Antiproliferative Effect of Sterols from Resin of *Commiphora habessinica*

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Abstract: *Commiphora habessinica* resin is used traditionally to treat various diseases in Ethiopia. In this study the cytotoxic effects of chloroform fraction and cholesterol and lathosterol isolated from the chloroform fraction of the resin of *C. habessinica* were evaluated. In the cytotoxicity assay on A549, A2780, MIA-Paca-2, and SNU-638 cell lines, the chloroform fraction showed cytotoxicity ranging from 0.77-3.35 µg/ml. The chloroform fraction significantly inhibited cell proliferation of A549, A2780, MIA-PaCa-2 and SNU-638, with dose-dependent relation *in vitro*. The chloroform fraction was more sensitive and has a strongest net growth as percent control effects on A549 cell lines. A mixture of cholesterol : lathosterol (47.9% : 52.1%) exhibited a moderate cytotoxicity which is greater than the individual compound towards A549 and A2780 with IC_{50} of 13.77 and 20.36 µg/ml, respectively that might be due to synergetic effect. The GC-MS analysis of chloroform fraction of the resin showed presence of pentacyclic triterpenes as major component (62.98%), sesquiterpene (4.27%), phytosterols (1.53%) and others in trace amount. The isolated compounds from the chloroform fraction were analysed by spectroscopic techniques such as NMR and MS.

Keywords: Chloroform fraction, cholesterol, lathosterol, A549 cell lines, GC-MS.

INTRODUCTION

The genus *Commiphora* Jacq., comprising 150-200 species, occurs in the dry bushlands of tropical Africa and Madagascar, Arabia, India and South America [1, 2]. *Commiphora* species are characterized as small trees or shrubs with spinescent branches, pale-gray bark and reddish-brown resinous exudates [3]. The genus is largely represented in Africa where it is confined to arid and semi-arid areas. South east lowland of Ethiopia is characterized by its high diversity of *Acacia* and *Commiphora* species. Soromessa [4] reported out of total reported species in the genus *Commiphora* about half of them are endemic to the small area of southeastern Ethiopia, northeastern Kenya and Somalia. *C. myrrha* (Nees) Engl. is the most well-known member of the genus *Commiphora*, yielding one of the most important resins of all times, commonly known as myrrh. Myrrh has also been used in the Ayurvedic medical system due to it therapeutic effects for treatment of inflammatory diseases, coronary artery diseases, gynecological disease, obesity, etc. [3].

Previously, we reported dammarane types of triterpenes from the resins of *C. confusa* [5]. Mansumbinone, mansumbinol (16S, 20R)-dihydroxydammar-24-en-3-one [6, 7] and two octanordammarane triterpenes [8] were reported from the resin of *C. kua* (J. F. Royle). Duwiejua *et al*. [9] showed mansumbinone possesses strong anti-inflammatory activity. Form the resin of *C. erlangeriana* we reported polygamain-type lignans, named erlangerin A and erlangerin B, and two lignans related to podophyllotoxin, namely erlangerin C and erlangerin D [10]. The effects of erlangerin C and D closely related to the activity profile of podophyllotoxin: they induced a concentration-dependent cytotoxicity in the murine macrophage cells (RAW 264.7) and a cytostatic effect in HeLa, EAhy926 and L929 cells. On the other hand, erlangerins A and B suppressed cell viability at relatively higher concentrations (EC_{50} values higher than 3 µM as compared with nM concentration range for erlangerins C and D and podophyllotoxin) and their activity appears to be consistent with a cytotoxic mode of action in all cell lines studied [11].

In the area of sample collections for this study, traditionally the gum-resins of *C. habessinica* (Berg) Engl is used to heal burn (in human beings), wound (in cattle), bathing a new born baby as disinfectant and to eradicate cattle ticks [12, 13]. Additionally, the information we gathered by interviewing the local people indicates resin of this plant is used as a detergent of cloths. As part of our ongoing research

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pharmacologically active compounds from the resins of genus *Commiphora* here we describe isolation, analysis and cytotoxic activities of chloroform fraction and sterols isolated from the resin of *C. habessinica*.

MATERIALS AND METHODS

General Experimental Procedure

Structural characterization of the constituents was based on the data obtained from IR, ^{1}D NMR (^{1}H-NMR, ^{13}C-NMR, DEPT-135), ^{2}D NMR (COSY, HSQC, HMBC) and Mass spectroscopy. LC-(TIC/EIC)-MS experiment was used for the accurate determination of isomers and overall purity of isolated sterols. NMR spectra were obtained using Bruker Avance 600 (600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR) and Avance 500 (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR) spectrometer. Chemical shifts (δ) are expressed in ppm relative to TMS at δ = 0 ppm for ^{1}H NMR and relative to $CDCl_3$ at δ = 77.16 ppm for ^{13}C NMR. The infrared (IR) spectra were obtained through Perkin Elmer FTIR spectrometer. LC-ESI-MS and GC-MS spectra were also generated. Flash column chromatography, also known as medium pressure chromatography, was performed on a glass column, using silica gel 60 (230-400 mesh size, E. Merck) as solid phase. Thin layer chromatography was performed on precoated silica gel glass plate (TLC Silica gel 60 F_{254} 20x20 cm, 0.25mm thick made in Germany Merck KGaA, 64271 Darmstad). For TLC staining Phosphomolybdic acid (PMA) (E. Merck) solution was prepared. After applying to TLC it has been heated gently to 120 °C until spots appear.

Chemicals

All solvents (extra pure) were purchased from Daejung chemicals and metals Co., LTD, Korea. Sodium sulfate anhydrous (extra pure) was purchased from Duksan pure chemical Co. LTD, Korea.

Plant Materials

Resins and other botanical specimens of *C. habessinica* were collected from surrounding of Yabello town, the Oromia regional state, Borena, Southern Ethiopia, in October, 2016. The plant was identified and a voucher herbarium specimen 072771 was deposited at Addis Ababa University, Ethiopia.

Extraction Yields and Isolation of Compounds

C. habessinica resins (218.6 g) were extracted three times (24 h x 730 ml) with methanol at room temperature and yielded (63.82 g, 29.19%) (Table **1**). After the solvent had been removed under reduced pressure, the residue of the crude methanol extract (60 g) was suspended in H_2O and then successively partitioned with solvents of different polarity such as *n*-hexane (1.87 g, 3.12%), chloroform (51.80, 86.33%), EtOAC (0.59 g, 0.98%), and *n*-BuOH (1.68 g, 2.80%) (Scheme **1**, Table **1**). Based on the bioactivity-guided assay result, the chloroform fraction was selected. The chloroform soluble fraction (5 g) was fractionated by flash column chromatography (FCC) on silica gel 60 (230-400 mesh), using increasing polarity of *n*-hexane:EtOAC (8:1 to 100% EtOAc), to yield one hundred and six sub-fractions of 18 ml each. Based on the TLC analysis those fractions with similar TLC profile were combined and further purified by flash column chromatography using an isocratic solvent system of *n*-hexane **:** EtOAC (4**:**1) yielded compounds **1**, **2** and a mixture **1** and **2** (47.85%**:**52.15%).

Table 1: Percentage Yield of Crude MeOH Extract and Fractions of the Resin of *C. habessinica*

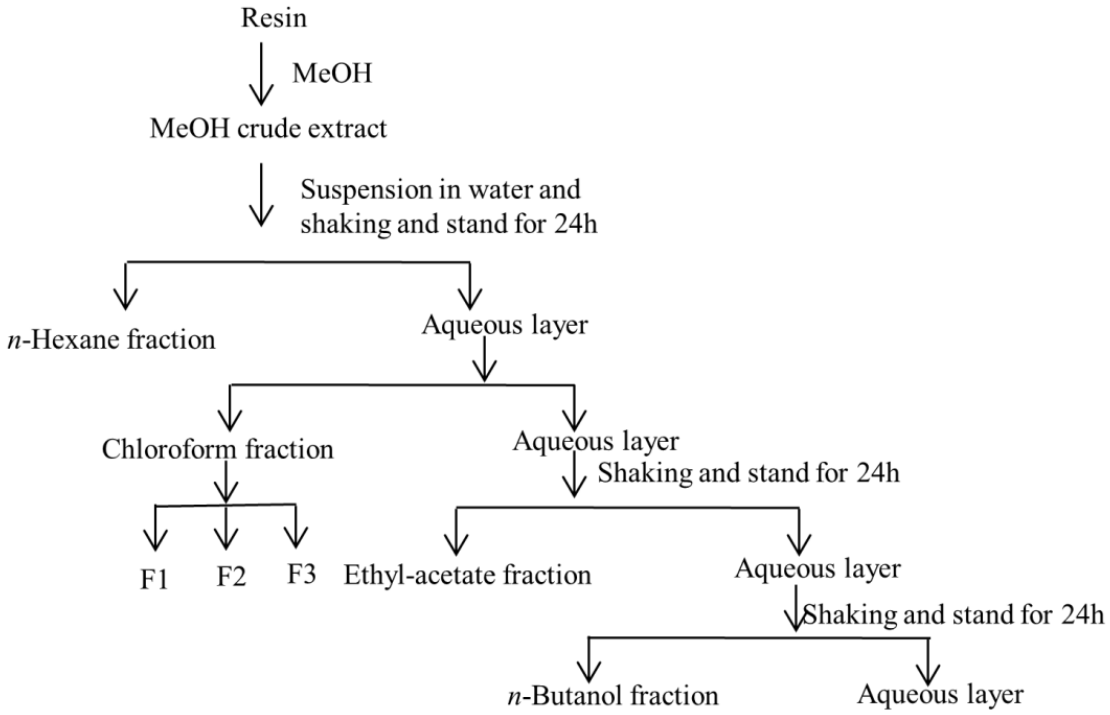
Extract/Fraction	% yield
Crude methanol extract	29.19
n-Hexane fraction	3.12
Chloroform fraction	86.33
EtOAC fraction	0.98
n-BuOH fraction	2.80

GC-MS Analysis

GC-MS analysis was done by using a GC (7890B, Agilent Technologies, USA) coupled with an MS (5977A Network, Agilent Technologies). The GC had an HP 5MS column (non-polar column, Agilent Technologies), 30 m × 250 µm internal diameter (i.d.) and 0.25 µm film thickness. The carrier gas was helium flowing at a rate of 1 mL/ min. The injector temperature was 230 °C and the injection mode was split mode with split Ratio 10:1. The initial oven temperature was 40˚C held for 5 min. It was raised to 250˚C at 6˚C/min held at this temperature for 20 min. The total run-time was 60 min. Mass spectra were recorded in EI mode at 70 eV, scanning the 50-500 m/z range. The identification of the chloroform fraction compounds was performed by comparing the mass spectra of the compounds with those in the database of NIST11 and literature data.

Cytotoxic Activity Assay

The cell lines used in this study are the human non-small cell lung cancer cell lines (A549), ovarian cancer



Scheme 1: Schematic representation of fractionation and separation of compounds from *C. habessinica* resins based on polarity.

cell line (A2780), pancreatic cancer cell line (MIA-Paca-2) and stomach cancer cell line (SNU-638) and were maintained using RPMI1640 cell growth medium (Gibco, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS) (Gibco), and grown at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Assessment

The cytotoxicity of the crude extract, fraction and compounds against cultured human tumor cell lines was evaluated by the sulforhodamine B (SRB) method [14]. Each tumor cell line was inoculated over standard 96-well flat-bottom microplates and then incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were then incubated with the serially diluted each samples. After continuous exposure to the compounds for 72 h, the culture medium was removed from each well and the cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again and then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader. Each experiment was conducted in triplicate. The IC_{50} values of compounds were calculated by the nonlinear regression analysis [14].

RESULTS

Characterization of Compounds

Mixture of Compounds 1 and 2

Fraction 1 was isolated as an amorphous powder; TLC analysis using *n*-hexane:EtOAC (4:1) showed a single spot. IR spectra of the mixture showed a broad absorption bands at 3334.72 cm^{-1} indicating the presence of hydroxyl group held together by intermolecular force, hydrogen bonding (Supporting information Figure **S1**). Bands 1650 and 1680 cm^{-1} show two weak absorption bands which are attributed to the presence of two unsaturated (C=C) moieties and bands at 2930 cm^{-1} indicate CH_3 and CH_2 absorptions in the molecules.

The HPLC analysis of fraction 1 indicates the presence of two major peaks at RT 1.32 and 1.94 min with area % of 47.85 and 52.15, respectively (Supporting information Figure **S2**) indicating two compounds though TLC showed a pure single spot. Its EI-MS spectrum exhibited molecular ion peak at *m/z* 386, with the molecular formula $C_{27}H_{46}O$ (Both sterols have the same molecular formula $C_{27}H_{46}O$, but a different retention time), suggesting they are isomers with different unsaturation pattern (Supporting information Figure **S3**).

Table 2: ^{1}H and ^{13}C NMR Spectroscopic Data of Compounds 1 and 2 ($CDCl_3$)

Position	Compound 1		Compound 2	
	δ_C, Type	δ_H (J in Hz)	δ_C, Type	δ_H (J in Hz)
1	37.3, CH_2		37.2, CH_2	
2	32.0, CH_2		31.5, CH_2	
3	71.8, CH	3.52 (1H, m)	71.1, CH	3.59 (1H, m)
4	42.3, CH_2		38.0, CH_2	
5	140.8, C		40.3, CH	
6	121.7, CH	5.35 (1H, m)	29.7, CH_2	
7	31.9, CH_2		117.4, CH	5.16 (1H, m)
8	31.7, C		139.6, C	
9	50.1, CH		49.5, CH	
10	36.5, C		34.2, C	
11	21.1, CH_2		21.6, CH_2	
12	39.8, CH_2		39.6, CH_2	
13	42.3, C		43.4, C	
14	56.8, CH		55.1, CH	
15	24.3, CH_2		22.9, CH_2	
16	28.2, CH_2		27.9, CH_2	
17	56.2, CH		56.2, CH	
18	11.9, CH_3	0.67 (3H, s)	11.8, CH_3	0.53 (3H, s)
19	19.4, CH_3	1.01 (3H, s)	13.0, CH_3	0.79 (3H, s)
20	35.8, CH		36.2, CH	
21	18.7, CH_3	0.91 (3H, d, 6.5 Hz)	18.8, CH_3	0.91 (3H, d, 6.5 Hz)
22	36.2, CH_2		36.1, CH_2	
23	23.8, CH_2		23.9, CH_2	
24	39.5, CH_2		39.5, CH_2	
25	28.0, CH		28.0, CH	
26	22.6, CH_3	0.86 (3H, d, 2.2 Hz)	22.6, CH_3	0.86 (3H, d, 2.8 Hz)
27	22.8, CH_3	0.87 (3H, d, 2.3 Hz)	22.8, CH_3	0.87 (3H, d, 2.8 Hz)

^{1}H NMR spectrum (Table **2**, Supporting information Figure **S4**) of the mixture exhibited five methyl signals at δ_H 0.53 (s), 0.68 (s), 0.80 (s), 0.86 (d), 0.87 (d), 0.91 (d) and 1.01 (s). An olefinic proton signals were observed at δ_H 5.16 (m) and 5.35 (m). The former and latter signals are characteristic for olefinic H-7 and H-6 in steroid compounds. The observation of a proton signal at δ_H 3.52 and 3.59 as a multiplet indicated the presence of a hydroxyl groups in the mixture. The ^{13}C NMR analysis revealed 50 carbon peaks (Supporting information Figure **S4**). Out of these, four carbon signals at δ_C 56.2, 28.0, 22.8 and 22.6 ppm are intensively displayed, these corresponds to C-17, C-25, C-26 and C-27 respectively . This is due to overlap of the side chain carbons of the two sterols at the specified carbon positions. Furthermore, the ^{13}C NMR spectrum showed intense signals at δ_C 121.7 and 117.4 and less intense ones at δ_C 140.8 and 139.6 attributed was to alkenyl carbon atoms. Additionally, assignment aided by the DEPT and 2D NMR data such as HSQC, COSY and HMBC (Supporting information Figure **S5**) and comparison of the spectra with literature data [15] the two C-27 sterol isomers were identified as cholesta-5-ene-3β-ol and cholest-7-ene-3β-ol which are commonly known as cholesterol and lathosterol, respectively.

Compound 1

The compound was isolated as a white amorphous powder from $CHCl_3$ fraction using column

chromatography eluting with *n*-hexane/EtOAc (4:1) followed by recrystallization with *n*-hexane. The compound has a broad and sharp IR band at 3434.07 cm^{-1} due to OH stretching. The characteristic strong peak at 2930.32 cm^{-1} is due to CH_2 symmetric stretching vibration (Supporting information Figure **S6**). HPLC analysis of the compound showed its purity (Supporting information Figure **S7**). The molecular formula of compound **1** was established as $C_{27}H_{46}O$ from IR, NMR spectral data. The ^1H NMR spectral data of the compound displayed proton signals due to five methyl groups comprising two tertiary methyl groups at δ_H 0.67 (3H, s, CH_3-18) and 1.01 (3H, s, CH_3-19), three secondary methyl groups at δ_H 0.91 (3H, d, J= 5.0 Hz, CH_3-21), δ_H 0.87 (3H, d, J = 2.3 Hz H_3-27) and δ_H 0.86 (3H, d, J = 2.2 Hz H_3-26) (Supporting information Figure **S8**). In the ^1H NMR spectrum a signal at δ_H 5.35 (H-6, m) and a downfield shift of C-5 and C-6 at δ_C 140.8 and 121.7, respectively in the ^{13}C NMR spectrum and one oxymethine proton at δ_H 3.52 and a downfield shift of C-3 at δ_C 71.8 were consistent with similar reported steroidal compounds [15]. The ^{13}C NMR spectral data with the aid of a DEPT experiment (Table **2**, Supporting information Figure **S8**), displayed the compound has 27 carbon signals, including five methyl, eleven methylene, eight methine, three quaternary carbons. Based the above spectral information and comparison of the NMR spectral data of the compound with literature data [15] compound **1** was identified as cholesta-5-en-3β-ol commonly known as cholesterol (Figure **1**).

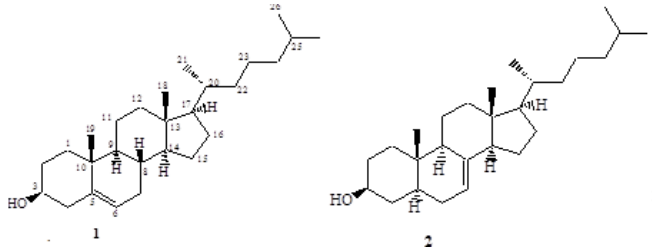


Figure 1: Structure of cholesterol (**1**) and lathosterol (**2**).

Compound 2

The compound was obtained as a white amorphous powder. The IR spectrum showed major absorption bands at 3387.51 cm^{-1} due to hydroxyl (OH) stretching (Supporting information Figure **S9**). The EI-MS spectrum showed a molecular ion peak at *m/z* 386 $[M]^+$ (Supporting information Figure **S10**). IR, ^1H NMR, ^{13}C NMR and DEPT-135 spectrum experiment enabled the determination of the molecular formula $C_{27}H_{46}O$ (Supporting information Figure **S11**). The ^1H NMR spectrum of compound **2** displayed proton signals due to five methyl groups comprising two tertiary methyl groups at δ_H 0.53 (3H, s, CH_3-18) and 0.79 (3H, s, CH_3-19)., three secondary methyl groups at δ_H 0.91 (3H, d, J= 6.5Hz, CH_3-21), δ_H 0.86 (3H, d, J = 2.2 Hz CH_3-26) and δ_H 0.87 (3H, d, J = 2.3 Hz CH_3-27) (Table **2**). One olefinic proton signal at δ_H 5.16 (1H, m) was indicative of a single unsaturation unit on ring B of the steroid. The ^1H NMR spectrum of compound **2** also evidenced one oxygenated methine group at δ_H 3.59 ppm (1H, m). Carbon signals in the olefnic region (δ_C 117.4 and 139.6) (Table **2**) are indicates at a sterol skeleton with a Δ7 double bond [15]. The location of Δ7 double bond was determined by a three bond HMBC correlations (Supporting information Figure **S12**). $^3J_{C\text{-}H}$ from H-7 to C-9 (δ_C 49.5), C-14 (δ_C 55.1) and a two bond correlation $^2J_{C\text{-}H}$ from H-7 to C-6 (δ_C 29.7), while the location of one hydroxyl group at C-3 was supported by both COSY correlations from H-3 to H-2, H-4 and a two bond HMBC correlation $^2J_{C\text{-}H}$ from H-3 to C-4 (δ_C 38.0) and vice versa, H-4 to C-3 (δ_C 71.1) and from H-4 to C-5 (δ_C 40.3). Based the above spectral information, and comparison of the NMR spectral data of the compound with literature data [15, 16] compound **2** was identified as cholest-7-en-3β-ol commonly known as lathosterol (Figure **1**).

GC-MS Analysis of the Chloroform Fraction

The chloroform fractions obtained the resin from *Commiphora habessinica* was analysed by means of GC/MS. The result showed the chloroform fraction constitute 37 compounds out of which pentacyclic triterpenes were dominant (62.98%). The main components of the chloroform fraction were pentacyclic triterpenes such as α-amyrin (24.13%), 3-epimoretenol (22.35%), β-amyrin (11.10%) and lupeol (5.40%). The fraction also contains 2(1H) naphthalenone, 3,5, 6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl) (3.52%), palmitic acid vinyl ester (2.29%), benzene, 1-(chloromethyl)-2-methyl (1.31%), lathosterol (1.04%) and 1,3-cyclopentadiene, 2,3,4,5-tetramethyl-1-(4-pentenyl) (1.04%) (Figure **2** and Table **3**). Structures of major pentacyclic tritepenes identified from the chloroform fractions were shown in Figure **3**.

Cytotoxicity of Chloroform Fractions

The emphasis of this work was to evaluate the cytotoxic activity of chloroform fraction against four human cancer cell lines, A549, A2780, MIA-Paca-2 and SNU-638. The IC_{50} value and the dose response curve of the chloroform fraction (Table **4**, Figure **4**) showed cytotoxicity ranging from 0.77µg/ml for A549 to 3.35µg/ml for MIA-PaCa-2. The chloroform fraction exhibited significantly inhibiting cell proliferation on the

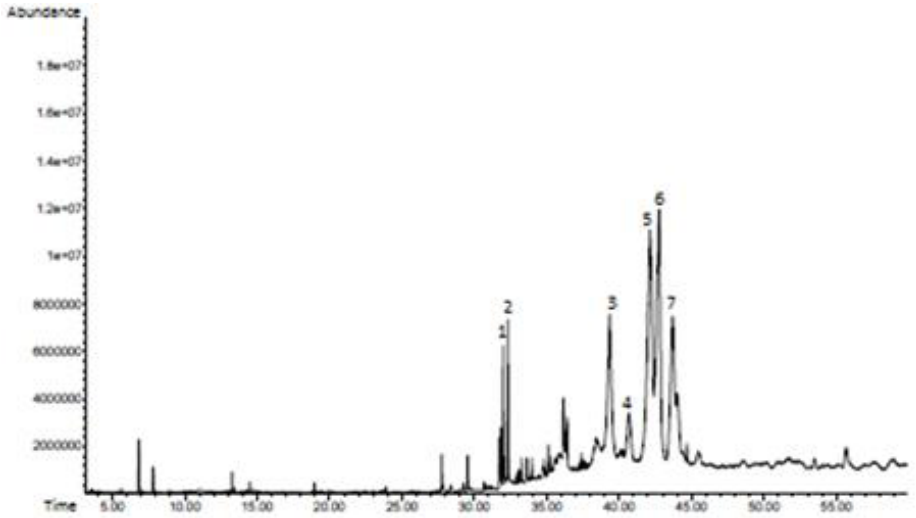


Figure 2: GC of chloroform fraction of *C. habessinica*, **1**: Isoquinoline (2.43%) **2**: Palmitic acid vinyl ester (2.29%) **3**: β-Amyrin (11.10%) **4**: 2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl) (3.52%) **5**: α-Amyrin (24.13%) **6**: 3-Epimoretenol (22.35%) **7**: Lupeol (5.4%).

Table 3: Relative Concentration (%) of Components of the Chloroform Fractions of *C. habessinica*

Compound name	RT (min)	Molecular Formula	Mol. Wt (amu)	% of Total
Ethylbenzene	6.835	C_8H_{10}	106.08	0.65
Styrene	7.819	C_8H_8	104.06	0.35
Oxalic acid, 2-ethylhexyl isohexyl ester	13.261	$C_{16}H_{30}O_4$	286.21	0.20
Decane, 3,6-dimethyl	14.502	$C_{12}H_{26}$	170.20	0.10
Pentadecanal-	27.749	$C_{15}H_{30}O$	226.23	0.62
Heptadecanal	29.540	$C_{17}H_{34}O$	254.26	0.48
2-[1-(4-Cyano-1,2,3,4-tetrahydronaphthyl)]propanenitrile	31.730	$C_{14}H_{14}N_2$	210.12	0.72
Isoquinoline	31.834	C_9H_7N	129.06	0.86
Quinoline	31.990	C_9H_7N	129.06	2.43
Palmitic acid vinyl ester	32.315	$C_{18}H_{34}O_2$	282.26	2.29
Indole	33.065	C8H7N	117.06	0.16
3-[1-(4-Cyano-1,2,3,4-tetrahydronaphthyl)]propanenitrile	33.266	$C_{14}H_{14}N_2$	210.12	0.32
3,6,9,12-Tetraoxatetradecane-1,14-diyl bis(2-methylbutanoate)	33.624	$C_{20}H_{38}O_8$	406.26	0.38
Tridecane	33.969	$C_{13}H_{28}$	184.22	0.29
Hexadecane	34.737	$C_{16}H_{34}$	226.27	0.17
Methyl 12-oxo-9-dodecenoate	34.810	$C_{13}H_{22}O_3$	226.16	0.27
2H-Pyran, 2-(8-dodecynyloxy)tetrahydro-	35.113	$C_{17}H_{30}O_2$	266.23	0.47
17-Pentatriacontene	35.250	$C_{35}H_{70}$	490.55	0.22
Octacosanoic acid	35.599	$C_{28}H_{56}O_2$	424.43	0.44
Benzene, 1-(chloromethyl)-2-methyl-	36.154	C_8H_9Cl	140.04	1.31
Cyclohexanecarboxylic acid, hexyl ester	36.217	$C_{13}H_{24}O_2$	212.18	0.87
(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	36.332	$C_{22}H_{20}OS$	332.12	0.61
Benzeneacetonitrile, .alpha.-methylene-	36.412	C_9H_7N	129.06	0.74
(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	37.385	$C_{22}H_{20}OS$	332.12	0.21
Octadecanoic acid	37.574	$C_{18}H_{36}O_2$	284.27	0.17

(Table 3). Continued.

Compound name	RT (min)	Molecular Formula	Mol. Wt (amu)	% of Total
13,27-Cycloursan-3-one	38.398	$C_{30}H_{48}O$	424.37	0.31
Ursa-9(11),12-dien-3-ol	38.489	$C_{30}H_{48}O$	424.37	0.18
β-Amyrin	39.347	$C_{30}H_{50}O$	426.39	11.10
2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-	40.675	$C_{15}H_{22}O$	218.17	3.52
α– Amyrin	42.117	$C_{30}H_{50}O$	426.39	24.13
3-Epimoretenol	42.769	$C_{30}H_{50}O$	426.39	22.35
5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S cis)-	43.679	$C_{15}H_{22}O$	218.17	0.13
Lupeol	44.011	$C_{30}H_{50}O$	426.39	5.40
Decanedioic acid, bis(2-ethylhexyl) ester	44.675	$C_{26}H_{50}O_4$	426.37	0.49
1,3-Cyclopentadiene, 2,3,4,5-tetramethyl-1-(4-pentenyl)-	45.476	$C_{14}H_{22}$	190.17	1.04
Lathosterol	55.678	$C_{27}H_{46}O$	386.36	1.04
1,4-Bis(6-methylpyridyl-3)butadiyne	58.957	$C_{16}H_{12}N_2$	232.10	0.64

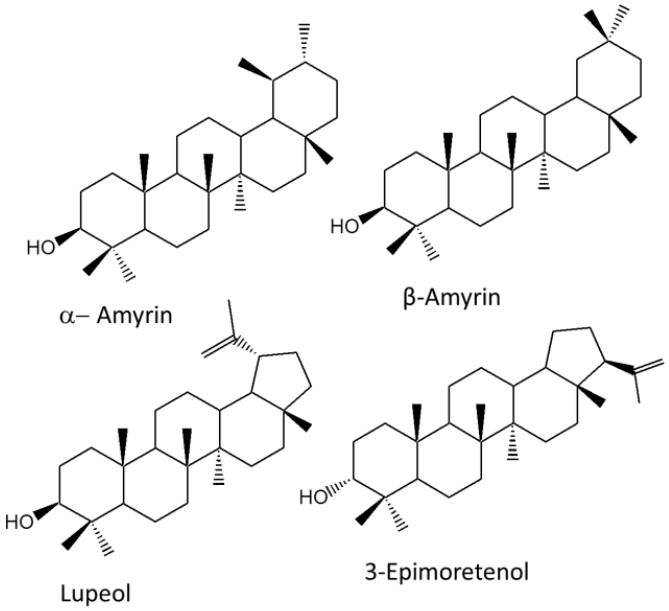


Figure 3: Structures of pentacyclic tritepenes identified by GC-MS from chloroform fraction of *C. habessinica.*

all four cell lines with dose-dependent relationship *in vitro*. Furthermore, the data exhibited the chloroform fraction was more sensitive and has stronger net growth as percent control effects on A549 cell lines and has comparable anti-proliferative effect to Etoposide, the positive control.

Cytotoxicity of Isolated Compounds

Dose response of chloroform fraction, pure compounds **1** and **2** and their mixture against the four cancer cell lines are summarized in Figure **4** and IC_{50} values were summarized in Table **4**.

DISCUSSION

Cancer continues to be a major health problem worldwide despite the enormous efforts that have been made in the search for novel drugs. In the study, In this study, we used methanol to extract the resin of the plant. The reason to use methanol as extraction solvent was that methanol can extract both polar and none-polar natural products, and its extraction effect is better than any other solvents. Additionally methanol can be removed easily compared with many other polar solvents.

Table 4: IC_{50} Values of the Chloroform Fraction and Isolated Compounds from Resin of *C. habessinica* Against four Cancer Cell Lines Using SRB Assay

Sample	Cell lines and IC_{50} (µg/ml)			
	A549	A2780	MIA-PaCa-2	SNU638
Chloroform fraction	0.77	3.03	3.35	3.02
Cholesterol (**1**)	N.C	N.C	N.C	N.C
Lathosterol (**2**)	22.25	N.C	N.C	N.C
Mixture * of **1** and **2**	13.77	20.36	N.C	N.C
Etoposide	0.09	0.08	1.1	0.67

IC_{50} (Inhibition of cell growth by 50). Data was generated by experiments performed in triplicates. N.C. No cytotoxic activity at highest concentration tested. *Mixture of **1** and **2** in a ratio of ca. 1:1.

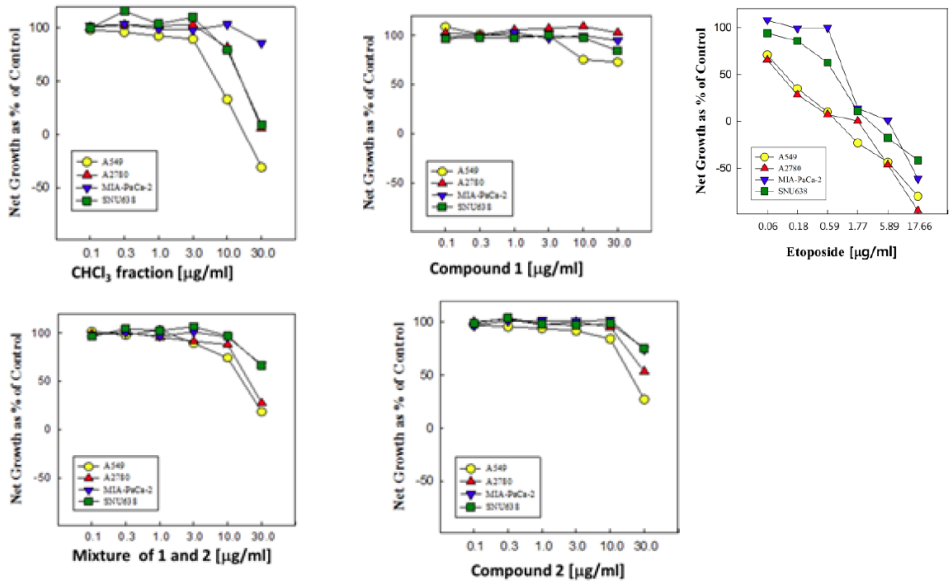


Figure 4: Dose response curve of the $CHCl_3$ fraction, a mixture, compound **1** and compound **2** isolated from the $CHCl_3$ fraction of *C. habessinica* resin using SRB assay. Etoposide was used as a positive control. *Mixture of **1** and **2** in a ratio of ca. 1:1.

Our bioassay results indicted the methanol crude extract and solvent fractions of the resin of *C. habessinica* that have potential cytotoxicity against four human cancer cell lines, A549, A2780, MIA-Paca-2 and SNU-638. Among the solvent fractions tested, the chloroform fraction exhibited significantly inhibiting cell proliferation on the all four cell lines with dose-dependent relationship *in vitro* and the chloroform extract was more sensitive and has strong net growth as percent control effects on A549 cell lines.

To the best of our knowledge there is no previous report on the cytotoxicity activity of the isolated sterols (cholesterol and lathosterol) on A549, A2780, MIA-Paca-2, and SNU-638 cancer cell lines. Compound **1** (cholesterol) was previously reported from *Commiphora abyssinica* [17]. Cholesterol was also previously reported from *Guggulu*, the gum resin exudates from the tree *Commiphora mukul* (Hook, ex Stocks) Engl. along with two other new C_{21} isomeric phytosterols, which were identified as 4,17(20)-(trans)-pregnadiene-3,16-dione (*Z*-guggulsterone) and 4,17 (20)-(cis)-pregnadiene-3,16-dione (*E*-guggulsterone). Guggulu is one of commercial resin in India because of its medicinal values [18]. *C. mukul* is extensively used in the Ayurvedic medicine to treat inflammation such as hyperlipidemia, obesity, and arthritis [19-22]. Additionally, guggulsterone has anticancer potential as indicated by its ability to suppress the proliferation of a wide variety of human tumor cell lines [23]. Bhat et al. [24] reviewed guggulsterone has been shown to possess cancer chemopreventive and therapeutic potential as established by *in vitro* and *in vivo* studies. Mencarelli et al. [23] also reported *trans*-guggulsterone protects mice against development of sign and symptoms of colon inflammation.

Cholest-7-en-3β-ol (lathosterol) was isolated here for the first time from the resin of *C. habessinica*. Previously, Erasto [25] reported cholest-7-en-3β-ol (**2**)

and 4α-methyl-cholest-7-en-3β-ol (**3**) from stem sap of *Commiphora eminii.* The sterols **1** and **2** were screened for antimycobacterial activity against *Mycobacterium madagascariense* and *Mycobacterium indicus pranii* and only cholest-7-en-3β-ol (**2**) exhibited antimycobacterial activity with MIC values of 1.6 mg/ml against both mycobacteria strains [25]. Additionally, cholest-7-en-3β-ol (**2**) was reported to have a potent antigenotoxic activity against mutagens both MNNG and NQO [16]. 4α-Methyl-cholest-7-en-3β-ol has previously been reported as a potential precursor in the biosynthesis of cholest-7-en-3β-ol (**2)** and other sterols in Echinoderms [26, 27]. It has been reported that the starfish *Asterias rubens* can convert mevalonic acid into cholest-7-en-3β-ol (**2**) by a pathway similar to that established for sterol biosynthesis in mammals [27-29]. An alternative route for Δ7 sterol production in starfish demonstrated that *Pisaster ochraceus* could convert ingested cholest-5-en-3β-ol (**1**) into cholest-7-en-3β-ol (**2**) [30]. Kamal-Eldin and Moazzami [31] reviewed the use of plant sterols and stanols as cholesterol - lowering ingredients in foods and nutraceuticals preparations. Therefore, cholest-7-en-3β-ol isolated here from the resin of *C. habessinica* and a mixture of cholesterol and lathosterol might have a cholesterol-lowering application in food industry.

The cytotoxicity of the isolated compounds here from the chloroform fraction of *C. habessinica*, displayed no significant anti-tumour activity in the SRB assay. The cytotoxic effects of the isolated compounds of *C. habessinica* are summarized in Table **4**. Interestingly, cholesterol had no significant effect on all cancer cell lines tested which is far less than 30 µg/ml. Besides this, lathosterol was cytotoxic on A549 cancer cell line with IC_{50} 22.25 µg/ml. Both cholesterol and lathosterol have the same chemical formula but differ only on the site of unsaturation (i.e position of the double bond) Δ5 for cholesterol and Δ7 for lathosterol. It may be that difference in the position of double bond account for the cell proliferative activity observed for lathosterol against A549 cancer cell line. Unlike the two independent sterols, a mixture of these two steroids showed a significant cytotoxicity towards A549 cancer cell line with IC_{50} 13.77 µg/ml. The cytotoxicity towards A549 cancer cell line by the mixture is nearly double than the cytotoxicity of lathosterol. Cytotoxicity was also observed by the mixtures on A2780 with IC_{50} 20.36 µg/ml. These results indicate that effective component(s) of *C. habessinica* resin, which were responsible for cytotoxicity of the chloroform fraction against the four cancer cell lines used in this study, have not been identified. As the about 50% of cholesterol and lathosterol mixture have a promising cytotoxic activity in aforementioned cell line, more study on cytotoxicity study of theses sterols at various concentrations might be important to find out their optimum cytotoxic activity. GC-MS analysis of the chloroform fraction revealed the presence of pentacyclic tritepenes such as α-amyrin, 3-epimoretenol, β-amyrin and lupeol as major compounds. Vázquez et al. [32] reviewed presence of α-amyrin and β-amyrin from various plant sources. α, β-Amyrins have been shown to exhibit various pharmacological activities *in vitro* and *in vivo* against inflammation, microbial, fungal, viral infections and cancer cells. The strong cytotoxicity of the chloroform fraction might be due to presence of these pentacyclic triterpenes in the plant.

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SUPPORTING INFORMATION

The supporting information can be downloaded from the journal website along with the article.

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