suggested that seed petroleum ether extract of *A. corrorima* containing the largest amount of 1, 8-Cineole has shown potential antiproliferative activity in a dose-dependent manner. Based on these preliminary results, *A. corrorima* could represent an important source of substances with antiproliferative activity. Therefore supplementing a balanced diet with *A. corrorima* seeds may have a beneficial effect in treating liver cancer.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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