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CYTOTOXICITY OF COMPOUNDS ISOLATED FROM *EUPHORBIA ABYSSINICA*: A COMBINED EXPERIMENTAL AND DOCKING STUDIES

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ABSTRACT. *Euphorbia abyssinica* Gmel. has been traditionally used in Ethiopia to treat various diseases, including swelling and cancer. This study assessed the cytotoxic effects of the ethanolic crude extract and its isolates from the aerial parts of *E. abyssinica* using the MTT assay method and molecular docking studies with the AutoDock Vina 4.2 program. *E. abyssinica* showed strong cytotoxicity against HeLa cells (IC_{50} = 18.6 ± 0.7 µg/mL) and weak cytotoxicity against PC3 cells, with percent inhibition of 55.2% and 23.7% at 30.0 µg/mL, respectively. Among the fractions, the Dichloromethane fraction exhibited strong cytotoxicity against PC3 cells (IC_{50} = 21.7 ± 0.9 µg/mL) and weak cytotoxicity against Hela cells, with 78.4% and 29.98% growth inhibition at 30.0 µg/mL, respectively. Dichloromethane fraction yielded *β*-sitosterol (**1**), friedelin (**2**) epifriedelanol (**3**), taraxerol (**4**) and glutinol (**5**). Friedelin (**2**) demonstrated strong cytotoxic activity against HeLa and PC3 cells. Similarly, epifriedelanol (**3**) showed strong cytotoxicity against PC3 cells (IC_{50} =28.4 ± 0.7 µM) and notable growth inhibition of HeLa cells (38.5% at 30 µM). Friedelin (**2**) showed better binding affinity and inhibition constant towards Human prostate specific antigen (PDB: 2ZCH) (-9.01 kcal/mol and 0.25 µM) and Alpha-actinin-4 (PDB: 6O31) (-7.61kcal/mol and 2.64 µM).

KEY WORDS: *Euphorbia abyssinica* Gmel., HeLa cells, PC3 cells, Cytotoxicity, MTT assay, Molecular docking

INTRODUCTION

Cancer is a general term used to refer to over 100 different diseases in which a group of cells becomes abnormal, divide without control, and invade other tissues, including organs. Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. A survey of epidemiological data from 184 countries suggested that the global burden of cancer will increase to 23.6 million new cases each year by 2030, an increase of 68% compared with 2012 [2] . It is estimated that 21 million people will be diagnosed, and 13 million will die of cancer in the year 2030. Low-income countries contribute up to 60% of this death [3]. In Ethiopia, cancer accounts for about 5.8% of total national mortality [4]. According to the clinical record finding in Tikur Anbessa Specialized Hospital, there is an estimate of 120,500 new cancer cases per year in Ethiopia [5]. It is estimated that the annual incidence of cancer, in Addis Ababa alone, is about 60,960 cases and the annual mortality is over 44,000 [4-5]. About two-thirds of reported annual cancer deaths occur among women. The most prevalent cancers in Ethiopia among the adult population are breast cancer (30.2%), cervical cancer (13.4%), and colorectal cancer (5.7%) [5].

Euphorbia abyssinica Gmel. (Euphorbiaceae), locally called "Kulkual", in Amharic, is a succulent leafless tree that grows up to 9 m high with erect branches. It occurs widely throughout

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dry-land Africa, where it is mostly used by the local population as a fence, firewood, and timber. In the eastern Africa region, *E. abyssinica* grows on altitude ranging between 1300-2400 m high [6&7]. It has been reported that, in Ethiopia, the plant is traditional used for the treatment of a number of diseases, including gonorrhea, leprosy[1], hemorrhoids, rabies, gastro-intestinal, venereal diseases, wounds, leshmaniasis, malaria, dandruff, skin wounds, warts, swelling and cancer [7-15]. That is mostly by rubbing the latex and the paste of the stem and bark to the affected area [14].

The phytochemical studies on the plant showed the presence of polyphenols, saponins, cardiac glycosides, flavonoids, carbohydrates, steroids, tannins and alkaloids [16, 17]. From the latex of *E. abyssinica,* compounds such as 8(R)-hydroxy-dec-3(E)-en-oic acid, lupeol, β-sitosterol (**1**), oleanolic acid and *β*-sitosterol glucoside were isolated [18]. *E. abyssinica* extract displayed a range of bioactivity, including chitinase [19], lysozyme [19], anti-malarial [20], antifungal [16, 21], antibacterial [17], anthelmintic activity [22], adhesive [23] and cancer chemopreventive activities[26]. In addition, the latex of the plant cures infections on skin of cattle caused by dermatophillosis, demodex, and ringworm [16]. The *E. abyssinica* fruit extract displayed chemopreventive activity against Hepa1c1c7, triggered protein expression of NQO1, a protein that is upregulated in response to oxidative stress [24]. However, the anticancer activity of *E. abyssinica* against cervical cancer (HeLa) and prostate cancer (PC3) cells was not studied and activity based isolation of compounds was not conducted. The current study was conducted to isolate cytotoxic compounds from *E. abyssinica* extracts and test them against HeLa and PC3 cells.

RESULTS AND DISCUSSION

Natural products are a rich source of cytotoxic anticancer compounds. Various plant extracts have exhibited significant cytotoxic activities against cancers, attributed to bioactive secondary metabolites such as polyphenols, flavonoids, steroids, and alkaloids. These compounds are believed to exert their anticancer effects through mechanisms such as inducing apoptosis, inhibiting cell proliferation, and enhancing the body's detoxification processes. As a result, natural products hold great promise as sources of new and effective anticancer agents. In cancer research, HeLa cells, derived from cervical cancer, and PC3 cells, originating from prostate cancer, are commonly used to evaluate the cytotoxicity of potential extracts and isolates.

E. abyssinica has been traditionally used to treat various ailments, including swelling and cancer [9, 24]. Recent studies have shown that *E. abyssinica* exhibits significant cytotoxicity against cancer cell lines, making it a promising candidate for anticancer drug development [24&26]. The ethanolic extract, fractions that are soluble in DMSO, and isolates from the cytotoxic fractions of the aerial parts of *E. abyssinica* were prepared, and their cytotoxicity against HeLa and PC3 cells was assessed using the MTT assay method. The cytotoxicity results of pure compounds isolated from the cytotoxic fraction, measured using the MTT assay method, were compared with molecular docking studies conducted using the AutoDock Vina 4.2 program. The results were analyzed, and possible discussions are provided. Researchers can raise insolubility issues related to some of the fractions as a major limitation of the study.

Yield of the plant extracts

The plant material of *E. abyssinica* yielded 15.75% crude ethanolic extract. While the active DCM extract was 10. 58%.

Cytotoxic activity (cell viability test)

The growth inhibitory effects of crude extracts were tested against two cancer cell lines, cervical cancer (HeLa) and prostate cancer (PC3) cells by MTT-assay. The experiments were conducted

by closely monitoring the viability of cultured cells that were exposed to the plant extracts. The anticancer agent, doxorubicin, was used as a positive control because it induces cytotoxicity in breast cancer, bladder cancer, Kaposi′s sarcoma, lymphoma, and acute lymphocytic leukemia [25]. The percent inhibition values were calculated after treating the cell lines for 72 h with ethanolic crude extracts at different concentrations.

The 80% EtOH extract of *E. abyssinica* exhibited cytotoxicity against cervical (HeLa) and prostate (PC3) cancer cell lines. The extract inhibited the proliferation of HeLa and PC3 cancer cells by 55.2 and 23.7%, at the concentration of 30 µg/mL respectively. *E. abyssinica* displayed potent cytotoxicity against HeLa cells with an IC_{50} value of 18.6 ± 0.7 µg/mL, which is comparatively lower than that of standard drug doxorubicin (IC_{50} value of 0.9 ± 0.14 µg/mL). According to Ahmed et al. 2022, *E. abyssinica* has a concentration-dependent cancer chemopreventive potential against murine hepatoma cell line (Hepa1c1c7) by inducing phase II detoxifying enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1), which plays an important role in cytoprotection against oxidative damage [23]. It is believed that the presence of secondary metabolites such as polyphenols, flavonoids, steroids, and alkaloids are responsible for the observed anticancer activities [16, 17, 26].

The ethanolic crude extract of *E. abyssinica* was further partitioned sequentially into eight fractions using different solvent polarities (Table 1). The cytotoxic activities of six of the eight fractions were conducted. However, petroleum ether (100%) and pet ether: DCM (1:1) eluted fractions were not tested due to insolubility in DMSO.

Table 1. Cytotoxic activities of *E. abyssinica* crude extract, fractions and standard drug.

Crude extract, fractions and standard drug	% inhibition on HeLa		% inhibition on PC3	
	30 µg/mL	IC_{50} ± SD	50 µg/mL	IC_{50} ± SD
Ethanolic Crude extract	55.2	18.6 ± 0.7	23.7	
Pet ether	INS		INS	
Pet ether: DCM (1:1)	INS		INS	
DCM	21.98		78.4	21.7 ± 0.9
DCM: EtOAc (1:1)	31.2		37.7	
EtOAc	-30.6		0.1	
EtOAc : MeOH (1:1)	9.78		-54.7	
MeOH	-54.6		-3.0	
MeOH : H_2O (1:1)	16.19		21.8	
Doxorubicin	101.2	0.9 ± 0.14	89.9	

DCM = dichloromethane, EtOAc = ethyl acetate, MeOH = methanol, H_2O = water, Pet ether = petroleum ether, INS= insoluble.

The results in Table 1 indicated the DCM (100%) eluted fraction has strongly inhibited the proliferation of PC3 cells compared to the remaining fractions and the crude extract. It inhibited the growth of PC3 by 78.4% at a concentration of 30 µg/mL. The cytotoxicity of *E. abyssinica* increased following fractionation, and it also showed a lower IC_{50} value of 21.7 ± 0.9 µg/mL compared to crude extracts, which exhibited an IC_{50} value of 34.2 ± 5.3 µg/mL. The EtOAc (100%) and MeOH (100%) fractions increased the viability of HeLa cells. Similarly, EtOAc: MeOH (1:1) and MeOH (100%) fractions increased the viability of PC3 cells.

The higher cytotoxicity of *E. abyssinica* ethanolic crude extract against HeLa cells might be due to the synergetic effects of the compounds found in the crude extract. The loss of synergetic effect might have to do with solubility of some of the constituents in DMSO and /or separation of the mixture into different individual components. When activity is lost in the process of bioassay-guided fractionation, quantified synergy can support the pursuit of alternate drug development directions such as the FDA botanical drug pathway [27, 28].

Cytotoxic activity of the isolated compounds

Among the tested fractions, the dichloromethane (DCM) fraction was selected for isolation of compounds due to its significant inhibitory effect on the proliferation of PC3 cells and weaker effect on HeLa cells, where the activity of the crude extract diminishes upon fractionation. The DCM fraction exhibited strong cytotoxic activity against PC3 cells with 78.4% inhibition at a concentration of 30 µg/mL (IC_{50} = 26.5 ± 0.9 µg/mL) and weak cytotoxic activity against HeLa cells with 21.98% inhibition at the same concentration. This suggests that the DCM fraction contains potent compounds contributing to its higher cytotoxicity, warranting further investigation and isolation of specific compounds from this fraction.

The bioassay-directed chromatographic fractionation of the *E. abyssinica* DCM (100%) fractions gave five compounds: *β*-sitosterol (**1**) [29], friedelin (**2**) [30], epifriedelanol (**3**) [31], taraxerol (**4**) [32] and glutinol (**5**) [33] (Figure 1). The isolated compounds (Figure 1) were tested for their cytotoxic activity against human cervical (HeLa) and prostate (PC3) cancer cells. Among the tested compounds, friedelin (**2**) and epifriedelanol (**3**) has demonstrated strong cytotoxic activity against HeLa/PC3 cells with an IC50 value < 30 µM. According to the American National Cancer Institute (NCI), the compound is said to have cytotoxic activity if the IC_{50} value is < 30 µg/mL [34].

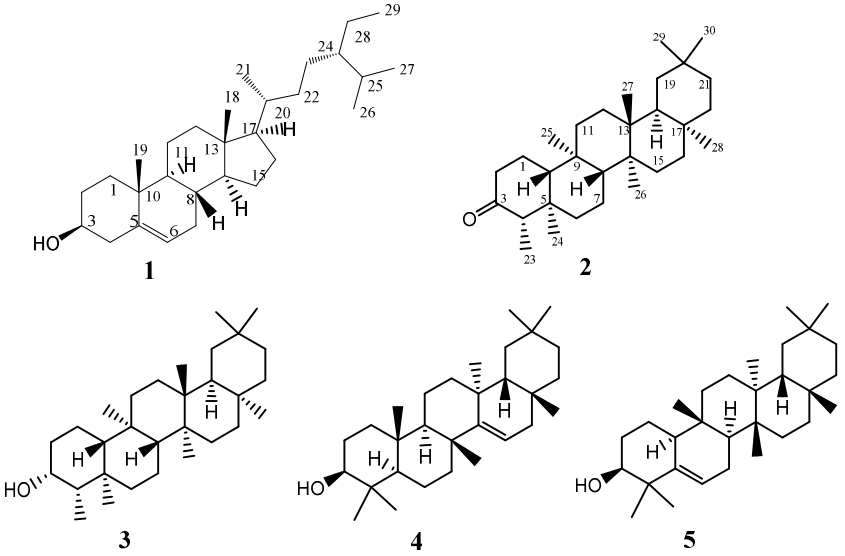

Figure 1. Structure of compounds isolated from cytotoxic fraction of *E. abyssinica*.

Among the isolated pentacyclic triterpnoids, friedelin (**2**) has demonstrated strong cytotoxic activity against HeLa and PC3 cells with IC_{50} values of 26.5 ± 0.9 and 24.2 ± 1.4 µM respectively. Similarly, epifriedelanol (**3**) has shown significant cytotoxicity to PC3 cells with IC_{50} values of 28.4 ± 0.7 µM and a notable growth inhibitory effect to HeLa cells with a percent inhibition of 38.5% at 30 µM. The cytotoxic effects of friedelin (**2**) and epifriedelanol (**3**) against PC3 cells were previously reported to be promising with IC_{50} values ranging from 3.54 ± 0.30 to 8.32 ± 1.92 µg/mL [37]. According to Lu et al. 2010, friedelin (**2**) exhibited a notable growth inhibitory effect against HeLa cells [36]. In addition, friedelin (**2**) inhibited the growth of prostrate

carcinoma PC3 cells by 69% at 31 μM [37]. Besides, it exhibited cytotoxic effects against nasopharyngeal cancer cells, breast cancer cells, and colon cancer [38, 39].

Taraxerol (**4**) and glutional (**5**) were shown to inhibit HeLa cell growth by 28.2% and 17.2%, respectively, at 30 μM. Taraxarol (**4**) showed a very weak cytotoxic effect on PC3 cells whereas, glutinol (**5**) was found to be non-toxic to PC3 cells. Previous studies indicated that both taraxerol (**4**) and glutionl (**5**) induce cell apoptosis through a mitochondria-mediated pathway in HeLa cells [40&41].

Table 2. Cytotoxicity of compounds isolated from cytotoxic fractions.

Crude extract, fraction, and Compounds	% inhibition on HeLa		% inhibition on PC3	
	30 μM	IC_{50} ± SD	30 μM	IC_{50} ± SD
Ethanolic Crude extract	55.2	18.6 ± 0.7	23.7	
DCM	21.98		78.4	21.7 ± 0.9
β-sitosterol (**1**)	8.4	-	16.5	-
Friedelin (**2**)	56.5	26.5 ± 0.9	63.2	24.2 ± 1.4
Epifriedelanol (**3**)	38.5	-	52.6	28.4 ± 0.7
Taraxerol (4)	28.8	-	4.8	-
Glutinol (**5**)	17.2	-	Inactive	-
Doxorubicin	101.2	0.9 ± 0.14	89.9	1.9 ± 0.08

β-sitosterol (**1**) demonstrated weak cytotoxic activity against PC3 and HeLa cancer cell lines. However, it has been reported to have anticancer properties against breast, prostate, colon, pancreatic, lung, stomach, and ovarian cancers by interfering with multiple cell signaling pathways including cell cycle and apoptosis [40-43].

Molecular Docking

Docking towards Human prostate specific antigen (PDB: 2ZCH)

Prostate-specific antigen (PSA) is an androgen-regulated serine protease, relevant as a biomarker to detect prostate cancer and to assess responses to treatment [44]. Prostate-specific antigen reported to activate single chain plasminogen activator, which is linked to prostate cancer invasion and metastasis [45]. The inhibition of PSA may be a useful tool to fight against prostate cancer.

Bioactive compounds extracted from medicinal herbs have been analyzed to possess cytostatic properties and can be used to combat cancer [46]. In this study, therefore, isolated compounds **2** and **3** were docked to human prostate specific antigen (PDB: 2ZCH) to test the ligand-receptor binding stability at the designated active site and compare the result with the *in vitro* cytotoxicity. Scored binding energies and inhibition constants (Ki), and amino acid residues involved in the ligand-protein interaction of the studied compounds and doxorubicin are given in Table 3, Figure 2.

Binding energies and inhibition constants of -9.01 and -8.26 kcal/mol, and 0.25 and 0.88 μM were scored for compound **2** and **3**, respectively. Compound **2** achieved better binding affinity (-9.01 kcal/mol) and smaller amount to inhibit (0.25 μM) than compound **3** (-8.26 kcal/mol, 0.88 μM), which is as good as the control (-9.29 kcal/mol, 0.15 μM). The docking plot of **2** (Figure 2) illustrated two π-Sigma/π-Alkyl interactions with residual amino acids Leu95D and His57, and four van der Waals type with Asn95F, Arg95G, Ser192 and Thr190. Similarly, compound **3** bind to receptor's active site through one H-bonding at Thr190, three Sigma/π-Alkyl at Cys220, His57 and Leu95D, and four van der Waals interactions at Ser192, Asn95F, Ser195 and Arg95G. The control doxorubicin displayed four H-bonding (His57, Ser195, Ser214 and Glu218), three Sigma/π-Alkyl (Leu95D, Cys191 and Cys220) and four van der Waals (Trp215, Gly216, Thr190 and Ser192). Despite the absence of H-bonding, compound **2** scored binding affinity (-9.01 kcla/mol) and inhibition constant (0.25 μM) significantly comparable to doxorubicin (-9.29 kcal/mol, 0.15 μM).

Docking towards Alpha-actinin-4 (PDB: 6O31)

Alpha-Actinin-4 (ACTN4) is an actin-binding protein that belongs to the spectrin gene superfamily and acts as an oncogene in various cancer types and also involved in tumorigenesis of cervical cancer [47]. The inhibition of Actin-4 result in the suppression of cell proliferation [47]. The docking of compound **2** and **3** toward *Alpha-actinin-4 (PDB: 6O31) (a biomarker for the precancerous state of cervical cancer) scored binding energy and inhibition constant of* -7.61 and -7.31 kcal/mol, and 2.64 and 4.37 µM, respectively (Table 3). Compound **2** scored lesser binding energy and inhibition constant (-7.61 kcal/mol and 2.64 µM) relative to the control, doxorubicin (-7.35 kcal/mol) and 4.06 µM), indicating better binding affinity and required smaller mount to inhibit the targeted protein. For **3**, binding affinity and inhibition constant (7.31 kcal/mol and 4.37 µM) comparable to the control (-7.35 kcal/mol and 4.06 µM). Based on the interaction plot (Figure 3), a total of eight interactions including three Sigma/π-Alkyl at Ala58, Lys54 and Arg53, four van der Waals at Gln70, Thr57, Pro238, Ser62 and Arg53, and one hydrogen boding at Arg65 were established for **2**. Compound **3** interact through one H-bonding at Ile91, three π-Sigma/π-Alkyl at Ala267, Ile237 and His266, and four van der Waals interactions at Val90, Gly93, His63 and Arg65.

Molecular docking result showed that isolated compound **2** and **3** scored stability with both human prostate specific antigen (PDB:2ZCH) and a*lpha-actinin-4 (PBD:6031) enzymes* comparable to that of the control, doxorubicin, that may encourage testing these compounds *in vivo* to verify their suitability in prostate cancer treatment.

Table 3. Molecular docking scores and the corresponding prominent residual amino acid interactions of the ligand against human prostate specific antigen and alpha-actinin-4 (one of the biomarkers for the precancerous state of cervical cancer).

Compounds	Binding energy (kcal/mol)	Inhibition constant (K_i)	H-bonding	π-Sigma/π-Alkyl	van der Waals
Human prostate specific antigen (PDB: 2ZCH)					
2	-9.01	0.25 µM	-	Leu95D,His57	Asn95F,Arg95G, Ser192,Thr190
3	-8.26	0.88 µM	Thr190	Cys220,His57, Leu95D	Ser192,Asn95F, Ser195,Arg95G
Doxorubicin	-9.29	0.15 µM	His57,Ser195, Ser214,Glu218	Leu95D,Cys191, Cys220	Trp215,Gly216, Thr190,Ser192
Alpha-actinin-4 (PDB: 6O31)[a]					
2	-7.61	2.64 µM	Arg65	Ala58,Lys54, Arg53	Gln70,Thr57, Pro238,Ser62
3	-7.31	4.37 µM	Ile91	Ala267,Ile237, His266	Val90,Gly93, His63,Arg65
Doxorubicin	-7.35	4.06 µM	ArG65,Gln70, Asn61,Lys239	Glu23,Pro238	Ala58,Met240, Ile237,Thr57

[a]Alpha-actinin-4 (ACTN4) is a biomarker for the precancerous state of cervical cancer.

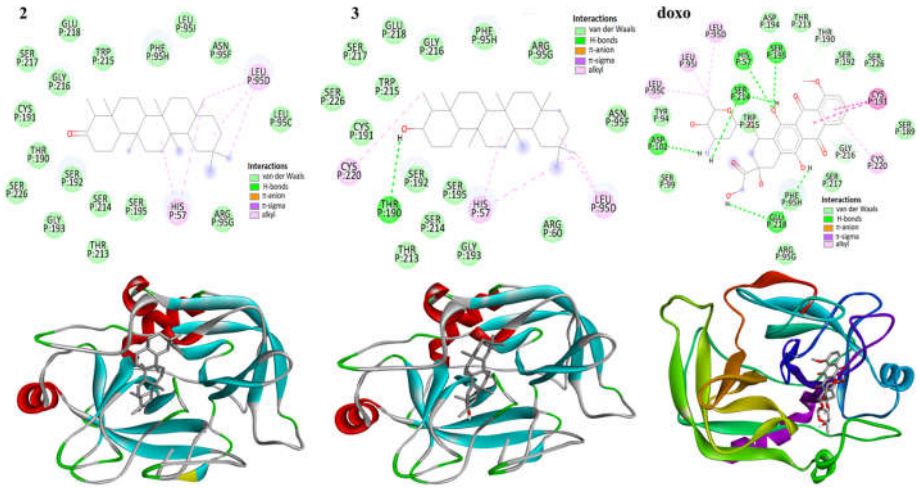

Figure 2. 2D (top) and 3D (bottom) binding interactions of compound **2**, **3** and **doxorubicin** against human prostate specific antigen (PDB: 2ZCH).

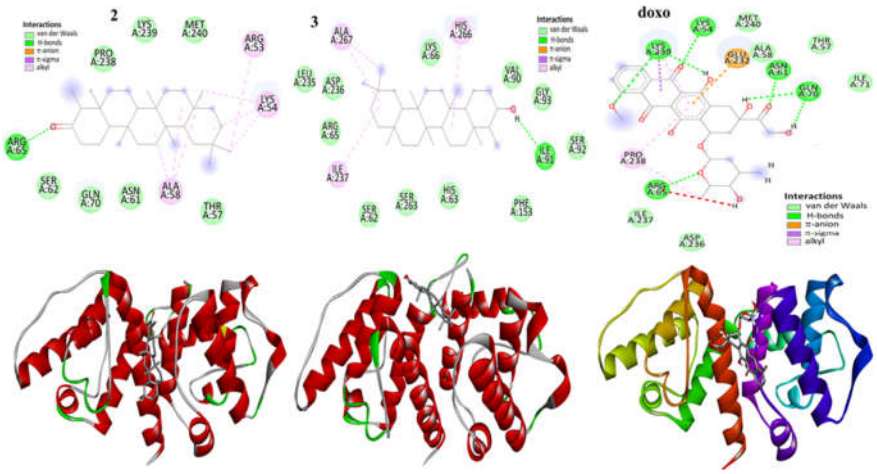

Figure 3. 2D (top) and 3D (bottom) binding interactions of compound **2, 3** and **doxorubicin** against *alpha-actinin-4 (PDB: 6O31).*

EXPERIMENTAL

Materials

All solvents were ACS grade (Carlo Erba reagents S.A.S, Val de Reuil, France); Dulbecco's Modified Eagle Medium (Gibco, ThermoFisher Scientific, USA); fetal bovine serum (Thermo-Scientific and ScienCell, USA); penicillin-streptomycin (Invitrogen, ThermoFisher Scientific, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Invitrogen, ThermoFisher

Scientific, ScienCell, USA); Sabouraud dextrose agar (Thermo-Scientific and ScienCell, USA); amphotericin B (Formepharma, Pakistan), Miconazole (Formepharma, Pakistan); doxorubicin (ICN, USA); Hanks Balanced Salt Solution ($HBSS^{++}$) (Sigma, St. Louis, USA); zymosan (Fluka, Buchs, Switzerland); Luminol (Research Organics, Cleveland, USA); 96-well white half area plates (Costar, NY, USA). A human cervical cancer (HeLa), human prostate cancer (PC3), and Swiss albino mouse embryo tissue (BJ) cells were obtained from the cell bank at Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), International center for chemical and biological sciences (ICCBS), University of Karachi, Karachi, Pakistan.

Apparatus and instrument

The compounds reported in this work were isolated using vacuum liquid chromatography (VLC) which carries 1kg silica gel 60HF, and three different sizes (big, medium, and small) column chromatography (CC) which can carry 200.0, 80.0, and 50.0 g silica gel respectively. Fractions collected from VLC were purified using column (50 mm x 50 cm) which can carry 200.0 g silica gel. TLC was performed on pre-coated plates (Silica gel 60 F254, 230-400 mesh, Merck) and Al_2O_3 plates. Spots were detected by observation under UV light (VILBER LOURMAT). Spraying agents used were 10% cerium (IV) sulphate ($Ce(SO_4)_2$) and 5% KOH. Melting point (mp) was determined in capillary tube with a digital electrothermal melting point apparatus. UV-Vis spectral measurements were done on a Shimadzu UV-VIS recording spectrophotometer, UV-160, spectronic genesys spectrophotometer. The IR (KBr) spectral measurements were done on a Perkin Elmer 1600 and Pye Unicam Infrared spectrophotometer SP3-300. Electron ionized mass spectrometry (EI-MS) was carried out using JEOL-600H-1. The nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents ($CDCl_3$ or $(CD_3)_2CO$) on a Bruker Avance III 400 and Avance Neo 600 MHz NMR spectrometers. All chemical shifts (δ) are reported in parts per million (ppm) with the solvent signal as a reference relative to TMS ($\delta = 0$) as internal standard, while the coupling constants (*J*) are given in Hertz (Hz).

Plant collection

The branch of *E. abyssinica* tree was collected in June, 2019, from Sendafa, Finfine Zuriya, Oromia, Ethiopia. This plant was identified and authenticated by a taxonomist and voucher specimen (JA-15-2019) was deposited at the National Herbarium, Department of Biology, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia. Botanical names have been transcribed according to the nomenclature system used by The World Flora Online (http: //www.theplantlist. org).

Extraction

Air-dried powdered plant material (1 kg) of *E. abyssinica* was extracted with 80% ethanol by maceration (plant material to solvent ratio of 1: 3) twice, each time for a period of one week. The macerated plant material was then filtered through Whatman No.1 filter paper under vacuum and the filtrates were evaporated to dryness under reduced pressure using a rotatory evaporator (BÜCHI, R-3000, Switzerland) at temperature not exceeding 40 ºC.

Fractionation

150.0 g *E. abyssinica* crude extract was pre-adsorbed on 200.0 g silica gel 60HF, and introduced to vacuum liquid chromatography (VLC) packed with 700.0 g silica gel 60HF as a stationary phase. The elution was carried out with different solvent gradient systems using (2000 mL each):pet. ether (100 %), pet. Ether:DCM (1:1), DCM (100%), DCM:EtOAc (1:1), EtOAc (100%), EtOAc: MeOH (1:1), MeOH (100%), and MeOH:H_2O (1:1) and the eluents were collected separately and concentrated using rotary evaporator.

Isolation of compounds

DCM eluted fraction of *E. abyssinica* (13.0 g) was pre adsorbed on silica gel 60HF(17.0 g) and applied to a CC (50 mm x 50 cm) packed with silica gel 60HF (170.0 g), and eluted with pet ether:EtOAc ((100.0:0.0, 99.0:1.0, 98.0:2:0, 96.0:4.0, 94.0:6.0, 90.0;10.0, 85.0:15.0, 80.0:20:0, 70:0:30.0, 50:0:50.0, 1000 mL each time). A total of 50 fractions (250 mL each) were collected (F1-F50). Fraction F24 afforded compound **5** (28 mg) as a white solid. Fraction F11-F16 (2.26 g) was pre-adsorbed on silica gel (2.50 g) and applied to a glass column (25 mm x 50 cm) packed with silica (50.0 g), and eluted with pet ether:EtOAc (100.0:0.0, 99.0:1.0, 98.0:2.0, 96.0:4.0; 200 mL each). Out of 16 fractions (50 mL each) collected, F11-F16 (f8-f14) afforded compound **2** (1.25 g) as a white powder after solvent removal under reduced pressure. Fraction F18-F26 (5.0 g) pre-adsorbed on silica gel (5.0 g) and applied to a glass column (25 mm x 60 cm) packed with silica gel (90.0 g), and eluted with pet. ether:acetone (100.0:0.0, 99.5:0.50, 99.0:1.0, 98.0:2.0, 96.0:4.0, 94.0:6:0, 92.0:8.0, 90.0:10.0, 80.0:20:0; 200 mL each). From thirty six fraction (50 mL each) collected, F18-F26 (f12-f15) and F18-F26 (f17-f22) afforded compound **3** (1.16 g) and **4** (1.63 g) as white solids after solvent removal under reduced pressure. Similarly, fraction F31-F39 (3.5 g) pre-adsorbed on silica gel (5.0 g) and applied to a glass column (25 mm x 50 cm) packed with silica (50.0 g), and eluted with pet ether : acetone (99.0:1.0, 98.0:2.0, 96.0:4.0, 94.0:6.0, 92.0: 8.0, 90.0:10.0, 80.0:10.0; 200 mL each). Out of 28 fractions (50 mL each) collected, F31-F39 (f18-f21) afforded compound **1** (0.83 g) as a clear white powder.

*β-sitosterol (**1**)*. White solid (0.83 g): mp 132-136 ºC; UV-Vis ($CHCl_3$) λ_{max} nm: no absorption between 600-200; IR (KBr) ν_{max} cm^{-1}: 3416, 2937, 2843, 1660, 1447, 1380, and 1048; EI MS: $[M]^+$ peak at m/z 414.4 corresponding to the molecular formula $C_{29}H_{50}O$ (calc. mass, 414.3862); ^{1}H-NMR (500 MHz, $CDCl_3$), chemical shift δ in ppm, coupling constant *J* in Hz: δ_H 5.33 (1H, m, H-6), 3.50 (1H, m, H-3), 0.99 (3H, *s*, H-19), 0.90 (3H, *d, J* = 6.5, H-21), 0.83 (3H, *d, J* = 7.5 Hz), 0.82 (3H, *d*, *J* = 6.85 Hz, H-26), 0.79 (3H, *d*, *J* = 6.8 Hz, H-27), 0.66 (3H, *s*, H-18); ^{13}C NMR (100 MHz, $CDCl_3$); δ_C 37.5 (C-1), 32.0 (C-2), 71.4 (C-3), 42.8 (C-4), 141.1 (C-5), 121.4 (C-6), 32.1 (C-7), 32.0 (C-8), 50.3 (C-9), 36.6 (C-10), 21.2 (C-11), 39.7 (C-12), 42.4 (C-13), 56.8 (C-14), 26.1 (C-15), 28.5 (C-16), 56.2 (C-17), 11.7 (C-18), 19.3 (C-19), 36.4 (C-20), 18.9 (C-21), 34.2 (C-22), 26.4 (C-23), 46.1 (C-24), 29.4 (C-25), 19.8 (C- 26), 19.1 (C-27), 23.3 (C-28), 12.0 (C-29).

*Friedelin (**2**)*. White crystal (1.25 g); melts at mp 260-262 °C; EI-MS: $[M]^+$ peak at *m/z* = 426.3 which are in agreement with the molecular formula $C_{30}H_{50}O$, calculated mass of 426.3862. UV-Vis (MeOH) at λ_{max} nm: no absorbtion between 600-200; IR (KBr) ν_{max} cm^{-1}: 2923, 2855, 1713, 1460 1390, 1050 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$), chemical shift δ in ppm, coupling constant *J* in Hz: δ_H 0.70 (3H, *s,* H-24), 0.85 (3H, *s,* H-25), 0.86 (3H, *d*, *J* = 6.72 Hz, H-23), 0.93 (3H, *s,* H-29), 0.97 (3H, *s,* H-30), 0.98 (3H, *s,* H-26), 1.03 (3H, *s,* H-27), 1.16 (3H, *s,* H-28), 1.67 (1H, *m,* H-1), 1.74 (1H, *m,* H-6), 1.94 (1H, *m,* H-1) 2.22 (1H, m, H-4), 2.28 (1H, *m*, H-2), and 2.37 (1H, *m*, H-2). ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C 213.3 (C-3), 59.5 (C-10), 58.2 (C-4), 53.1 (C-8), 42.8 (C-18), 42.2 (C-5), 41.4 (C-2), 41.3 (C-6), 39.7 (C-13), 39.3 (C-22), 38.3 (C-14), 37.4 (C-9), 36.0 (C-16), 35.6 (C-11), 35.3 (C-19), 35.0 (C-30) 32.8 (C-21), 32.4 (C-12), 32.1 (C-28), 31.8 (C-29) 30.5 (C-15), 30.0 (C-17), 28.2 (C-20), 22.3 (C-1), 20.3 (C-27), 18.7 (C-26), 18.2 (C-7), 18.0 (C-25), 14.7 (C-24), 6.8 (C-23).

*Epifriedelanol (**3**)*. White needle-shaped crystal (1.16 g); mp 274-276 ºC); EI-MS: $[M]^+$ peak at *m/z* = 428.3 corresponding to the molecular formula $C_{30}H_{52}O$ (calc. mass, 428.4018); UV-Vis (MeOH) λ_{max} nm: no absorption between 600-200; IR (KBr) ν_{max} cm^{-1}: 3473, 2925, 2858, 1462, 1379; ^{1}H-NMR (500 MHz, $CDCl_3$), chemical shift δ in ppm, coupling constant *J* in Hz: δ_H 0.84 (3H, *s*, H-25), 0.91(3H, *d*, *J* = 7.3 Hz, H-23), 0.92 (3H, *s*, H-29), 0.94 (3H, *s*, H-24), 0.97 (3H, *s*, H-26), 0.98 (3H, *s*, H-30), 0.99 (3H, *s*, H-27), and 1.15 (3H, *s*, H-28), 1.71 (1H, *dt*, *J* = 12.9, 3.05

Hz, H-4), 1.87 (1H, *dt*, *J* = 10.05, 2.5 Hz, H-2), 3.72 (1H, *br. d, J* = 2.5, H-3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 16.1 (C-1), 35.5 (C-2), 72.0 (C-3), 49.3 (C-4), 37.2 (C-5), 42.0 (C-6), 17.8 (C-7), 53.4(C-8), 38.0 (C-9), 61.7 (C-10), 35.7 (C-11), 30.8 (C-12), 38.4 (C-13), 39.8 (C-14), 33.0 (C-15), 36.3 (C-16), 30.1 (C-17), 43.0 (C-18), 35.8 (C-19), 28.2 (C-20), 32.5 (C-21), 39.4 (C-22), 11.7 (C-23), 16.5 (C-24), 18.3 (C-25), 18.7 (C-26), 20.2 (C-27), 32.1 (C-28), 35.0 (C-29), 31.8 (C-30).

*Taraxerol (**4**)*. White crystals (1.63 g); mp 278-280 °C; EI-MS: $[M]^+$ peak at *m/z* = 426.3 corresponding to the molecular formula $C_{30}H_{50}O$ (calc. mass, 426.3862); UV-Vis ($CHCl_3$) λ_{max} nm: no absorption between 600-200. IR (KBr) ν_{max} cm^{-1}: 3447, 2930 and 2843, 1693. ^{1}H NMR (400 MHz, $CDCl_3$), chemical shift δ in ppm, coupling constant *J* in Hz: δ_H 5.51 (1H, *dd*, *J* = 8.12, 3.12 Hz, H-15), 3.18 (1H, *dd*, *J* = 10.92, 4.52 Hz, H-3), 2.01 (1H, *dt, J* = 12.6, 3.0 Hz, H-7), 1.90 (1H, *dd, J* = 14.72, 2.76 Hz, H-16), 0.96 (3H, *s*, H-23), 0.93 (3H, *s*, H-29), 0.91 (3H, *s*, H-25) 0.89 (3H, *s*), 0.89 (3H, *s*, H-30), 0.80 (3H, *s*, H-26), 0.78 (3H, *s*, H-24); ^{13}C NMR (100 MHz, C_6D_6): δ_C 158.0 (C-14), 117.0 (C-15), 78.3 (C-3), 55.5 (C-5), 49.3 (C-18), 49.0 (C-9), 41.34 (C-19), 38.8 (C-4), 39.1 (C-8), 37.6 (C-17), 38.0 (C-1), 37.8 (C-13), 36.8 (C-16), 35.7 (C-10), 37.9 (C-12), 33.9 (C-21), 33.3 (C-29), 33.3 (C-22), 29.9 (C-28), 29.9 (C-26), 28.8 (C-20), 28.0 (C-23), 27.5 (C-2), 26.0 (C-27), 21.3 (C-30), 18.8 (C-6), 17.7 (C-11), 15.6 (C- 25), 15.4 (C-24).

*Glutinol (**5**)*. White solid (28 mg); mp 202-204 °C; EI MS: $[M]^+$ peak at *m/z* = 426.3 corresponding to the molecular formula $C_{30}H_{50}O$ (calc. mass, 426.7174); UV-Vis (MeOH) λ_{max} nm: no absorption 600-200; IR (KBr) ν_{max} cm^{-1}: 3447, 2930, 2843, 1693; ^{1}H-NMR (400 MHz, $CDCl_3$), chemical shift δ in ppm, coupling constant *J* in Hz: δ_H 5.61 (1H, *d, J* = 6.06 Hz, H-6), 3.44 (1H, *d, J* = 2.8, H-3), 1.14 (3H, *s*, H-28), 1.12 (3H, *s*, H-23), 1.07 (3H, *s*, H-26), 1.02 (3H, *s*, H-24), 0.98 (3H, *s*, H-27), 0.97 (3H, *s*, H-30), 0.93 (3H, *s*, H-29), 0.82 (3H, *s*, H-25); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 141.6 (C-5), 122.1 (C-6), 76.3 (C-3), 49.7 (C-10), 47.4 (C-18), 43.1 (C-8), 40.8 (C-14), 39.3 (C-4), 39.0 (C-22), 37.8 (C-13), 36.0 (C-16), 35.1 (C-19), 34.8 (C-9), 34.6 (C-11), 34.5 (C-30), 33.1 (C-21), 32.3 (C-28), 32.1(C-15), 32.0 (C-29), 30.4 (C-12), 30.1 (C-17), 28.9 (C-23), 28.2 (C-20), 27.8 (C-7), 25.4 (C-4), 23.6 (C-1), 19.6 (C-27), 18.4 (C-26), 18.2(C-2), 16.2 (C-25).

Biological activity

Cell lines and Culture Conditions

The human cervical cancer (HeLa) and prostate cancer (PC3) were separately cultured in Dulbecco's Modified Eagle Medium (DMEA), supplemented with 5% for HeLa and PC3 cells of fetal bovine serum (FBS), 100 IU/mL of penicillin, 100µg/mL of streptomycin in 75 cm^2 flasks, and kept in 5% CO_2 incubator at 37 ºC.

Cytotoxic activity

The cytotoxicity assays (cell viability test) on HeLa and PC3 was performed according to microculture MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as mentioned by Mosmann (1983) [48]. Briefly, 100 µL per well of cell solutions (6×10^4 cells per mL HeLa cells, 1×10^5 cells per mL PC3 cells), were added into 96-well plate and incubated for 24 h at 37 °C. After overnight incubation, medium was removed and 200 µL of fresh medium was added with extract (30 µg/mL)/fraction (30 µg/mL)/compound (30 µM)/ standard (30 µM) for 48 h. After this, 200 µL MTT (0.5 µg/mL) was added to each well and incubated further for 4 h at 37 °C. Then 100 µL DMSO was added to each well to dissolve the formazan crystal. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 570 nm (PC and HeLa) or 550 nm (BJ) using a microplate reader (Spectra Max Plus, Molecular Devices, CA, USA). Standard drug doxorubicin as a positive control and DMSO as a negative

control was used to find the percent growth inhibition or decrease in cells viability. The IC_{50} value of active compounds (inhibition > 50% at 30 µg/mL) was calculated using the EZ-Fit software. The percent growth inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = \frac{100-(\text{Mean of O.D of test substance} - \text{Mean of O.D of NC}}{(\text{Mean of O.D of PC} - \text{Mean of O.D of NC})} * 100 \quad (1)$$

where, O.D is optical density, NC is negative control and PC is positive control.

Molecular docking

Molecular docking analysis was conducted to compute the binding affinity and inhibition constant of isolated compounds **2** and **3** against human prostate specific antigen (PDB: 2ZCH) and *alpha-actinin-4 (PDB: 6O31) (one of the biomarkers for the precancerous state of cervical cancer) receptors.* AutoDock Vina with previously reported protocol [49] was used to dock the isolated compounds towards the active site of receptors. The structure of studied compounds with appropriate 2D and minimized energy were managed by ChemOffice tool (Chem Draw 16.0) and ChemBio3D, respectively.

Crystal structures of the proteins (PDB 2ZCH and PDAB: 6O31) were obtained from the respective Protein Data Bank (PDB), and adjusted by removing the co-crystallized substrates and water molecules, and added polar hydrogen and cofactors using AutoDock 4.2 (MGL tools1.5.7) following the same protocols as previously reported [50]. The grid box was adjusted with graphic user interphase program in such a way that the grid surrounds the key amino acids in the region of interest. With the use of the Lamarckian genetic algorithm (LGA) program and an adaptive entire method search in the AutoDock Vina, different conformations for each molecule were determined. Nine different conformations were generated and the one with the most favorable (least) free binding energy were selected to visualize the interactions between the residual amino acids and ligands by Discovery studio visualizer and PyMOL. The ligands are represented in different colors, H-bonds and the interacting residues are represented in stick model representation. The molecular docking results were compared with the positive controls doxorubicin.

CONCLUSION

The bioassay-guided fractionation and isolation of compounds from *E. abyssinica* against cancer cells resulted in isolation of cytotoxic compounds friedelin (**2**) and epifriedelanol (**3**) that could be a potential therapeutic agents in the treatment of various types of cancers. The lesser binding free energy and inhibition constant of friedelin (**2**) and epifriedelanol (**3**) towards both Human prostate specific antigen (PDB:2ZCH) and *Alpha-actinin-4 (PDB:6O31)* show that the compounds form stable complex with each receptor, and small amount is required to inhibit the activity of the proteins. The molecular docking results are in line with the *in vitro* cytotoxicity activity. The findings of this study suggest that *E. abyssinica extract and the isolated compounds* are a potential candidates in the treatment of cancer-like symptoms, and for cancer management.

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