Egypt. J. Chem. **Vol. 65,** No. SI13B pp. 1465 - 1476 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Cytotoxic Activity of Some Egyptian Plants against Hepatic Human Cancer Cell Line, In-vivo Anticancer Activity and Bio-guided Isolation of Active Extracts

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Abstract

Plants are valuable source of a variety of drugs for human diseases including cancer. Most of Egyptian plant species have not been studied for their biological activities. In the continuing effort to screen Egyptian plants for anticancer activity, 16 plants were collected from different areas in Egypt. Plants extracts were tested for their cytotoxic activity against human hepatocellular carcinoma cell line (Hep-G2). According to the National Cancer Institute NCI protocols, *Sesamum indicum and Aberia caffra* are considered as strongly active plants against Hep-G_2 cell line (less than IC_{50} 20 µg/ml) while *Conocarpus erectus a*nd *Bombax ceiba* showed moderate cytotoxicity (IC_{50} 21 -50 µg/ml). The rest of tested plant extracts showed no activity.

Bio-guided isolation of petroleum ether extract of *Sesamum indicum*, resulted in isolation and identification of two active compounds, methyl linoleate and sesaminol (IC_{50} = 14.7 and 17.9 µg/ml, respectively). Moreover, column chromatographic separation of alkaloids containing fraction from *Aberia caffra* resulted in the isolation of an active compound with IC_{50} 5.4 µg/ml which was identified as Aberiamine. *In-silico* analysis of *Hep-G2* cytotoxic compounds revealed their ability to bind with caspase-3 via hydrogen bonds and/or arene-cation interactions to initiate apoptosis of cancer cells. *In vivo* antitumor study against diethylnitrosamine (DEN)-induced liver hepatocellular carcinoma in rats of *Sesamum indicum* showed improvement in liver enzymes serum level, Alpha-fetoprotein (AFP) hepatic content and in histopathological findings. On the other hand, treatment with *Aberia Caffra* showed minimal improvement in liver enzymes, AFP hepatic content and histopathological findings.

Keywords: *Aberia caffra;* Aberiamine; Cytotoxicity; Human hepatocellular carcinoma; Methyl linoleate; Sesaminol; *Sesamum indicum.*

1. Introduction

Liver cancer disease is considered potential threats to human life [1-2]. Hepatocellular carcinoma (HCC) or liver cancer is the third most common cause of cancer mortality and the fifth most common cancer worldwide [3]. Natural plant products have attracted the notice of chemists and biologists around the world over the last 50 years. Natural antitumor products are considered to be a goldmine because of the diversity of bioactive metabolites and continue to provide novel natural products with varied biological and pharmacological activities[4].Consequently, in recent years, there has been much interest in the antitumor activity of naturally occurring substances from which we investigated two plants in this study namely, *Sesamum indicum* and *Aberia caffra* from list of 16 Egyptian plants for anticancer activity were collected from different areas in Egypt Table 1&2.

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Receive Date: 16 May 2022, Revise Date: 11 March 2023, Accept Date: 18 July 2022

DOI: 10.21608/EJCHEM.2022.132760.5865

Sesamum indicum L. "Sesame" seed is commonly known as the 'Queen of the oil seeds', due to its resistance to oxidation and rancidity[5]. Sesame oil has antioxidant activity, blood pressure and serum lipid-lowering potential due to presence of sesamol or sesamin which increases the antioxidant effect of γ-tocopherol by three times[6]. In previous studies, sesame seeds extract exhibited antiproliferative activity against HepG2 cell[7]. *Aberia caffra* (Harv. & Sond), *Dovyalis caffra* (Warb.), Kei apple, Kai apple, or Kau apple, is a small to medium-sized tree, native to southern Africa. The ripe fruits are tasty, reminiscent of a small apple[8]. It is considered as a valuable medicinal anticancer plant. According to literature, the methanol extract of *A. caffra*, showed significant cytotoxic activity against four human cancer cell lines namely (MCF-7) breast, (HCT-116) colon, (HepG-2) hepatocellular and (A-549) lung [9]. Diethylnitrosamine (DEN) is a carcinogenic N-nitrosamine compound well known as a hepatocarcinogen [10]. DEN causes metabolic catalyzation by liver cytochrome P450 enzymes to form DEN-DNA adduct and gives carcinogenicity [11]. Also, DEN induced reactive oxygen species (ROS) gives oxidative stress leading to hepatocarcinogenesis [12-13].

In the current study, we investigated the anti-hepatocarcinoma activity of *Sesamum indicum* and *Aberia caffra* extracts and their fractions which led to the isolation of bioactive compounds from the most active fractions. The bioactivity was tested against (Hep-G2) using the MTT assay and the mechanism of action was postulated by molecular docking of the bioactive compounds and *in-vivo* antitumor study.

2. Materials and methods

2.1. Plant material

The wild plants and weeds were collected from Zagazig farms, Moshtohor farm, Eastern Desert, Mit-Ghammr, Marsa Matroh and desert vicinity of Sharkia governorate area (60 kilometers west to Sues Canal, Egypt) in 2016. The collected samples were kindly identified by Dr. Ahmad Saeed, Prof. of Horticulture, Moshtohor Agriculture Faculty and Dr. Mohamad Abd-Elkader, Assist. Prof. of Plant Horticulture, Zagazig Agriculture Faculty.

2.2. Extraction and cytotoxic screening against Hep-G2 cells

The fresh plants included in this study (50 g each) were cut and extracted by cold maceration using 70 % ethyl alcohol (0.5 L × 3) till complete exhaustion. The combined extract of each plant was separately evaporated under reduced pressure at 45-50 °C. Each extract was screened for its cytotoxicity against Hep-G_2 cell line using MTT-assay.

2.2.1 Large scale extraction and fractionation of active plants

The aerial parts of *Aberia caffra* (Kei apple, Salicaceae) (4.5 kg) was extracted by cold maceration using 70% ethyl alcohol (3 × 8L) till complete exhaustion. The combined extracts were evaporated under reduced pressure at 45-50 °C to give 150 g greenish brown viscous alcoholic extract. The alcoholic extract was successively fractionated by petroleum ether (60-80 °C) then methylene chloride and finally with ethyl acetate, (3 × 6 L) for each. The three fractions dried over anhydrous sodium sulphate and concentrated under reduced pressure at 45-50 °C to afford a greenish brown viscous residue of 25g, a greenish viscous residue of 21g and a yellow viscous residue of 8 g, for petroleum ether, methylene chloride and ethyl acetate, respectively.

Also, about (1Kg) from the fresh fruits of *Aberia caffra* was extracted by cold maceration using 70% ethyl alcohol (2L × 3) till complete exhaustion. The combined extract was evaporated under reduced pressure at 45-50 °C to give a total residue of 10 g brown viscous extract.

Finally, 100gm of the alcoholic extract of the aerial parts of *Aberia caffra* was suspended in 1.5 L of 1% aqueous H_2SO_4, filtered, concentrated to 500 ml (under reduced pressure), and then partitioned with petroleum ether (500 ml × 3). The aqueous layer was separated, alkalinized with 25% Na_2CO_3 solution till pH 11.5, and extracted with dichloromethane-DCM (500 ml × 4). The DCM-layer was evaporated under reduced pressure to give 10.5 gm of crude alkaloid extract. The crude alkaloid extract was dissolved in 500 ml DCM and extracted with 10% aqueous acetic acid (500 ml × 3). The acetic acid layer was concentrated (under reduced pressure), alkalinized with Na_2CO_3 powder (till no more effervescence), and extracted again with DCM (500ml × 4). The DCM-fraction was evaporated under reduced pressure to give 5 gm of purified crude alkaloid extract (Alkaloid Fraction).

On the other hand, the powdered seeds of *Sesamum indicum* L. (6 kg) were defatted with petroleum ether (10L × 3) after crushing then extracted with 90 % methanol (12 L × 3) till complete exhaustion. The methanolic extract was evaporated under reduced pressure at 45-50 °C to give 600 g brown viscous residue. Methanol extract was kept in refrigerator to remove remains of fats.

The aerial parts of *Conocarpus erectus* and *Bombax ceiba* (1.5 kg) each were extracted by cold maceration using 70% ethyl alcohol (3 × 3L) till complete exhaustion**.** The extracts were evaporated under reduced pressure at 45-50 °C to give 15 and 12

g of alcoholic extracts, respectively. The alcoholic extracts were successively fractionated by Petroleum ether then methylene chloride and finally with ethyl acetate, (3 × 3L) for each. All fractions were dried over anhydrous sodium sulphate and concentrated under reduced pressure at 45-50 °C to afford 5 and 4 g of petroleum ether soluble fractions, 4 and 4.5 g of methylene chloride soluble fraction and residue of 3 and 5 g of ethyl acetate soluble fraction, respectively.

Samples (20 mg each) from the petroleum ether, methylene chloride, ethyl acetate, aqueous fractions, alkaloid extract and total extract of fruit of *Aberia caffra* , collected petroleum ether fraction, syrupy methanol fraction and oily methanol fraction of *Sesamum indicum* also, Petroleum ether, methylene chloride, ethyl acetate, aqueous fractions of *Conocarpus erectus* and *Bombax ceiba* were screened for their cytotoxicity against Hep-G_2 cell line using MTT-assay.

2.3. Bio-guided column chromatography for isolation:

2.3.1. Bio-guided column chromatographic isolation of petroleum ether extract of Aberia caffra.

About 20 g of petroleum ether fraction was subjected to silica gel 60 ((0.063 – 0.200 mm), Merck) column chromatography (4 × 100cm, 850g) packed in petroleum ether, the polarity was increased gradually using methylene chloride then methanol. Resulted fractions were examined by TLC (silica gel precoated, kieselgel 60 F254, silica 0.25 mm, Germany) using solvent systems **I** (Petroleum ether, 100%), **II** (Petroleum ether: methylene chloride, 1:1) and **III** (Petroleum ether: methylene chloride: methanol, 15:15:1). Developed chromatograms were sprayed with anisaldehyde/sulphuric acid reagent. The similar fractions were pooled, crystallized to afford six compounds, 0.52 (Comp.1), 0.67(Comp.2), 0.72(Comp.3), 0.61(Comp.4), 0.54(Comp.5) and 0.71(Comp.6). The six isolated compounds were examined for their cytotoxicity against *Hep-G_2* cell line. For all the isolated compounds, melting point (electro-thermal LTD, England), IR (FT/IR-6100typeA (JASCO-Germany), EI-MS (ISQ LT, USA), ^{1}H - and ^{13}C-NMR analyses (Bruker, Switzerland; 100 and 400 Hz, respectively) were performed.

2.3.2. Bio-guided column chromatographic isolation of methylene chloride extract of Aberia caffra

About 20 g of methylene chloride extract was subjected to silica gel 60 ((0.063 – 0.200 mm), Merck) column chromatography (4x100cm, 650g) packed in petroleum ether), the polarity was increased gradually using methylene chloride then methanol. Resulted fractions were examined by TLC (silica gel precoated, kieselgel 60 F254, silica 0.25 mm, Germany) using solvent systems **IV (**Ethyl acetate, 100%). The isolated compounds **7**, 0.26 (Comp.7) were examined for their cytotoxicity against Hep-G_2 cell line.

2.3.3. Bio-guided column chromatographic isolation of alkaloid extract of Aberia caffra

About 5 g of methylene chloride extract was subjected to silica gel 60 ((0.063 – 0.200 mm), Merck) column chromatography (2x100cm, 100g) packed in methylene chloride, the polarity was increased gradually using methylene chloride then methanol. Resulted fractions were examined by TLC (silica gel precoated, kieselgel 60 F254, silica 0.25 mm, Germany) using solvent systems **V (**CH_2CL_2: CH_3OH: Ammonia
7:3:5 drops). The isolated compounds **8**, 0.56 (Comp.8) was examined for its cytotoxicity against Hep-G_2 cell line.

2.3.4. Bio-guided column chromatographic isolation of oily petroleum ether extract of *Sesamum indicum* L.

About 10g of oily petroleum ether extract was subjected to silica gel 60 ((0.063 – 0.200 mm), Merck) column chromatography (2.5x80cm, 320g) packed in petroleum ether, the polarity was increased gradually using methylene chloride then methanol. Resulted fractions were examined by TLC (silica gel precoated, kieselgel 60 F254, silica 0.25 mm, Germany) using solvent systems **II** (Methylene chloride: Petroleum ether 1:1) and **V** (Methylene chloride 100%). The two separated compounds **9** (0.2 g), **10** (0.6 g) were examined for their cytotoxicity against *Hep-G_2* cell line.

2.4. Cytotoxic activity

Human hepatocellular carcinoma (HepG-2), obtained from VACSERA Tissue Culture Unit, were used to evaluate the cytotoxic effect of the different extracts, fractions and isolated compounds using cell viability MTT assay [14-15] Percentages of relative viability as well as 50% inhibitory concentrations (IC_{50}) were calculated for cell line.

2.5. Statistical analysis

Linear regression was performed for the calculation of IC_{50} in the case of the *in-vitro* assay. The Microsoft EXCEL 2010 program was used for data analysis and to draw the figures. Data were performed in triplicates and presented as mean± SD.

2.6. Molecular docking study

Molecular docking of potent isolated compounds was performed using Molecular Operating Environment 2009 (MOE) as previously described [16]. Briefly, the compounds were constructed in 3D structure, their energies were minimized and saved to MDB file. Caspase-3 (responsible for *Hep-G2* apoptosis)

X-ray crystallographic structure coded as 2J30 was downloaded from the Protein Data Bank (R.P.D. Bank, RCSB PDB: Homepage, 2020. https://www.rcsb.org/ (accessed 2 December 2020). Hydrogens were added to the protein structure and missed connections and their types were corrected automatically. The receptor and its atoms potential were fixed. The active site of the enzyme was determined based on co-downloaded natural ligand using surfaces and maps.

The constructed compounds' database was docked against caspase-3 using the following parameters: Placement, triangle matcher; scoring, London dG with ten retains; refinement, focefield. The resulted poses were investigated based on their energy, root-mean square deviation (rmsd) and formed interactions (bonds).

2.7. *In-vivo* Anti-tumor study

2.7.1 Animals

Male Wister rats (110-160 g) were purchased from the Modern veterinary office for laboratory animals, Giza, Egypt. The animals were housed in plastic boxes and left to acclimatize a week at the animal facility of Faculty of Pharmacy, Cairo University (Egypt). Rats were kept under controlled temperature (23 ± 2°C) as well as, constant relative humidity (60% ± 10%) and a light/dark cycle (12/12 h) throughout the experimental period.

All animals were allowed free access to standard diet and water and libitum during the investigation period. The experimental work was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

2.7.2. Plant extract and drugs

The dried alcoholic extract of aerial parts of *Aberia caffra* and seeds of *Sesamum indicum* L**.** were separately suspended in 0.5% Tween-80 solution (ADWIC, Egypt). The dose used in the present work was 300 mg/kg body weight.

DEN (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) injected intraperitoneally as a solution in normal saline.

Design of experiments

Animals were allocated into three groups (n=15) as following:

Group 1 (Negative control): rats received saline by i.p. injection, five times weekly for 6 consecutive weeks

Group 2 (DEN control): rats received DEN (20 mg/kg) five times weekly for 6 consecutive weeks to induce hepatocellular carcinoma (HCC).

Group 3: rats received DEN (20 mg/kg) five times weekly for 6 consecutive weeks to induce HCC followed by administration of alcoholic extract of *Aberia caffra* and *Sesamum indicum* L (300 mg/kg; p.o "per. Os. mean oraly in latin ") daily starting from the 6th week and till the 10th week.

Samples collection and storage

At the end of experimental period (10 weeks), blood and liver samples were collected as following:

1. Blood sampling

Blood samples were collected from the retro-orbital sinus of anaesthetized rats using non heparinized capillary tubes. Serum was separated by centrifugation at 3000 rpm for 15 minutes at 4°C using cooling centrifuge (Hettich universal 32A, Germany). Each sample was divided into two aliquots, one for each estimated parameter to avoid repeated freezing and thawing. Plasma aliquots were stored at -20ºC until assessment of ALT & AST activities.

2. Tissue sampling

After collecting blood samples, animals were sacrificed by cervical dislocation; the liver samples were immediately removed as whole livers and finely dissected from adjacent tissues and big blood vessels, washed with ice-cold saline, dried between two filter papers, weighed for determination of liver index and rapidly frozen at -20°C. Part of liver samples were then weighed and homogenized in cold saline using a homogenizer (Heidolph Diax 900, Germany) to prepare 10% tissue homogenate for estimation of liver α-fetoprotein while the other part was fixed in 10% formalin for 24 hours, specimens were processed by paraffin embedding and 5 μm sections were prepared for histopathological examination.

Liver index calculation:

Liver index was calculated using the following formula: (liver weight/body weight×100).

Biochemical determinations

Liver function tests: alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities were assessed using Biodiagnostic kits (Egypt). Procedures were performed according to manufacturer's instructions and results were expressed as U/l.

Alpha-fetoprotein (AFP): Hepatic AFP content was assessed using rat AFP ELISA kit (eBioscience, San Diego, USA). The procedure of the used kit was performed according to the manufacturer's instructions and results were expressed as ng/mg protein.

Histopathological examinations: Parts of the liver were obtained from different levels (base, middle and apex), rinsed in ice-cold saline and immediately fixed in 10% formalin for 24 h. Specimens were then processed for paraffin embedding.

Statistical analysis:

Data were expressed as means ± standard error (SEM) and percentage of normal. Analysis of the results was done using one-way-analysis of variance

test (ANOVA) followed by Tukey's post-hoc multiple comparison's test. GraphPadPrism® software package, version 6 (GraphPad Software, Inc., USA) was used to carry out all statistical tests.

3. Results and discussion

In this study, the methanolic extracts of sixteen Egyptian plants were tested for their cytotoxic activity against Hep-G2 cell line. According to the National Cancer Institute NCI protocols, the extracts of ***Sesamum indicum*** seeds is considered as strongly active against Hep-G_2 cell line with IC_{50} 14.1 µg/ml, respectively. On the other hand, ***Conocarpus erectus***, ***Aberia caffra*** aerial parts *a*nd ***Bombax ceiba*** **s**howed moderate cytotoxicity (IC_{50} 22.7, 24.4 and 39.9 µg/ml, respectively). All the other tested plant extracts showed no activity. Tested plants, their botanical sources and related IC_{50} were summarized in table 1.

Four active extracts subjected to fractionation using petroleum ether, methylene chloride and ethyl acetate as shown in table 2. According to the protocols of the National Cancer Institute NCI [17], the alkaloid extract of *Aberia caffra* and petroleum ether fraction of *Sesamum indicum* were considered as active fractions against Hep-G_2 cell line with IC_{50} 12 and 5.5µg/ml, respectively. So, column chromatographic fractionation was carried out for each of these fractions

Aberia caffra bio-guided fractionation and isolation revealed that petroleum ether and methylene chloride fractions significantly inhibit the growth of Hep-G2 cell line. Six compounds were isolated and identified from petroleum ether fraction of *Aberia caffra* besides one compound from methylene chloride fraction (Figure 1). The isolated compounds **(1-7)** from petroleum ether and methylene chloride extract afforded no activity upon comparison with cisplatin & vinblstin reflecting that the activity of each extract is attributed to synergistic effect of its components. On the other hand, the active compound (8) from alkaloid extract was identified as aberiamine (Figure 2). Aberiamine exhibited cytotoxic activity with IC_{50} 5.4 µg/ml indicating a high cytotoxic activity upon comparison with cisplatin & vinblstin**.** Also, two active compounds (9&10) from petroleum ether extract of *Sesamum indicum* were obtained and identified as methyl linoleate and sesaminol (Figure 3) with IC $_{50}$ 14.7 and 17.9 µg/mL, respectively.

Table (1): Screening the cytotoxic activity of some Egyptian plant against Hep-G2 cell line:

Plant (Name, Family)	IC $_{50}$ (µg/mL)	Site of collection
1. *Sesamum indicum* **(Sesame seed, Pedaliaceae)**	14.1	Zagazig farm Mushtuhur
2. *Conocarpus erectus* (**buttonwood** or **button mangrove** ,**Combretaceae**)	22.7	Agriculture faculty
3. *Aberia caffra arial parts; fruit* (***Dovyalis caffra,*** **Salicaceae**)[a]		Mit-Ghammr
4. *Bombax ceiba aerial part* (**red silk-cotton**; **red cotton tree**, **Malvaceae**)	24.4**;** 60	
5. *Euphorbia tirucalli* (**Indian tree spurge**, **naked lady**, **pencil tree**, **milk bush**, **Euphorbiacea**)	39.9	Zagazig University
6. *Dracocephalum moldavica* (**Moldavian dragonhead, Lamiaceae**)	87.8	Mushtuhur Agriculture faculty
7. *Urgenia maritima* aquous and alcohol (**Red squill, Liliaceae**)		
8. *Thevetia peruviana* (**Apocynaceae**)	59.2	
9. *T*erminala catappa (**Combretaceae**)		Zagazig University
10. *Shinus terebintifolius* (**Anacardiaceae**)	203&94	
11. *Cedrela odorata* (**Meliaceae; Cedreleae**)	134	Zagazig University
12. *Ruta graveolens* (**common rue, Rutaceae**)	120	Mushtuhur
13. *Alocasia regina* (**Araceae**)	109	Mushtuhur Agriculture faculty
	210	
14. *Trachspermum ammi* (Ajowan caraway, **Apiaceae** or **Umbelliferae)**	159	
15. *Sesbania sesban aeial part and fruits* **(Egyptian riverhemp**,**Fabaceae** or **Leguminosae**)	121	
16. *Asphodelus macrocarpus seed and root* (**Asphodelaceae**)	132	Mit-Ghamr
	179& 83.4	Marsa Matroh
	117 &123	

Table (2): Half maximal inhibitory concentration of different fractions of the potent and moderately active extract against Hep-G2:

Plant	Total extract IC_{50} (µg/ml)	Fractionation	Fraction IC_{50} (µg/ml)
1- *Sesamum indicum* L.	14.1	Petroleum ether	5.5
		Methanol	16
		Oily layer of methanol	26
		Syrupy layer of methanol	56
2- *Aberia caffra* (Harv.& Sond) *Dovyalis caffra* (Warb.) Arial part	24.4	Petroleum ether	20.6
		Chloroform	7.35
		Ethyl acetate	18.5
		Aqueous	25.9
		Alkaloid extract from aqueous	12
3- *Conocarpus erectus*	22.7	Petroleum ether	34.1
		Chloroform	27.8

		Ethyl acetate Aqueous	119 120
4- *Bombax ceiba*	39.9	Petroleum ether Chloroform Ethyl acetate Aqueous	95.9 30.8 384 >500

3.1. Structural elucidation of isolated compounds from petroleum ether extract of Aberia caffra

N-hexacosan (1). ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): 0.86 (2H, t, J=6.5 Hz, 2CH3), 1.33-1.25 (2H, m, 2CH2), 1.25 (44H, brs, H-4 to H-25). **^{13}C-** APT (CD_3OD**, 100 MHz):** δC, 14.17, 14.27 for 2 (CH3) & 22.76 to 22.87 and 29.23 to 29.86 for 22(CH2). **IR spectrum: (ν_{max} KBr cm^{-1}):** 2915&2847 (-CH2), 1392, 1165. **EI-Mass spectrum: m/z (% relative abundance)**: 366 $(M)^+$(3.6), 336 (5.2), 153 (23.0), 139 (28.6), 125, 111 (83.1), 97 (100.0), 83 (88.3), 71 (48.1), 57 (61.3) [18].

Hentriacontan (2). ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): δH, 0.90 (2H, t, J=6.5 Hz, 2CH3), 1.54-1.75 (2H, m, 2CH2), 1.33 (54H, brs, C-3 to C-29). **^{13}C-** APT (CD_3OD**, 100 MHz):** 13.62, 14.27 for 2 (CH3) and 22.26 to 24.87and 28.53 to 30.54 for 27(CH2). **IR absorption: (ν_{max} KBr cm^{-1}):** 2921& 2852 (-CH2), 1463, 1379 and 1245. **EI-Mass spectrum: m/z (% relative abundance):** 434 (M^+) (25), 406 (12), 378 (10), 51(10) [19]**.**

N- Hexacosanol (3). ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): 0.88 (3H, br.t), 1.25 (46H, 23 CH2, s), 1.96 (2H, CH2, m), 3.62 (2H, CH2-OH, t) for two protons of CH2 next to hydroxyl. **^{13}C-NMR (δ, $CDCl_3$, 100 MHz):** 63.3, 33.08, 22.9-29.6, 14.3. **IR spectrum: (ν_{max} KBr cm^{-1}):** 3421 (-OH), 2916 & 2848 (-CH2), and 1462 (C-O). **EI-Mass spectrum: m/z (% relative abundance):** 382 (M^+) (10), 366 (M^+-H2O), 131(20), 105 (60), 91 (20), 77 (30), 51(10) [20]**.**

Hentriacontan-1-ol (4). ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): 0.87 (3H, t, H-31), 1.24 (52H, m, H-5 to H-30), 1.44 (6H, m, H-2 to H-4), 3.48 (2H, t, H1). **^{13}C-APT (δ, CDOD3, 100 MHz):** δ 63.15(C-1), δ 22.61(C-30), 25.2(C-3), 29.9 for $(-CH_2)_{25}$, 32.2 (C-29), 34.5(C-2), 14.27 for (C-31). **IR spectrum absorption: (ν_{max} KBr cm^{-1})**: 3480 (-OH), 2915& 2847 (-CH2), and 1462 (C-O). **EI-Mass spectrum: m/z (% relative abundance):** 452 (M^+) ($C_{31}H_{64}O$) (10), 434 (M^+-H_2O) (25), 406 (12), 378 (10), 51 (10) [21].

***β*-sitosterol (5). ^{1}H-NMR (δ, $CDCl_3$, 400 MHz):** 1.16 (m, 2H),1.25 (m,2H),3.51(m,1H),2.31 (m,2H),--,5.34 (bs,1H), 1.83 (m,2H),1.23 (m,1H), 1.25 (m,1H), --, 1.45(m,2H), 1.51(m,2H), --, 1.23(m,2H), 1.83(m,2H), 1.78(m,2H), 1.79(m,2H), 0.85(s,3H), 0.93(s,3H), 1.42(m,1H), 0.80(d,3H,), 1.96(m,2H), 1.99(m,2H), 1.18(m,1H), 2.23(m,1H), 0.80(d,3H,j=7.5Hz), 0.85(d,3H,j=1.7Hz), 1.36(m,2H), 0.86(t,3H,j=6.8Hz). **^{13}C-APT (δ, CDOD3, 100 MHz):** 37.51, 29.40, 72.05, 42.56, 141.02, 121.97, 31.92, 32.16, 50.39, 36.70, 21.34, 40.03, 42.38, 56.31, 24.56, 28.50, 56.31, 12.11, 19.65, 36.40, 20.09, 34.20, 26.33, 45.09, 29.40, 19.23, 19.65, 23.32, 12.23. **IR spectrum absorption: (ν_{max} KBr cm^{-1}):** 3377 (-OH), 3070 (C=C-H), 2916 & 2848 (-CH2), 1662 (C=C) and 1462 (C-O). **EI-Mass spectrum: m/z (% relative abundance):** 414 and peak at m/z 399 (M-CH_3) for molecular formula $C_{29}H_{50}O$. Other fragments at m/z 396 (M^+- H_2O), 273, 231 [22-23].

***β*-sitosterol acetate (6). ^{1}H-NMR (δ, CDOD3, 400 MHz):**1.43 (m, 2H), 1.50 (m,2H), 4.78(m,1H), 2.4, 2.31 (m,2H), --, 5.36 (bs,1H), 1.79 (m,2H), 1.22 (m,1H), 1.25 (m,1H), --, 1.58(m,2H), 1.46(m,2H), --, 1.23(m,2H), 1.87(m,2H), 1.79(m,2H), 1.84(m,2H), 0.65(s,3H), 1.06(s,3H), 1.43(m,1H), 0.91(d,3H,), 1.79(m,2H), 1.85(m,2H), 1.11(m,1H), 2.43(m,1H), 0.89(d,3H,j=7.5Hz), 0.78 (d,3H,j=1.7Hz), 1.25 (m,2H), 0.96 (t,3H,j=6.8Hz), ---, 2.20(S,3H). **^{13}C-APT (δ, 100 MHz):** 37.30, 29.74, 77.37, 42.33, 140.93, 121.70, 31.84, 31.90, 50.07, 36.69, 21.07, 38.78, 42.53, 56.65, 24.34, 28.27, 55.63, 12.15, 19.58, 35.95, 20.19, 33.81, 25.90, 45.63, 29.74, 19.04, 19.38, 23.07, 12.26, 180.93, 21.07. **IR spectrum: (ν_{max} KBr cm^{-1}):** 2932&2866 (-CH2), 1753 (C=O), 1653 (C=C) and 1463 (C-O). **EI-Mass spectrum: m/z (% relative abundance)**: 456, 414 (M-acetate) and peak at m/z 399 (M-CH_3) suggesting the molecular formula $C_{31}H_{52}O_2$. Other fragments at m/z 396 (M^+ - H_2O), 328, 273, 231 [24].

Cytotoxic activity

The six isolated compounds were examined for their cytotoxicity against Hep-G_2 cell line with IC_{50} 147, 222, 199, 94.8, **176**, 163 ug/ml, respectively.

Cell viability percentages of different concentrations of those isolated compounds were shown in Figure 4 in comparison with cisplatin and vincristine standards.

3.2. Structural elucidation of isolated compounds from methylene chloride extract of Aberia caffra

***β*-sitosterol glucoside (7). ^{1}H-NMR (δ, CDOD3, 400 MHz):**1.46 (m, 2H), 1.49 (m, 2H), 3.06 (m, 1H), 2.88 (m, 2H), 5.46 (bs, 1H), 1.78 (m, 2H), 1.23 (m, 1H), 1.25 (m, 1H), 1.49 (m, 2H), 1.46 (m, 2H), 1.23 (m, 2H), 1.80 (m, 2H), 1.78 (m, 2H), 1.79 (m, 2H), 0.65 (s, 3H), 0.93 (s, 3H), 1.46(m, 1H), 0.85(d, 3H, j=6.5 Hz), 1.79 (m, 2H), 1.78 (m, 2H), 1.13 (m, 1H),

2.88 (m, 1H), 0.76 (d, 3H, j=7.5Hz), 0.75 (d, 3H, j=1.7Hz), 1.25 (m, 2H), 0.76 (t, 3H, j=6.8Hz), 4.85 (d, 1H, j=7.9Hz), 3.06 (m, 1H), 3.06 (m, 1H), 3.14 (m, 1H), 3.06 (m, 1H), 2.88 (m, 1H). **^{13}C-APT (δ,, 100 MHz):** 37.30, 29.72, 70.51, 42.33, 140.93, 121.70, 31.84, 31.90, 50.07, 36.69, 21.07, 38.78, 42.33, 56.65, 24.34, 28.27, 55.90, 12.15, 19.58, 33.81, 33.81, 25.90, 45.61, 29.17, 19.09, 19.35, 23.07, 12.26, 101.24, 73.91, 77.24, 70.58, 77.24, 61.58. **IR spectrum: (ν_{max} KBr cm^{-1}):** 3420 (-OH), 2933&2864 (-CH2), 1641 (C=C) and 1463 (C-O). **EI-Mass spectrum: m/z (% relative abundance)**: (M^+-glucose), 414 (20%), 369 (100), 273 (20%)[22].

Cytotoxic activity

The isolated compounds **7** was examined for its cytotoxicity against *Hep-G_2* cell line with IC_{50} **177 ug/ml.** Cell viability percentage of compound 7 was represented in figure 4 in comparison with cisplatin and vincristine standards.

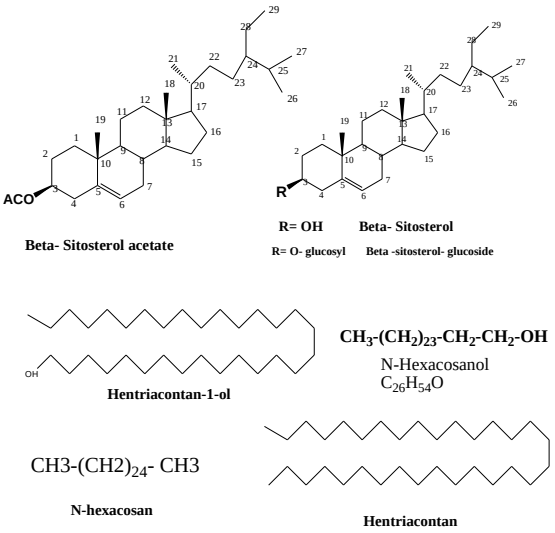


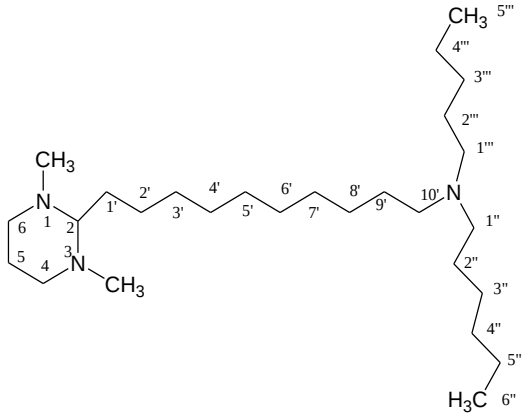
Figure 1. Isolated compounds from petroleum ether and methylene chloride fractions extract of *Aberia caffra*

3.3. Structural elucidation of isolated compounds from alkaloid extract of Aberia caffra

Aberiamine (8). ^{1} H-NMR (CDCl$_3$, δ ppm, 400 MHz): 3.76 (t, 1H), 2.76 (t, 2H), 1.33 (m, 2 H), 2.76 (t, 2H), 1.33 (m,2H), 1.32 (m,2H), 1.22 (m,2H), 1.23 (m, 2H), 1.13 (m,2H), 1.33 (m,2H), 1.33(m, 2H), 1.23(m,2H), 1.33(m,2H), 2.76 (t, 2H), 2.76 (t, 2H), 1.22(m,2H), 1.33(m, 2H), 1.33(m, 2H), 1.33 (m,2H), 0.97 (t,3H), 2.76 (t, 2H), 1.32(m,2H), 1.33(m, 2H), 1.27(m,2H), 0.97 (t,3H), 2.97 (s, 6H). **^{13}C-NMR (δ, CDCl$_3$, 100 MHz):** 77.63, 55.32, 20.74, 55.48, 33.37, 24.38, 29.75, 30.43, 30.75, 31.39, 31.49, 32.27, 33.55, 46.30, 46.36, 24.85, 24.93, 25.30, 26.56, 14.03, 46.85, 23.77, 26.96, 27.11, 14.29, 44.30, 44.33. **IR spectrum**: **(ν_{max} KBr cm^{-1})**: 3425, 2924, 2854, 1465. **EI-Mass spectrum: m/z (% relative abundance)**: (M^+) 423 (35%), (M^++1) 424 (13%), 409 (90%), 352, 253, 239, 141,113, 98, 84 [25].

Cytotoxic activity

The isolated compounds **8** was examined for its cytotoxicity against *Hep-G_2* cell line with IC_{50} **5.4 ug/ml.**



Aberiamine

Figure 2. The active isolated compounds from alkaloid extract of *Aberia caffra*.

3.4. Structural elucidation of isolated compounds from oily petroleum ether extract of Sesamum indicum

Methyl linoleate (9). ^{1} H-NMR (CDCl$_3$, δ ppm, 400 MHz): 2.35 (t, 2H), 1.63 (p, 2H), 1.34, 2(m, 4H), 5.33(m, 8H), 2.71(t, 2H), 1.35(m, 6H), 0.9(t, 3H), 3.85(S, 3H). **^{13}C-NMR (δ, CDCl$_3$, 100 MHz):** 174.17, 77.2, 77.1, 76-68.1, 77.3, 29.8, 127-129, 31.5, 25.2-29.7, 14.2, 51.3{Díaz, 2007}.**IR spectrum**: **(ν_{max} KBr cm^{-1})**: 3082, 3062(C=C-H), 2924, 2854 (-CH2), 1743 (C=O), 1600(C=C) and 1440 (C-O). **EI-Mass spectrum: m/z (% relative abundance)**: 294(M^+) (12), 263(5), 220(5), 152(8), 137 (6), 123(10), 109(30)[26]**.**

Sesaminol (10). ^{1} H-NMR (CDCl$_3$, δ ppm, 400 MHz): 3.11 (m, 2H), 4.74 (d,2H), 4.28, 4.24(dd,2H) (J=7,10, J=7, 9), 3.89 , 3.88(dd, 2H), (J=4,10, J=2, 9), 6.87(1H,S), 6.84(1H, S), 6.82(1H,S), 6.81(1H,S), 6.79(1H,S), 5.97(2H,S), 5.94(2H,S). **^{13}C-NMR (δ, CDCl$_3$, 100 MHz):** 54.35, 80.80, 85.80, 70.72, 71.73, 119.36, 147.99, 97.1, 146.1, 139.09, 106.51, 132.29, 119.36, 115.2, 145.1, 147.12, 108.20, 101.08, 101.08. **IR spectrum: (ν_{max} KBr cm^{-1}):** 3478(OH), 3078(C=C-H), 2976 (-CH2), 1607(C=C) and 1499 (C-O). **EI-Mass spectrum: m/z (% relative abundance):** 369 (M^+-1) (2), 339 (20), 219 (15), 150 (14), 151(8), 191 (6), 105 (60), 77 (75) **[27]**

Cytotoxic activity

The two isolated compounds **9, 10** were examined for their cytotoxicity against *Hep-G₂* cell line with IC_{50} 14.7 and 17.9 ug/ml.

Cell viability percentages of the two compounds isolated from *Sesamum indicum* were shown in Figure 5 in comparison with cisplatin and vincristine standards.

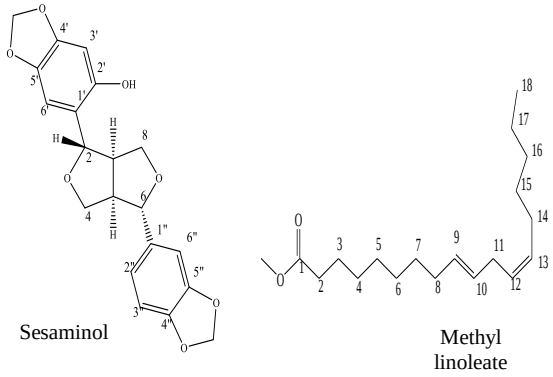


Figure 3. The two active isolated compounds from oily petroleum ether extract of *Sesamum indicum*

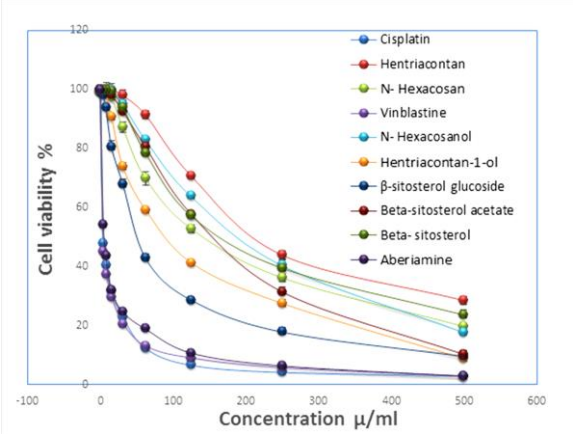


Figure 4: Cell viability percentage of isolated compounds from *Aberia caffra* L. with cisplatin and vinblasine standards against *Hep-G2* cell lines.

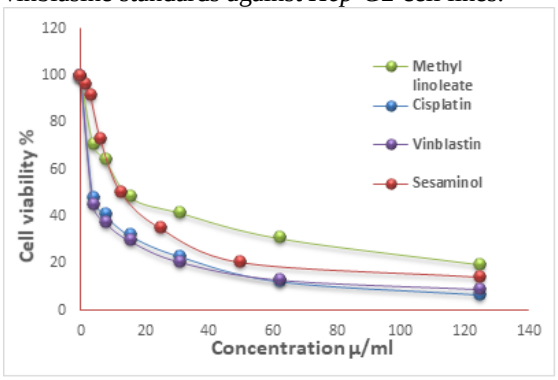


Figure 5: Cell viability percentage of isolated compounds from *Sesamum indicum* L with cisplatin and vinblasine standards against *Hep-G2* cell lines.

3.5. Mechanism of action of Hep-G2 cytotoxic components based on molecular docking study

Caspasa-3 represents one of the main enzymes that initiate apoptosis in cancer cells including *Hep-G2* [28]. Using molecular docking study, we aimed to postulate the mechanism by which our potent compounds can stimulate caspase-3 and consequently initiate apoptosis of *Hep-G2*. The results showed that the docked compounds were able to form interactions with amino acids responsible for caspase-3 activity. The interactions include formation of hydrogen bonds and arene-cation interactions. As to *Aberia caffra*, aberiamine was able to form 2 hydrogen bonds with ASsn208 with bonding energy (-20.49048) (**Figure 6**). Concerning *Sesamum indicum*, methyl linoleate interacted with Arg207 via hydrogen bond formation at energy of -13.5116 (**Figure 7**). Sesaminol exhibited the best binding energy (-17.6654) through interaction by hydrogen bonds with Thr62 and Arg207 and arene-cation interaction with Arg207 (**Figure 8**).

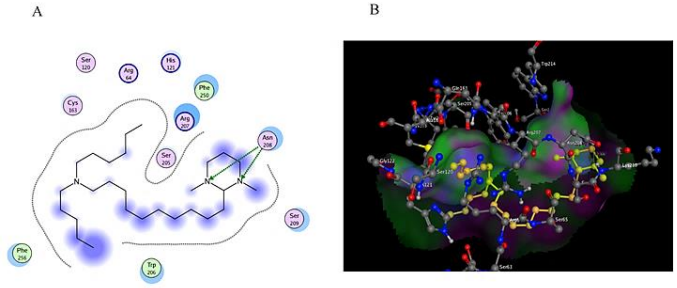


Figure 6: Molecular docking of aberiamine to caspase-3 using MOE (A: two dimensional, B: three dimensional).

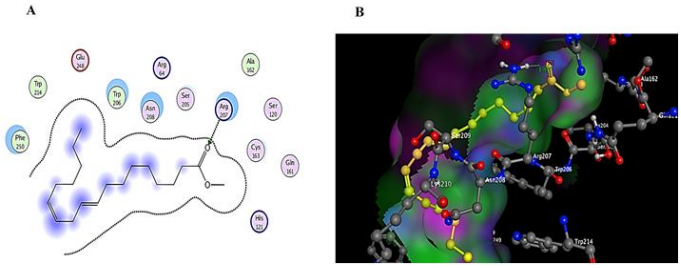


Figure 7: Molecular docking of Methyl linoleate to caspase-3 using MOE (A: two dimensional, B: three dimensional).

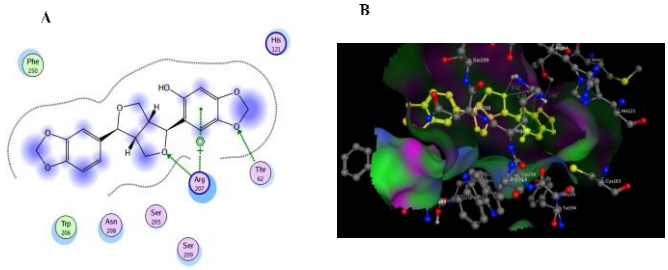


Figure 8: Molecular docking of Sesaminol to caspase-3 using MOE (A: two dimensional, B: three dimensional).

3.6. *In-vivo* antitumor study

3.6.1. Effect of *Sesamum Indicum* and *Aberia Caffra* on liver integrity and plasma aminotransferases activities in DEN-induced liver hepatocellular carcinoma in rats.

Results are presented in Table 3, 4 and graphically illustrated in Figure 9 & 10.

Following DEN (20 mg/kg, i.p.) administration, hepatocellular carcinoma was manifested as an increase in liver index (liver weight/ body weight %) as well as an elevation in plasma ALT and AST levels.

On the other hand, treatment with *Sesamum Indicum* (300 mg/kg/day, p.o.) succeeded to decrease liver index and suppress the plasma levels of ALT and AST when compared to DEN group. However, Treatment with *Aberia Caffra* (300 mg/kg/day, p.o.) showed minimal improvement in liver index as well as plasma ALT and AST levels.

Table 3: Effect of* Sesamum Indicum *and* Aberia Caffra *on liver index DEN-induced liver hepatocellular carcinoma in rats.

Parameter / Groups	Liver index (%) Mean ± SEM
Normal	2.867±0.176
DEN (20mg/kg)	5.233± 0.2404*
DEN+SI (300 mg/kg)	4.033 ± 0.088*#
DEN+AC (300 mg/kg)	4.300 ± 0.230*#

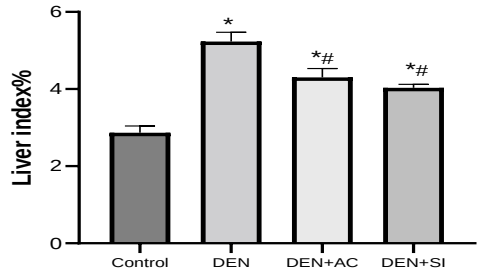


Figure 9. Effect of *Sesamum Indicum* and *Aberia Caffra* on liver index in DEN-induced liver hepatocellular carcinoma in rats. Each value represents the mean of 5 experiments ± S.E. M. *p<0.05 vs. normal, #p<0.05 vs. DEN. SI: *Sesamum Indicum*, AC: *Aberia Caffra* and DEN: diethylnitrosamine

3.6.2. Effect of *Sesamum Indicum* or *Aberia Caffra* on liver Alpha-Fetoprotein content in DEN-induced liver hepatocellular carcinoma in rats.

Results are presented in Table **5** and graphically illustrated in Figure 11

Induction of hepatocellular carcinoma by DEN (20 mg/kg, i.p.) significantly elevated the liver contents of AFP.

Administration of *Sesamum Indicum* (300 mg/kg/day, p.o.) to diseased rats significantly reduced the rise in the

liver content of AFP. On the other hand, *Aberia Caffra* (300 mg/kg/day, p.o.) produced non-significant changes in liver AFP content when compared to the DEN group.

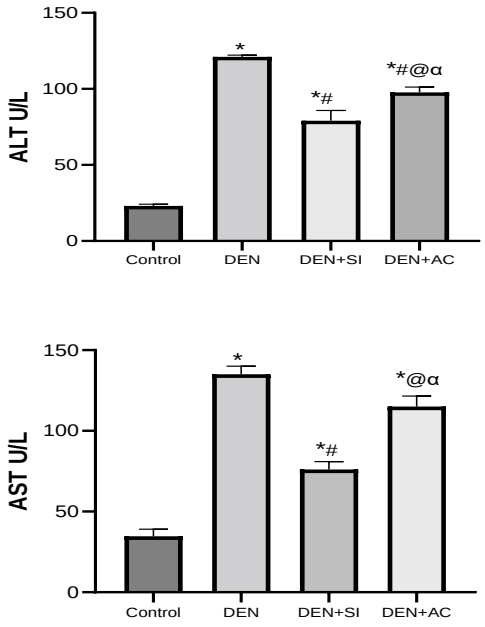


Figure 10. Effect of *Sesamum Indicum* or *Aberia Caffra* on ALT and AST in DEN-induced liver hepatocellular carcinoma in rats. Each value represents the mean of 5 experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. DEN. SI: *Sesamum Indicum*, AC: *Aberia Caffra* and DEN: diethylnitrosamine.

Table 4: Effect of* Sesamum Indicum *or* Aberia Caffra *on plasma aminotransferases activities DEN-induced liver hepatocellular carcinoma in rats

Parameters / Groups	ALT (U/l) Mean ± SEM	AST (U/l) Mean ± SEM
Normal	23.00±1.155	34.67± 4.333
DEN (20mg/kg)	121.0± 1.155*	135.0± 5.033*
DEN+SI (300 mg/kg)	79.00± 6.807*#	76.00± 4.933*#
DEN+AC(300 mg/kg)	97.67± 3.528*#@α	115.0± 6.557*@α

SI: *Sesamum Indicum*, AC: *Aberia Caffra* and DEN:diethylnitrosamine

Table 5: Effect of *Sesamum Indicum* or *Aberia Caffra* on alpha-fetoprotein liver content in DEN-induced liver hepatocellular carcinoma in rats.

Parameter / Groups	Alpha-fetoprotein(ng/mg protein) Mean ± SEM
Normal	6.400±0.568
DEN (20mg/kg)	18.37± 0.866*
DEN+SI (300 mg/kg)	14.73± 0.895*#
DEN+AC (300 mg/kg)	16.10± 0.907*

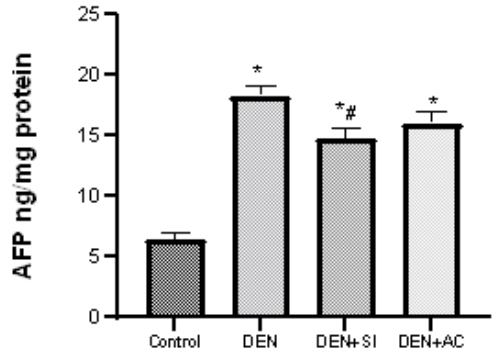


Figure 11. Effect of *Sesamum Indicum* or *Aberia Caffra* on AFP in DEN-induced liver hepatocellular carcinoma in rats. Each value represents the mean of 5 experiments ± S.E.M. *p<0.05 vs. normal, #p<0.05 vs. DEN. SI: *Sesamum Indicum*, AC: *Aberia Caffra* and DEN: diethylnitrosamine.

3.6.3. Effect of *Sesamum Indicum* or *Aberia Caffra* on hepatic histological changes in DEN-induced liver hepatocellular carcinoma in rats.

Results are graphically illustrated in Figure 12

Normal control samples demonstrated normal histological features of rat liver parenchyma with many apparent intact well organized hepatocellular architecture with intact subcellular details (arrow), intact hepatic vasculatures (star) as well as hepatic sinusoids were showed.

Induction of hepatocellular carcinoma by DEN (20 mg/kg, i.p.) showed many records of multiple areas of focal hepatocellular alteration and dysplasia with significant cytomegally, hyperchromatic binucleated cells with prominent nucleoli and many mitotic figures (red arrow), accompanied with moderate dilatation of hepatic Bvs (black star) and hyperplasia of bile ducts (red star). Abundant mononuclear inflammatory cells infiltrate in perivascular areas (yellow arrow). Significant fibroblastic activity was observed with moderate pseudolobulation of hepatic lobules by newly formed collagen fibers.

Sesamum Indicum (300 mg/kg/day) showed persistent records of multiple areas of focal hepatocellular alterations. However; minimal records of mitotic figures were shown with mild cytomegally (red arrow) and vacuolar degenerative changes. Minimal fibroblastic activity was shown. Mild inflammatory cells infiltrates were recorded (yellow arrow).

Aberia Caffra (300 mg/kg/day) showed few focal small sized areas of dysplastic hepatocellular alterations with mild cytomegally or mitotic figures records (red arrow) accompanied with mild fibroblastic activity and inflammatory cells infiltrates (yellow arrow). However; diffuse vacuolar degenerative changes were showed allover hepatic lobules at different zones (black arrow). Moderate dilatation and congestion of hepatic BVs (black star)

4. Conclusion

Aberia caffra, bio-guided fractionation and isolation showed that **petroleum ether**, methylene chloride and alkaloid fractions were potent inhibitors HCC cell lines. The ethyl acetate and aqueous extracts exhibited no cytotoxic activity against *Hep-G2*. Seven compounds **(1-7)** were isolated from **petroleum ether**, methylene chloride extracts afforded no activity. The activity of total **petroleum ether**, methylene chloride extract can be attributed to synergistic effect of their compounds.

While, active compound **(8)** from alkaloid extract was obtained and identified as aberiamine which has a promising cytotoxic activity against *Hep-G2*. Moreover, two active compounds (9-10) from petroleum ether extract of *Sesamum indicum* were obtained and identified as methyl linoleate and sesaminol which have high inhibitory effect on the growth of HCC cell lines. Molecular docking of *Hep-G2* cytotoxic constituents to caspase-3 indicated their ability to bind with the enzyme active site and consequently start apoptosis process of cancer cells.

Sesamum indicum showed improvement in liver enzymes serum level, AFP hepatic content and also in histopathological findings. The effect of *Sesamum indicum* on liver may be due to lignans which considered one type of polyphenolic compounds present in sesame. Although *Aberia Caffra* alcoholic extract and aberamine, the major alkaloid isolated from the extract, exhibit high cytotoxic activity against Hep-G2 cell line, the extract showed minimal improvement in liver enzymes, AFP hepatic content and histopathological findings. *Aberia Caffra* showed similar results as DEN-group. As the correlation between *in-vitro* and *in-vivo* studies depends mostly on pharmacokinetic properties of the administered drug, the weak *in-vivo* activity of *Aberia Caffra* alcoholic extract may be due to poor bioavailability of orally administered drug or rabid drug clearance**.**

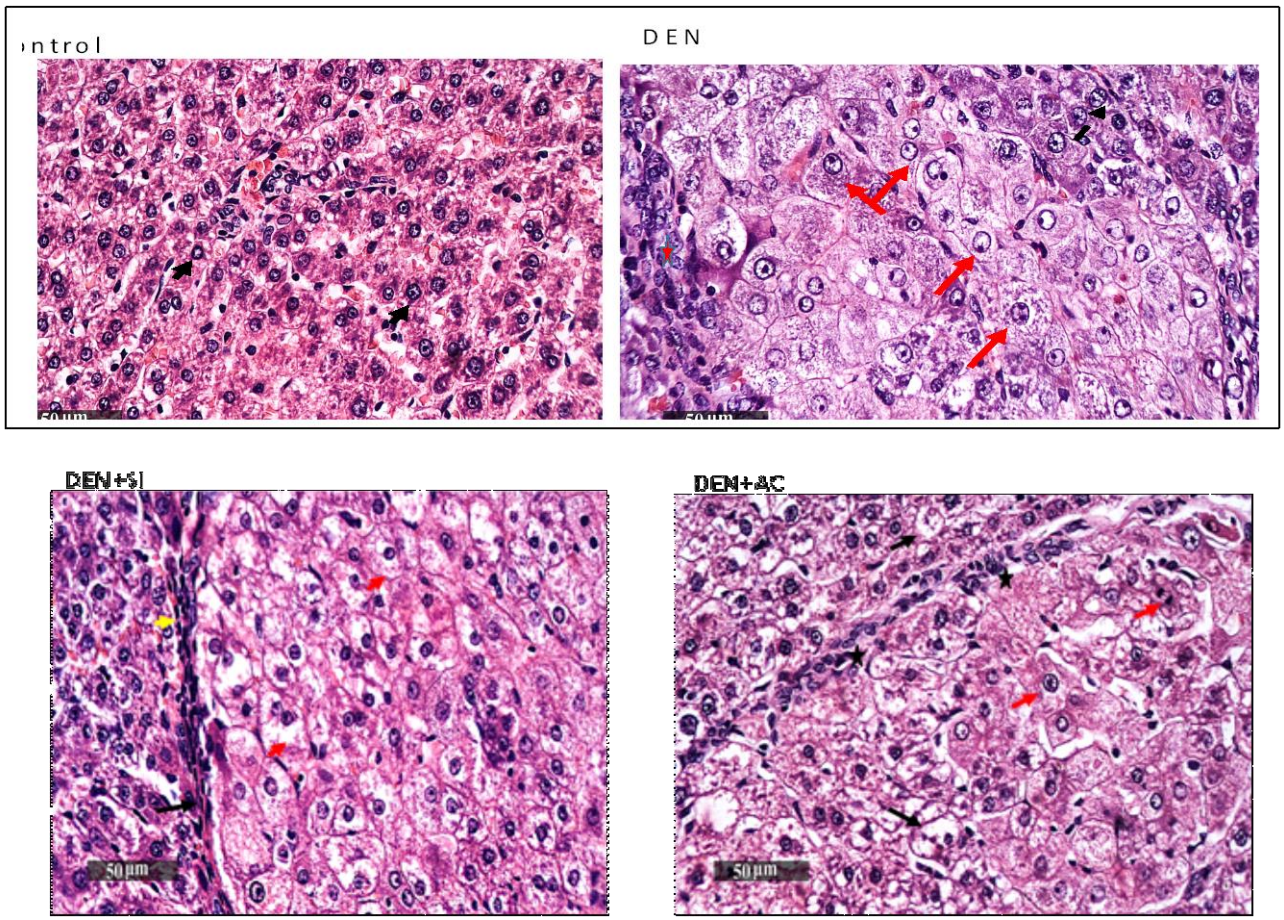


Figure 12. Effect of *Sesamum Indicum* or *Aberia Caffra* on hepatic histological changes in DEN-induced liver hepatocellular carcinoma in rats. (H & E staining) (magnification x40)

5. Acknowledgment

Authors are indebted to Dr. Ahmad Saeed, Prof. of Plant Horticulture, Mushtuhur Agriculture faculty for his kind collection and identification of the plant.

6. Funding sources

No list of funding sources

7. Conflicts of interest

There are no conflicts to declare.

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