Investigating *Volutaria abyssinica* as a potential source for cytotoxic sesquiterpenoids

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Abstract: Phytochemical investigation of *Volutaria abyssinica* (A. Rich.) C. Jeffrey led to the isolation of three sesquiterpene lactones, *viz*., amberboin, lipidiol and cynaropicrin along with daucosterol and 20-hydroxy ecdysone. All these compounds are identified for the first time in the plant. Their structures were determined based on chemical and spectroscopic data. The sesquiterpene lactones were evaluated for their *in vitro* cytotoxicity against four human tumor cell lines and the results were explained through a molecular modeling study.
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Key words: *Volutaria abyssinica*, Asteraceae, Centaureinae, sesquiterpenoids, amberboin, lipidiol, cynaropicrin, ecdysteroids, cytotoxicity, *in silico*, molecular modeling.

1. Introduction

Volutaria abyssinica (A. Rich.) C. Jeffrey (syn *Centaurea hochstetteri* Oliv. & Hiern, *Amberboa hochstetteri* Oliv. & Hiern, buching. ex Soják) (Wood 1997; Greuter et al., 2005; Greuter 2008) belongs to the genus *Volutaria* Cass., tribe Cardueae, subtribe Centaureinae of the Asteraceae. Plants are annual herbs. The genus comprises eighteen species growing in dry stony places in the Mediterranean and Irano-Turanian region from Arabia and Iran to Morocco (Kadereit and Jeffrey, 2007). According to Bruno et al. (2013), the majority of genera in Centaureinae have not yet been investigated for their sesquiterpene profile. Sesquiterpenes have a recognized potential as anticancer agents. To date, several members are in ongoing clinical trials, such as artemisinin, dimethyl-amino-parthenolide and thapsigargin (reviewed in Ghantous et al., 2010; van Haaften et al., 2015). So, this work was conducted in contribution to the investigation of the sesquiterpene content of one of the species in this subtribe; *viz*., *V. abyssinica* (A. Rich.) C. Jeffrey, which has not been examined so far. Previous investigations of other *Volutaria* species revealed the presence of sesquiterpenoids and flavonoids (Mezache et al., 2010; Zaabat et al., 20011). In this work we report the isolation of sesquiterpene lactones and other secondary metabolites identified for the first time from *V. abyssinica*. The complete structure elucidation and determination of the relative stereochemistry of two of the isolated sesquiterpenes are described here for the first time. The isolated sesquiterpenes were evaluated for their *in vitro* cytotoxicity against four human tumor cell lines using MTT assay and the results were explained through *in silico* molecular modeling study.

2. Results and discussion:

Phytochemical investigation of *V. abyssinica* (A. Rich.) C. Jeffrey, led to the identification and chemical characterization of five compounds reported for the first time from this species. These belonged to various structural classes, *viz*., sesquiterpene lactones, sterols and phytoecdysteroids. Column chromatography of the CH_2Cl_2 extract on silica gel afforded compounds **1**-**4**, while that of the n-butanol extract afforded compound **5** (Figure 1).

Compound **1** was obtained as crystalline solid, m.p 148-149 ^{o} C. EI mass spectrum showed a quazi-molecular ion peak at m/z 265 $[M+H]^+$, corresponding to the molecular formula $C_{15}H_{20}O_4$. ^{13}C-NMR spectrum displayed 15 carbon resonances attributed to a sesquiterpene lactone planar structure (experimental section 3.7). These included: signals due to two methyls at δ 10.2 and 13.6 corroborated to proton signals at δ 1.32 (d, J = 7.7 Hz) and 1.15 (d, J = 6.9 Hz) and assigned by HMBC and HMQC experiments (Figure 2) to CH_3-13 and CH_3-15, respectively; a lactone carbonyl carbon at δ 180.5 and a saturated keto carbonyl at δ 220.0. The ^{1}H-NMR spectrum displayed two broad singlets at δ 4.74 and 4.80 attributable to a pair of vinyl hydrogens of an exomethylene group which were assigned to C-14 (δ 113.7), based on observed HMBC correlations between these protons and each of the carbon resonances at δ 40.0 (C-1) and 48.3 (C-9) (Figure 2).

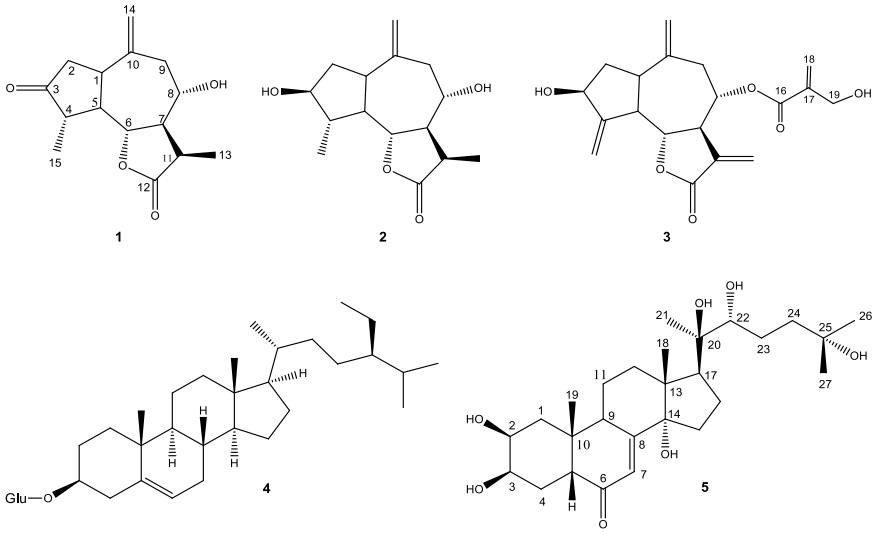

Figure 1. Structures of compounds **1**- **5**.

Another two downfield signals in the ^{1}H-NMR spectrum at δ 3.73 (ddd, *J* = 9.9, 13.8, 6.1 Hz) and 4.22 (dd, *J* = 8.5, 9.0 Hz) indicated vicinity to oxygenated carbons and were assigned to H-8 and H-6, respectively. The large coupling constants of H-6 as well as the absence of NOE correlations with H-5 and H-7 (Figure 3) indicated the *trans* fusion of the lactone ring, while correlation peaks in the NOESY spectrum between the signals at δ 3.18 (m , H-1) and 2.33 (m, H-5) indicated *cis* fusion of the two rings at these positions. NOE correlations between H-11 (δ 2.81, m) and H-9α (δ 2.18, dd, *J* = 13.8, 10 Hz) allowed the determination of CH_3-13 as β-oriented. Another NOE correlation between H-5 (δ 2.33, m) and CH_3-15 (δ 1.15, d, *J* = 6.9) allowed the determination of CH_3-15 as α-oriented. The collective data of **1** confirmed its identity as amberboin isolated before from *Amberboa lippii* D. C. (*syn Centaurea lippii* L.) (Gonzalez et al., 1967; 1970; Bermejo et al., 1969) and *Centaurea sinaica* (Al-Easa et al., 1990).

Compound **2** was obtained as crystalline solid, m.p 192-193 ° C. EI mass spectrum showed a molecular ion peak at m/z 266 $[M]^+$, corresponding to the molecular formula $C_{15}H_{22}O_4$.The observed mass which was two mass units higher than that of **1** suggested possible reduction at C-3, C-14 or opening of the lactone ring. Analysis of the NMR spectral data (experimental section 3.8) revealed close resemblance to those of **1**, except for the disappearance of the carbon signal due to the keto group at C-3 (δ 220.0). Instead, a new signal due to a methine carbinolic carbon appeared at δ 77.5. This was correlated to the multiplet at δ 3.61 in ^{1}H-NMR spectrum by HMQC experiment and assigned to position 3, based on observed HMBC correlations between C-3 and CH_3-15 (δ 1.26, d, *J* = 6.0 Hz) and H-3 (δ 3.61, m) and C-2 (δ 37.7). The planar structure of **2** was established through ^{1}H-^{1}H COSY (Figure 2). The relative stereochemistry of the OH group at C-3 and the CH_3 at C-11 (CH_3-13) were determined to be β-oriented, while that at C-4 (CH_3-15) was α-oriented based on observed correlations in the NOESY spectrum of **2** between H-11and H-9α; between CH_3-15 and each of H-3 and H-5; also between H-3 and H-5 (Figure 3). The above data clearly identified **2** as lipidiol, previously isolated from *Amberboa lippii* (Gonzalez et al., 1970; Rybalko et al., 1975) and *Teucrium viscidum* (Hao et al., 2013). It is worth noting that the full structure assignment and determination of stereochemistry through spectroscopic methods including 2D-NMR of compounds **1** and **2** are described in this work for the first time. Previous identification based on limited ^{1}H-NMR analysis and chemical derivatization (Gonzalez et al., 1970).

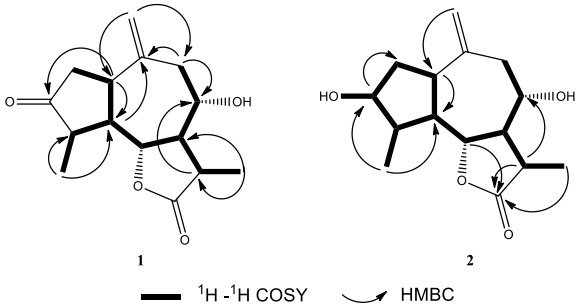

Figure 2. Significant ^{1}H-^{1}H COSY and HMBC correlations of **1** & **2**.

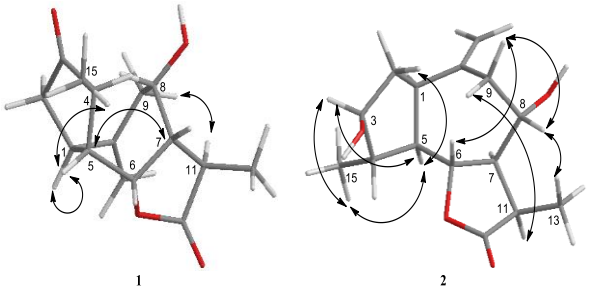

Figure 3. Significant NOESY correlations of 1 & **2.**

Compound **3** was obtained as amorphous gum and had an intermediate polarity between **1** and **2**. Its EI mass spectrum showed a molecular ion peak at m/z 346 $[M]^+$ corresponding to the molecular formula $C_{19}H_{22}O_6$. Its ^{13}C-NMR spectrum (experimental section 3.9) displayed 19 carbon resonances, of which 15 resonances could be assigned to sesquiterpene lactone nucleus and four resonances were due to an ester at C-8. The acylation at C-8 was evidenced by the observation of a downfield shift of H-8 in **3** by 1.36-1.51 ppm, as well as deshielding of C-8 by 4.4-4.7 ppm compared to the same position in **1** and **2**. The ^{1}H-NMR spectrum showed four sets of signals due to exomethylene groups. Three of them could be assigned to positions 13, 14 and 15, while the fourth set was assigned to the ester function which was defined as 4'-hydroxymethacrylate. The collective data of **3** were almost identical to those published for cynaropicrin (Yayli et al., 2006; Zaabat et al., 2011). Cynaropicrin has been reported from other species in the subtribe *Centaureinae* (reviewed in Nowak et al., 1986) and has been reported from one *Volutaria* species *viz*., *V. crupinoides* (Desf.) Maire (Zaabat et al., 2011).

Compound **4** was isolated as amorphous solid from the more polar fractions of the CH_2Cl_2 extract and was identified as daucosterol by co-chromatography with authentic sample.

Compound **5** (Figure 1) was isolated as crystalline solid, m.p 242-243 ^{o}C from the n-butanol extract. Its EI mass spectrum gave a quazi molecular ion peak at m/z 481 $[M+H]^+$ which is compatible with the molecular formula $C_{27}H_{44}O_7$. ^{1}H and ^{13}C-NMR spectral data suggested a highly oxygenated steroid nucleus. The ^{13}C-NMR spectrum (section 3.11) displayed 27 carbon resonances including those due to unsaturated ketone at δ 205.6 (C-6), an olefinic carbon at δ 121.3 (C-7), in addition to six oxygenated carbons distributed between the steroid ucleus and the side chain. UV absorption maxim at 240 nm, characteristic of the 14α-hydroxy-7-en-6-one chromophore (Dinan, 2001) indicated that **5** is an ecdysteroid. Its spectral data (experimental section 3.11) were in good agreement with those published for 20-hydroxy-ecdysone (Harmatha et al., 2002; Satoh et al., 2003; Zaghloul and Marzouk, 2006).

The cytotoxic activity of compounds **1-3** was evaluated on four human cancer cell lines using MTT assay (Table 1). The tested compounds showed some selectivity towards the cell lines. All tested compounds showed weak activity against hepatocellular carcinoma, HePG-2. Only compound **3** was strongly active against Epidermoid carcinoma of larynx, HeP-2 with IC_{50} of 22.81 ± 5.80 μM, a result which is statistically significant from that of the positive control used in this assay (5-flourouracil, IC_{50} 32.05 ± 0.96 μM). Compound **1** was the best active against colorectal carcinoma (HCT116), while compound **2** showed the best activity against mammary gland carcinoma, MCF-7 cell lines, among tested compounds.

Table 1. Results of cytotoxicity assay of compounds **1-3** on different human tumor cell lines.

	IC_{50} (μM)			
	HePG2	HeP2	HCT-116	MCF-7
1	22.13 ± 0.57	52.49 ± 1.04	32.39 ± 0.65	37.83 ± 0.71
2	32.30 ± 0.28	78.30 ± 1.03	48.21 ± 0.62	23.77 ± 0.23
3	25.08 ± 0.43	22.81 ± 5.80*	45.89 ± 0.39	32.65 ± 0.34
5- FU	10.76 ± 1.03	32.05 ± 0.96	26.84 ± 0.61	15.94 ± 0.98

Data expressed as means ± SE. * Significant from 5FU at $p > 0.05$.

A molecular docking study was carried out to find out a possible explanation for the experimentally observed cytotoxicity results, as two of the tested compounds (**1** and **2**) lacked the α-methylene-γ-lactone moiety first suggested by Kupchan (1970) to be responsible for the cytotoxicity of sesquiterpene lactones and then widely used by many researchers to explain such activity. PharmMapper server demonstrated a variety of putative targets that might exhibit considerable binding affinity to the test compounds. These targets are implicated in cancer therapy in a diversity of approaches. Their interactions with the tested compounds might explain the experimentally observed cytotoxicity. Table 2 lists the scores with the top ranked targets. The results of docking analysis with the receptors (Table 3) shows that all the tested compounds exhibited the strongest affinity towards

Table 2. Fit scores of the tested compounds against the top ranked targets

Target Name	Fit score		
	Amberboin	Lipidiol	Cynaropicrin
Proto-oncogene tyrosine-protein kinase LCK (3BYU)*	3.06	3.34	3.33
Insulin-like growth factor 1 receptor (2ZM3)	3.25	3.39	-
Androgen receptor (1GS4)	3.09	3.40	3.29
Epidermal growth factor receptor (1M17)	3.02	3.04	-
Vitamin D3 receptor (1DB1)	2.77	3.37	3.4
Estrogen receptor (1YIN)	2.81	2.83	-

* Protein Data Bank (PDB) code (-) Target not included in the top 300 targets for this compound

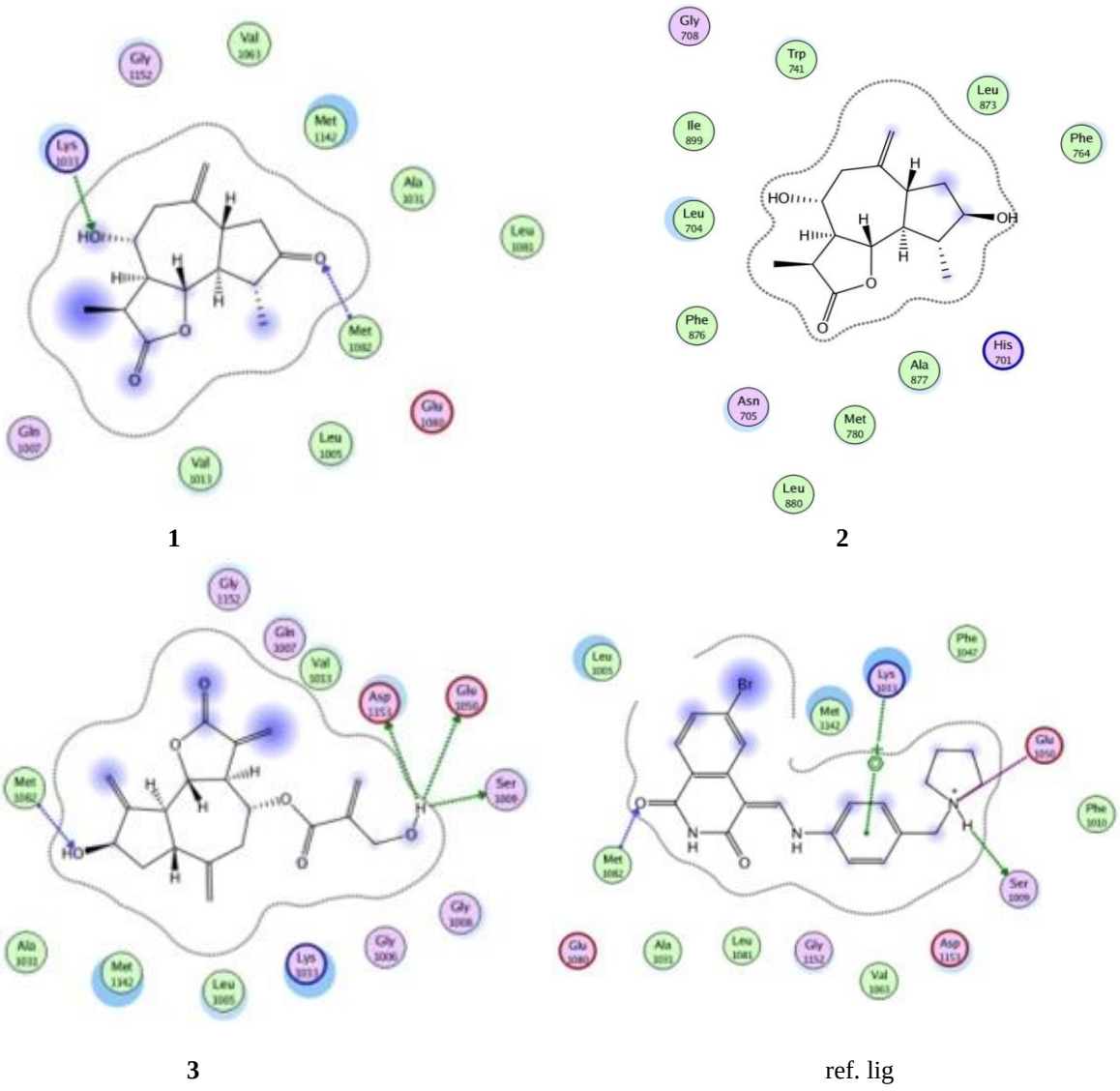

Figure 4. Best Docking poses calculated by MOE for the tested compounds **1-3** and referece ligand (ref. lig.) with (2ZM3) receptor.

insulin-like growth factor 1 receptor (2ZM3) with cynaropicrin having the highest score. The best scoring poses of all compounds showed H-bonding to several amino acid residues within the active site of the receptor (Figure 4). These results together with the experimentally observed cytotoxicity, supported the conclusion that the presence of α-methylene-γ-lactone moiety is not always, but usually responsible for cytotoxicity of many sesquiterpene lactones and other reactive centres are required for high degree of activity (Ivie and Witzel, 1983). This conclusion is acceptable, especially when observing that amberboin was more cytotoxic to HepG2 and HCT-116, also lipidiol to MCF-7 cell lines than cynaropicrin (Table 1) which possess the α-methylene-γ-lactone moiety. Similar results to those obtained in this work could be traced in the literature for cytotoxic sesquiterpene lactones lacking α-methylene-γ-lactone moiety. Examples are reported by McDonald et al. (2004); Williams et al. (2005); Yang et al. (2007); Chicca et al. (2011) and Zhang et al. (2014). The compounds isolated in this work might be considered in combinatorial therapy of drug resistant cancers. Enzyme assays of the tested compounds for insulin-like growth factor 1 inhibitory activity are essential to verify these results. Insulin-like growth factor 1 which is a member of the tyrosine-kinases family is known to be implicated in many cancers. Its antiapoptotic properties allows cancerous cells to resist the cytotoxic properties of chemotherapeutic drugs or radiotherapy, so inhibitors of this receptor would be promising in treating such types of cancers (Warshamana-Greene et al., 2005; Negi et al., 2013).

3. Experimental:

3.1. Plant material:

V. abyssinica (A. Rich.) C. Jeffrey was collected from Yemen, near Ibb province in 2009. The identity of the plant was confirmed at the Pharmacognosy Department, Faculty of Pharmacy, Sana'a University, Yemen. Voucher specimens (1547) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Sana'a University and at the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt.

3.2. General experimental procedures:

Melting points (uncorr.) were recorded on Yamagimoto micro-melting point apparatus MP-500D (Japan). UV spectra were recorded on Shimadzu UV-1601 Spectrophotometer (Japan). IR spectra were recorded on a Shimadzu FTIR-8100 spectrometer (Japan). ^{1}H, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMBC, HMQC and NOESY spectra were obtained with JEOL JNM-LA 400 ((Japan) and Bruker dpx 500 (USA) high-field spectrometers, operating at 400 and 500 MHz (^{1}H) and 100 and 125 MHz (^{13}C). EI mass spectra (70 eV) were recorded on a Shimadzu Qp-2010 plus mass spectrometer (Shimadzu, Japan). Column chromatography was performed on silica gel (230-400 mesh, Merck, Germany). TLC was carried out on precoated Si gel 60 GF_{254} (0.25 mm, Merck, USA) plates. Developed chromatograms were visualized by spraying with 0.01% vanillin/H_2SO_4 followed by heating until maximum development of the spots colour. Solvents were reagent grade. Cell lines for cytotoxicity assay were obtained from ATCC *via* a Holding company for biological products and vaccines (VACSERA, Egypt). RPMI-1640 medium, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT), DMSO and 5-fluoro-uracil (Sigma, USA), Fetal Bovine serum (GIBCO, UK).

3.3. Extraction and isolation:

The air dried powdered aerial parts of *V. abyssinica* (2.0 kg) were extracted with MeOH (10 L x 5) at room temperature. Evaporation of the solvent under reduced pressure gave the crude extract (162.7 g) as a semi-solid residue. The concentrated MeOH extract was resuspended in 200 ml MeOH, diluted to 500 ml with dist. H_2O, defatted with pet ether and then successively extracted with CH_2Cl_2, EtOAc and finally n-butanol. Evaporation of the solvents gave 8.30 g of pet ether, 18.2 g of CH_2Cl_2, 11.1 g of EtOAc and 14.1 g of n-butanol extracts, respectively.

A portion of the CH_2Cl_2 extract (18.0 g) was chromatographed on a silica gel column (90 x 2.5 cm) and eluted with mixtures of EtOAc/pet ether of increasing polarity (5-50%), collecting 100 ml fractions. The collected fractions were monitored by TLC, visualized by spraying with vanillin/H_2SO_4 and similar fractions pooled to give four main fractions (A-D). Fraction A (3.60 g) was purified by rechromatography on silica gel column (70 x 1.5 cm) eluted with CH_2Cl_2/MeOH mixtures. Sub-fractions eluted with 1% MeOH (1.90 g) afforded pure **1** (1.46 g) after recrystallization from pet ether/CH_2Cl_2 (70:30). Fraction B (1.26 g) was also rechromatographed on silica gel column (70 x 1.5 cm) isocratically eluted with 2% MeOH in CH_2Cl_2 to yield pure **3** as colourless gum (490.0 mg). Fraction C (500.0 mg) was purified on silica gel column (50 x 1.0 cm) eluted with CH_2Cl_2/MeOH mixtures to yield pure **2** as crystalline solid (170.0 mg) in sub-fractions eluted with 3% MeOH. Rechromatography of

Table 3. Free energy of binding in kcal/mol calculated by MOE for the tested compounds.

Target Name (PDB Code)	Free energy of binding (kcal/mol)			
	Amberboin	Lipidiol	Cynaropicrin	Ref. Ligand
Proto-oncogene tyrosine-protein kinase LCK (3BYU)	- 3.84	- 4.72	- 5.05	- 9.76
Insulin-like growth factor 1 receptor (2ZM3)	- 5.09	- 4.73	- 7.86	- 4.51
Androgen receptor (1GS4)	- 6.71	- 6.19	- 8.53	- 9.84
Epidermal growth factor receptor (1M17)	- 4.42	- 4.61	- 5.18	- 4.54
Vitamin D3 receptor (1DB1)	- 4.89	- 5.04	- 7.23	- 14.04
Estrogen receptor (1YIN)	- 6.79	- 6.91	- 8.50	- 12.44

fraction D (300.0 mg) on silica gel column (50 x 1 cm) eluted with CH_2Cl_2/MeOH mixtures afforded pure **4** as amorphous solid (134.0 mg) in sub-fractions eluted with 6% MeOH.

The n-butanol extract (12.7 g) was also chromatographed on silica gel column (85 x 2.5 cm) eluted with CH_2Cl_2/MeOH mixtures to afford crude **5** (227.0 mg) in fractions eluted with 15% MeOH.These sub-fractions were purified by rechromato-graphy on silica gel column eluted with the same solvent system used above. Pure **5** was obtained as colourless needles (89.0 mg).

3.4. Cytotoxicity assay:

Compounds **1**-**3** were screened for *in vitro* cytotoxic activity against four human tumor cell lines namely; hepatocellular carcinoma, HePG-2; Epidermoid carcinoma of larynx HeP-2; Colorectal carcinoma, HCT-116 and Mammary gland carcinoma, MCF-7. Cell viability was assessed by MTT assay of the studied compounds, as described previously (Mosmann 1983; Mauceri et al. 1998). 5-flourouracil was used as positive control.

3.5. Statistical analysis:

The results are expressed as mean values ± SE from three separate experiments. The IC_{50} values were calculated from the dose response curves using non-linear regression analysis that gave a percentage of the inhibition values. Group differences were determined by analysis of variance (ANOVA). When statistically significant differences indicated by ANOVA, the values were compared by the Tukey test. The differences were considered statistically significant from the controls at $p < 0.05$.

3.6. *Molecular modeling study:*

3.6.1. In silico target fishing:

PharmMapper server which is designed to identify potential target candidates (receptors or active binding sites of enzymes) for small molecules was used to identify potential targets involved in cancer therapy for the tested compounds *via* a reverse pharmacophore mapping approach (Liu et al., 2010). PharmMapper is freely accessed at http://59.78.96.61/pharmmapper. The output of a PharmMapper run is demonstrated in the form of a ranked list of hit target pharmacophore models that are sorted by fit score in descending order. PharmMapper adopts semi-rigid pharmacophore mapping protocol. As a result, multiple conformations of the query molecule are required prior to mapping which can be achieved by online service provided by the server. PharmMapper finds the best mapping poses of the uploaded molecules against all the targets in PharmTarget DB (data bank) and top N potential drug targets (default value is 300), as well as respective molecule's aligned poses are output.

3.6.2. *Docking using MOE:*

The potential targets proposed by pharmaco-phore mapping approach were used to investigate their interaction with the test compounds. Molecular Operating Environment (MOE) Version 2009.10 (http://www.chemcomp.com) was employed in the search for favorable binding configurations between ligands and macromolecular target. The bound ligand was given as the reference for defining the binding site. Validation of the docking simulation protocol was performed based on their ability to reproduce the reference ligand pose observed experimentally. The reference ligand was extracted from the crystal structure of complex and re-docked in the protein binding site. After docking, Root Mean Square Deviation (RMSD) value of the predicted pose to experimentally verified pose is calculated. RMSD value indicates the measure of spatial similarity between two structures. If the RMSD value is found to be less than 2.00 Å, the prediction of binding mode is considered as successful. Consequently, all docking algorithms were well suited for the experiments conducted here. After that, all the target compounds were built using the MOE builder

interface. Docking studies of the test compounds were performed by MOE using the x-ray crystallographic structure of target protein obtained from the protein data bank. The ligand co-crystallized with the selected target protein is used as a reference for assessing the affinity of the tested compounds. We performed 100 docking interactions for each ligand and the top scoring configuration of each of the ligand-enzyme complexes was selected on energetic ground. In Table 3, the output of docking simulation is the scoring function which reflects the binding free energy *dG* in kcal/mol.

*3.7. Amberboin (**1**):*

Colourless crystals, m. p. 148-149 ° C (lit. 147-149 ° C). R_f: 0.36 (CH_2Cl_2 / MeOH, 95:5). IR (KBr) ν_{max}: 3495, 1770, 1745, 1640, 920. ^{1}H NMR (500 MHz, $CDCl_3$-CD_3OD): δ 3.18 (m, H-1), 2.44 (dd, *J* = 3.8, 18.4 Hz, H-2α), 2.53 (d, *J* = 9.2, 18.4 Hz, H-2β), 2.31 (m, H-4), 2.33 (m, H-5), 4.22 (dd, *J* = 8.5, 9.0 Hz, H-6), 2.65 (ddd, *J* = 7.9, 8.5, 10.0 Hz, H-7), 3.73 (ddd, *J* = 9.9, 13.8, 6.1 Hz, H-8), 2.18 (dd, *J* = 13.8.0, 10 Hz, H-9α), 2.79 (m, H-9β), 2.81 (m, H-11), 1.32 (d, *J* = 7.7 Hz, CH_3-13), 4.74 (br s, H14a), 4.80 (br s, H-14b), 1.15 (d, *J* = 6.9 Hz, CH_3-15) . ^{13}CNMR (125 MHz, $CDCl_3$-CD_3OD): δ 40.0 (C-1), 43.2 (C-2), 220.0 (C-3), 47.1 (C-4), 50.9 (C-5), 83.4 (C-6), 50.3 (C-7), 69.5 (C-8), 48.3 (C-9), 144.9 (C-10), 39.3 (C-11), 180.5 (C-12), 10.2 (C-13), 113.7 (C-14), 13.6 (C-15). EI-MS: m/z (rel. int.) 265 (2.3) $[M+H]^+$, 246 (2.3) $[M-H_2O]^+$, 203 (2.4) $[M-H_2O-COO]^+$, 149 (4.4), 71 (71.3), 69 (100).

*3.8. Lipidiol (**2**):*

Colourless crystals, m. p. 192-193 ° C (lit. 189.5-191 ° C). R_f: 0.23 (CH_2Cl_2 / MeOH, 95:5). IR (KBr) ν_{max}: 3285, 1780, 1645, 908. ^{1}H NMR (500 MHz, $CDCl_3$-CD_3OD): δ 2.80 (m, H-1), 1.65-1.68 (m, H-2a), 2.75 (m, H-2b), 3.61 (m, H-3), 1.76 (m, H-4), 1.97 (m, H-5), 4.08 (dd, *J* = 10.7, 10.4 Hz, H-6), 2.28 (ddd, *J* = 10.0, 10.0, 8.0, H-7), 3.58 (m, H-8), 1.65-1.68 (m, H-9a), 2.04 (m, H-9b), 2.72 (m, H-11), 1.62 (d, *J* = 7.7 Hz, H-13), 4.87 (br s, H-14a), 4.98 (br s, H-14b), 1.26 (d, *J* = 6.0 Hz, H-15). ^{13}CNMR (125 MHz, $CDCl_3$-CD_3OD): δ 42.0 (C-1), 37.7 (C-2), 77.5 (C-3), 47.5 (C-4), 50.3 (C-5), 82.3 (C-6), 54.8 (C-7), 69.8 (C-8), 47.7 (C-9), 144.1 (C-10), 45.5 (C-11), 180.5 (C-12), 9.7 (C-13), 113.2 (C-14), 17.5 (C-15) . EI-MS: m/z (rel. int.) 266 (73.7) $[M]^+$, 248 (59) $[M-H_2O]^+$, 216 (56.4) $[M-2H_2O-CH_2]^+$, 150 (69), 69 (57.3), 57 (100).

*3.9. Cynaropicrin (**3**):*

Amorphous gum. R_f: 0.34 (CH_2Cl_2 / MeOH, 95:5). IR (KBr) ν_{max}: 3421, 1751, 1730, 920. ^{1}H NMR (500 MHz, $CDCl_3$-CD_3OD): δ 2.90 (m, H-1), 1.66 (m, H-2a), 2.10 (m, H-2b), 4.46 (m, H-3), 2.78 (m, H-5), 4.24 (m, H-6), 3.16 (m, H-7), 5.09 (m, H-8), 2.33 (m, H-9a), 2.63 (m, H-9b), 5.58 (br s, H-13a), 6.15 (br s, H-13b), 4.87 (br s, H-14a), 5.07 (br s, H-14b), 5.31 (br s, H-15a), 5.41 (br s, H-15b), 5.94 (br s, H-18a), 6.29 (br s, H-18b), 4.27 (s, 2H, H-19) . ^{13}CNMR (125 MHz, $CDCl_3$-CD_3OD): δ 45.0 (C-1), 36.6 (C-2), 73.0 (C-3), 151.8 (C-4), 51.0 (C-5), 78.8 (C-6), 47.3 (C-7), 74.2 (C-8), 38.7 (C-9), 141.8 (C-10), 137.4 (C-11), 169.9 (C-12), 122.7 (C-13), 118 (C-14), 113.0 (C-15), 165.4 (C-16), 139.7 (C-17), 125.9 (C-18), 60.8 (C-19). EI-MS: m/z (rel. int.) 346 (1.7) $[M]^+$, 246 (1.9) $[M\text{-ester}]^+$, 232 (2.2) $[M\text{-ester-}CH_2]^+$, 203 (3.2),193 (48.4), 168 (41.9), 71 (100), 69 (81.5).

*3.10. Daucosterol (**4**):*

Amorphous solid, R_f: 0.27 (CH_2Cl_2/MeOH, 90:10).

*3.11. 20-hydroxyecdysone (**5**):*

Colourless needles, m.p 242-243 ° C (lit. 242-244 ° C). R_f: 0.25 (CH_2Cl_2-MeOH, 85:15). UV λ_{max} (MeOH) 240, 207 nm.^{1}H NMR (400 MHz, CD_3OD): 3.85 (m, H-2), 3.98 (m, H-3), 5.82 (s, H-7), δ 0.91 (s, H-18), 0.99 (s, H-19), 1.21 (s, H-21), 3.32 (dd, *J* = 9.0, 2.0 Hz, H-22),1.22 (s, 6H, H-26, 27),.^{13}CNMR (100 MHz, CD_3OD): δ 36.5 (C-1), 67.7 (C-2), 67.9 (C-3), 31.6 (C-4), 51.0 (C-5), 205.6 (C-6), 121.3 (C-7), 167.2 (C-8), 34.3 (C-9), 38.5 (C-10), 23.6 (C-11), 31.7 (C-12), 48.6 (C-13), 84.4 (C-14), 30.9 (C-15), 21.2 (C-16), 49.7 (C-17), 17.3 (C-18), 21.2 (C-19), 77.3 (C-20), 20.5 (C-21), 77.6 (C-22), 26.5 (C-23), 41.6 (C-24), 70.5 (C-25), 28.2 (C-26), 28.9 (C-27). EI-MS m/z (rel. int.) 481(2) $[M+H]^+$, 463 (46) $[M+H-H_2O]^+$, 445 (10.5) $[M+H-2H_2O]^+$, 355 (48), 264 (47), 59 (100).

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