

Metabolite Profiling and Cytotoxic Investigation of *Senecio biafrae* Oliv. & Hiern (Compositae) on Nauplii Brine Shrimps and Cancer Cell Lines

Funke Toyin Akinola,^{a*} Vincent Olumayowa Oriyomi,^b Seun Bayonle Ogundele,^c Babatunde Mukaila Adekola^d, Joseph Morounfolu Agbedahunsi^e Olusegun Olubunmi Babalola^a.

^aDepartment of Biochemistry and Molecular Biology, Obafemi Awolowo University, Ile-Ife, Nigeria.

^bDepartment of Biochemistry and Forensic Science, First Technical University, Ibadan, Nigeria.

^cDepartment of Pharmacognosy and Natural Products, College of Pharmacy, Afe Babalola University, Ado-Ekiti, Nigeria

^dDepartment of Environmental Management and Toxicology, Federal University of Agriculture, Abeokuta, Nigeria.

^eDrug Research and Production Unit, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.

***Corresponding Author**

Akinola, Funke Toyin
Biochemistry and Molecular Biology
Faculty of Science
Obafemi Awolowo University, Ile-Ife, Nigeria

Email: akinolafunke054@gmail.com

Abstract

The study investigated the cytoactivities of methanolic leaf extract (MLE) and fractions of *Senecio biafrae*, profiled, and elucidated their metabolites. Hydromethanolic extract of *S. biafrae* obtained by maceration was partitioned and subjected to cytotoxicity using brine shrimp lethality assay (BSLA), human cervical adenocarcinoma (HeLa) and prostate cancer cell (PC3) lines. The bioactive principles of most active fractions were profiled using GC-MS. The dichloromethane fraction (DMF) was purified using column and high-performance liquid chromatography to afford compounds 1 and 2 identified by ¹H and ¹³C, DEPT-90, DEPT-135, COSY, NOESY and EIMS spectra. The MLE and its fractions exhibited good toxicity on nauplii brine shrimp. The HF exhibited a pronounced cytoactivity on PC-3 cell line with 92.8% followed by DMF. The GC-MS profiling of HF and DMF identified 17 and 6 constituents respectively comprising saturated and unsaturated fatty acids, diterpenoidal alcohol, phytol and aromatic compounds. Hexadecanoic acid, cis, cis, cis-9, 12, 15-octadecatrienoic acid, and phytol accounted for the highest percentage of the constituents in both fractions. Structure elucidation confirmed compounds 1 and 2 as stigmasterol and ergosterol with cytoactivity which compared favourably with doxorubicin. The study revealed that *S. biafrae* contains cytotoconstituents evidenced by proliferation inhibition of HeLa and PC-3 cell lines. The cyto-activities and bioconstituents indicate that *S. biafrae* has the potential to be considered as a candidate for drug development in the management of cancer-related conditions. Further investigation into the specific bioactive compounds and their mechanisms of action could provide valuable insights into the therapeutic potential of *S. biafrae* in cancer treatment.

Keywords: *Senecio biafrae*, Cytotoxicity, HeLa cell line, Prostate cell line, GC-MS Chemical Profiling

INTRODUCTION

From time immemorial, Man have depended on nature for basic needs of life like food, shelter, clothing, etc. with majority of these natural products coming from plants, animals and minerals needed for treating human diseases^[1, 2]. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies^[3]. This system continues today because of its biomedical benefits and cultural beliefs in many parts of the world which made it a great contribution towards maintaining human health^[4]. The World Health Organization estimated that 80% of the population in the developing countries continues to use traditional medicine to treat infectious diseases^[5]. Medicinal plants are known to contain inherent active ingredients used to manage disease or relieve pain^[6]. The use of traditional medicines and medicinal plants as therapeutic agents for the maintenance of good health in most developing countries has been widely observed^[7]. According to World Health Organization, medicinal plants are the best sources for obtaining variety of drugs. Therefore, plants should be investigated to better understand their properties, safety and efficacy^[8]. The efficacy of medicinal plants in the management of diseases is indubitable as it occupies a unique place in human life and provides information about the use of plants or plant parts as medicine^[9]. *Senecio biafrae* Oliv. & Hiern (Compositae), commonly known as life root plant, is one of the most important medicinal plants with edible leaves and stems, widely consumed as vegetables among the Yoruba tribe of South-western, Nigeria^[10]. There is a resurgent of interest in the pharmacological efficacy of medicinal plants in the management of various human carcinomas and other pathologies, especially in the face of increasing failure and toxicity associated with the use of some anticancer agents^[11]. Numerous bioactive compounds or their derivatised complexes from medicinal plants have been reported for chemopreventive and anticancer activity^[12]. Phytochemical investigations

of *Senecio biafrae* revealed the presence of phytometabolites belonging to different classes of chemical diversities such as terpenoids, flavonoids, alkaloids, anthocyanins, coumarins, glycosides and dihydroisocoumarins which have been reported to possess antimicrobial properties^[13]. The leaf of *S. biafrae* is ethnomedicinally used for the treatment of wounds, sore eyes, infertility, rheumatism, pulmonary defects, heart problems, cough and diabetes and promotion of milk secretion in lactating mothers^[14-15]. *S. biafrae* has been reported to be a good source of protein having high levels of essential amino acids and vitamins A, C and E, and has been recommended as a food supplement^[16]. In this study, we investigated the cytotoxic activity of *Senecio biafrae* leaf extract and its fractions on nauplii brine shrimp, human cervical adenocarcinoma (HeLa) and prostate cancer cell (PC-3) lines and carried out the chemical profiling, isolation, purification and structure elucidation of the active chemical principles on the two most active fractions.

MATERIALS

Chemicals and Reagents

Fetal bovine serum (FBS), trypan blue solution and antibiotics (streptomycin/penicillin) were purchased from Gibco (California, USA). RPMI-1640 medium, D-Glucose, HEPES, Dulbecco modified eagle medium (DMEM), Triton X100, F12-DMEM and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfuroxide (DMSO) was purchased from Fisher Scientific (Leicestershire, UK). *Escherichia coli* Lipopolysaccharide (LPS) was purchased from DIFCO Laboratories (Michigan, USA). PC-3 (human epithelial, prostate cancer) and HeLa (human epithelial adenocarcinoma) cells were obtained from European Collection of Cell Cultures, Salisbury ECACC (UK). HPLC-graded methanol, chloroform, hexane and ethyl acetate were purchased from Merck (Darmstadt, Germany). Tween 20, ethanol, pre-coated silica gel (F₂₅₄) plates, silica gel (SiO₂; 230-400 mesh), butanol, dichloromethane and chloroform were purchased from Merck, (Darmstadt, Germany). De-ionized

water was obtained from a Milli-Q UV-Plus water purification system (Millipore Corp., Billerica, MA, USA).

Collection and Identification of Sample

Fresh leaves of *Senecio biafrae* were collected in January, 2019 from Akamo Village Iperindo, Osun State, Nigeria (Latitude 07°29'40"; Longitude E04°49'26"). The plant was identified and authenticated at the IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile – Ife, Nigeria. Voucher specimen (voucher number (IFE-17936) was prepared and deposited in the Herbarium of the department of Botany, Obafemi Awolowo University, Nigeria. The nomenclature of the plant was further confirmed at www.worldfloraonline.org.

METHODS

Extraction and Partitioning of Plant

Powdered leaf (2 kg) was macerated (3X) with 80% (v/v) MeOH: H₂O (10 L) at room temperature for 48 h with continuous agitation on a shaker. The combined extract was then filtered and evaporated *in vacuo* at 40°C to afford a dry crude MeOH extract (45 g) which was subsequently suspended in H₂O and successively partitioned to afford the respective fractions: *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), butanol and aqueous residue which were also concentrated to dryness *in vacuo* at 40°C. The crude extract and respective fractions were then tested for their cytotoxic activities. The *n*-hexane and dichloromethane fractions exhibited the most cytotoxic activity and were selected for further analysis.

GC-MS Metabolite Profiling of Most Active Fractions

The metabolite profiling of fractions that exhibited the most cytoactivities was carried out in order to identify the chemical constituents responsible for their cytotoxic activity. They were analyzed on a fused silica AT-5MS capillary column (30m x 0.25mm internal diameter, 0.25 µm film thickness)

equipped on a gas chromatograph Agilent 5975C combined with inert XL EICI MSD fitted with a triple-axis detector source (Agilent Technologies, Santa Clara, CA, USA). The split ratio of injection was set at 1:30 with injection pot temperature of 270°C, while the column temperature was set at 60°C upon injection. The temperature was increased at rate of 3°C/minute to 270°C and held for 30 minutes. Purified helium was the carrier gas at flow rate of 1mL/minute. The mass spectrometer was operated in EI mode at 70eV, ion source temperature of 230°C, quadrupole temperature 150°C, mass range (m/e) of 29-550 with scan rate of 6.35 scan/s and electron multiplier voltage 1341V. The MS transfer line temperature was set at 280°C. Identification of components was performed based on their retention time and by mass spectra matching with National Institute of Standard and Technology (NIST) MS library 2009, while concentration of identified components was calculated using area normalization over flame ionization detector response.

Chromatographic Fractionation of DCM Fraction

The DCM fraction (10g) was chromatographed and fractionated over silica gel on open column and eluted with *n*-hexane with increasing 10% gradient of EtOAc up to 100% EtOAc followed by increasing gradient elution with MeOH up to 100% MeOH to afford 13 fractions A-M. Sub-fraction G was further fractionated over sephadex LH 20 column and isocratically eluted with DCM:MeOH (9:1) to afford sub-fractions 1-30 of 10 mL each, which were analyzed by TLC. Sub-fractions 6-15 were combined and further chromatographed on silica gel column to afford Compound 1 as a white powder, which precipitated out and was recrystallized with CHCl₃. Sub-fraction H was also chromatographed on open column chromatography and eluted with dichloromethane up to 100% followed by gradient with EtOAc up to 100% followed by gradient with MeOH to give sub-fractions H1-H10. Following TLC analysis, sub-fractions H6-H10 were combined and was further chromatographed on open column with silica gel 230-400 mesh as stationary phase and eluted with

dichloromethane with increasing EtOAc up to 100% and MeOH solvent systems to afford an off-white powder, which was dissolved in CH₂Cl₂ and purified on sephadex column to afford Compound 2 which was recrystallized from CHCl₃ to give a white solid.

Brine Shrimp Lethality Bioassay

The extract and fractions of *S. bialafrae* leaf were evaluated in a test for lethality to brine shrimp larvae according to Meyer *et al.* (1982). Toxicities of compounds were tested at 10, 100, and 1000 µg mL in 10 mL sea-water solutions with 1% DMSO (v/v). Ten nauplii were used in each test and survivors counted after 24 h. Three replications were used for each concentration. The blank control is conducted with Distilled water. The median lethal concentration of the fractions for 50% mortality after 24 h of exposure was calculated using Probit Analysis (Finney, 1971) using the mathematical expression, % mortality = (no. of dead nauplii/initial no. of live nauplii) × 100.

***In vitro* Cell Culture**

Human cervical adenocarcinoma and prostate cancer cell lines were used to evaluate the cytotoxic effect of extract and fractions while Compound 1 was investigated on HeLa cell line. The cells were grown in Dulbecco modified eagle medium (DMEM) (Sigma-Aldrich (St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin (Gibco, USA). Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

***In vitro* Cytotoxicity Assay**

The cytotoxic effect of extract, fractions and isolated compounds on PC3 and HeLa cells was evaluated by colorimetry assay using 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) ^[17]. Extract, fractions and isolated compounds were individually dissolved in 1% DMSO and diluted with cell culture medium to give final concentration of 30 µg/mL. A 100 µL of HeLa and PC3 cells suspensions (6x10⁴ cells/mL) was seeded in a 96-well plate and incubated

overnight at 37°C in 5% CO₂. The medium was carefully aspirated and the cells were challenged with the prepared solutions of extract, fractions and isolated compounds and the plate was further incubated for further 48 hours. An aliquot of 50 µL MTT at concentration of 0.5 mg/mL was then dispensed into each well and incubated for 4 hours, followed by the addition of 100 µL of DMSO. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 570 nm using a micro-plate reader (Spectra Max Plus, Molecular Devices, CA, USA). The cytotoxicity was recorded as percentage inhibition of the sample and standard on the cell lines. Doxorubicin (standard drug) was used as a positive control, while DMSO served as negative control. The percent inhibition was calculated by using the following formula:

$$\% \text{Inhibition} = 100 - \frac{(\text{Mean OD of Test Compound} - \text{Mean OD of Negative Control})}{(\text{Mean OD of Positive Control} - \text{Mean OD of Negative Control})} \times 100$$

Statistical Analysis

Data obtained were expressed as mean ± S.E.M using Graph Pad Instat Graphical Statistical Package version 5 (GraphPad Software Inc, USA). Significance of the results were evaluated using one-way analysis of variance (ANOVA) and the means was compared using Turkey's test. Values of p < 0.05 was considered as statistically significant

190 3. RESULTS

191 Brine Shrimp Lethality Assay of *S. bialfrae* Plant

192 The result of brine lethality assay of *S. bialfrae* extract and fractions is represented in Table 1.

193 Table 1: Percentage Mortality of shrimp nauplii after treating with *Senecio bialfrae* extract and fractions.

Plant Extract	Concentration (µg mL)	Number of Surviving Nauplii (after 24 h)			Total Number of Nauplii Survivor	% Mortality	LC50 (µg/mL)
		R1	R2	R3			
Control (Distilled water)	10	10	10	10	30	0%	0
	100	10	10	10	30	0%	
	1000	10	10	10	30	0%	
Standard (Etoposide)	10	10	9	9	28	6.67%	465.64
	100	7	6	6	19	36.67%	
	1000	1	1	0	2	93.33%	
<i>S. bialfrae</i> (Methanolic Extract)	10	10	10	9	29	3.33%	571.32
	100	10	10	9	29	3.33%	
	1000	9	9	9	27	10%	
<i>S. bialfrae</i> (Hexane Fraction)	10	10	10	10	30	0%	610.74
	100	10	10	9	29	3.33%	
	1000	3	3	3	9	70%	
<i>S. bialfrae</i> (DCM Fraction)	10	10	10	10	30	0%	610.75
	100	10	9	9	28	6.66%	
	1000	0	0	0	0	100%	
<i>S. bialfrae</i> (EA Fraction)	10	10	10	10	30	0%	572.73
	100	10	9	9	28	6.66%	
	1000	9	9	9	27	10%	
<i>S. bialfrae</i> (Butanol Fraction)	10	9	9	9	27	10%	483.98
	100	8	8	8	24	20%	
	1000	8	8	8	24	20%	
<i>S. bialfrae</i> (Aqueous Fraction)	10	10	10	10	30	0%	576.95
	100	10	9	9	28	6.66%	
	1000	8	8	8	24	20%	

Cytotoxicity of *S. bialafrae*

The cytotoxic activity of extract, fractions and isolated compounds on PC-3 and HeLa cell lines are presented in Table 2. The hexane showed better activity than the crude extract, and other fractions tested on the PC-3 cell lines. The cytotoxic activity revealed that *n*-hexane fraction of the methanolic extract *S. bialafrae* leaf exhibited the best cytotoxic activity with % inhibition of 92.8 on PC-3 cell lines and 20.2 on HeLa cell lines, and therefore is the most active fraction of the crude extract. Thus, in this study, the hexane fraction particularly demonstrated a better cytotoxic activity on PC-3 cell line followed by doxorubicin, dichloromethane, compounds 1 and 2. Compound 1 was found to exhibit 45.9 and 21.7 % inhibition on the proliferation of HeLa and PC-3 cell lines respectively compared to doxorubicin with 89.9 and 100 % inhibition at the same concentration of 30 µg/mL. Also compound 1 exhibited a better cytotoxic activity than compound 2.

Table 2: Cytotoxic Activity of Extract, Fractions and Compounds 1, 2 on PC-3 and HeLa cell

Fraction/Drug	PC3	HeLa
	% Inhibition at 30µg/ml	% Inhibition at 30µg/ml
Crude extract	0.73	6.9
Hexane	92.8	20.2
DCM	17.1	6.8
Ethyl acetate	-17.7	-8.4
Butanol	-24.1	6.11
Aqueous	-12.0	-0.6
Compound 1	21.9	45.9
Compound 2	13.4	15.2
Doxorubicin	89.9	101.2
DMSO	0	0

GC-MS Metabolite Profiling of Hexane and Dichloromethane Fractions

The GC-MS profiling of the most active fractions of methanolic extract identified seventeen (17) bioactive compounds in the hexane and six (6) in the dichloromethane fractions respectively. The total ion chromatogram is presented in Figures 1 and 2 while Tables 3 and 4 show the identified constituents, their names, molecular weight, molecular formula, retention time and abundance (peak area in percentage). The major compounds identified were n-Hexadecanoic acid (35.90%), 9, 12, 15-Octadecatrienoic acid, (Z, Z, Z) (21.85%), Phytol (9.40%), Hexadecanoic acid, methyl ester (7.38%), 9, 12-Octadecadienoic acid (Z,Z)- (6.08%) and 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (5.97%) while the minor compounds identified were 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (2.79%), Octadecanoic acid (1.68%), 2-Pentadecanone, 6,10,14-trimethyl (1.52%), Octadecanoic acid, methyl ester (0.97%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (0.69%), 1,2-Benzenedicarboxylic acid, diisooctyl ester (0.59%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (0.46%), 2H-Pyran-2-one, tetrahydro-6-nonyl (0.32%), Eicosanoic acid, methyl ester (0.28%), 1,4-Benzenediol, 2,6-bis(1,1-dimethylethy (0.11%) and 3-Butoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane (0.09%).

Figure 1: GC-MS Chromatogram of Hexane Fraction

Figure 2: GC-MS Chromatogram of Dichloromethane Fraction

S/N	Compounds	Formula	MW	RT(min)	Area
-----	-----------	---------	----	---------	------

					(%)
1	2-Pentadecanone, 6,10,14-trimethyl	C ₁₈ H ₃₆ O	268	35.01	1.52
2	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270	41.00	7.38
3	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	43.90	35.90
4	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C ₁₉ H ₃₄ O ₂	294	48.70	2.79
5	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C ₁₉ H ₃₂ O ₂	292	48.87	5.97
6	Phytol	C ₂₀ H ₄₀ O	296	49.27	9.40
7	Octadecanoic acid, methyl ester	C ₁₉ H ₃₈ O ₂	298	49.51	0.97
8	9,12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	280	49.73	6.08
9	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	C ₁₈ H ₃₀ O ₂	278	49.91	21.85
10	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	284	50.32	1.68
11	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	296	51.52	0.69
12	Eicosanoic acid, methyl ester	C ₂₁ H ₄₂ O ₂	326	53.40	0.28
13	2H-Pyran-2-one, tetrahydro-6-nonyl-	C ₁₄ H ₂₆ O ₂	226	53.90	0.32
14	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	296	54.39	0.46
15	3-Butoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane	C ₁₃ H ₃₆ O ₄ Si ₄	368	56.26	0.09
16	1,2-Benzenedicarboxylic acid, diisooctyl ester	C ₂₄ H ₃₈ O ₄	390	56.61	0.59
17	1,4-Benzenediol, 2,6-bis(1,1-dimethylethyl)	C ₁₄ H ₂₂ O ₂	222	56.93	0.11

Table 3: Constituents Identified in Hexane Fraction of *S. biafrae* Leaf Extract

MW= Molecular weight, RT= Retention time

Table 4: Metabolite Constituents Identified in the Dichloromethane Fraction of *S. biafrae*

S/N	Compounds	Formula	MW	RT(min)	Area (%)
1	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	296	34.63	4.70
2	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	43.63	17.24
3	Phytol	C ₂₀ H ₄₀ O	296	49.28	12.67
4	9,12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	280	49.67	9.52
5	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C ₁₉ H ₃₂ O ₂	292	49.87	52.35
6	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	C ₁₈ H ₃₀ O ₂	278	50.29	3.51

Chromatographic Fractionation of Dichloromethane (DCM) Fraction

Chromatographic fractionation to isolate and purify the chemical components in the DCM fraction afforded two known triterpenoids.

Compound 1 (Stigmasterol) Figure 3

Nature: off-white crystalline powder

Yield: 73 mg

¹H-NMR (500 MHz, CDCl₃) and ¹³C-NMR (600 MHz, CDCl₃). See (Table 5).

EIMS [M]⁺ m/z 421.2 (100) calculated for C₂₉H₄₈O, [M-CH₄]⁺ m/z 396.2 (43), [M-C₂H₇]⁺ m/z 381.2, 300.1 (41), 271.1 (54), 255.1 (78), 159.0 (42), 83 (40), 69 (27), 55.0 (41) 43 (20).

Compound 2 (Ergosterol) Figure 4

Nature: white powder

Yield: 45 mg

¹H-NMR (500 MHz, CDCl₃) and ¹³C-NMR (600 MHz, CDCl₃) (Table 6).

EIMS [M]⁺ m/z 396.4 (100) calculated for C₂₈H₄₄O, [M-CH₂]⁺ m/z 382 (23), 255 (21), 147 (19).

Figures 3 and 4: Structures of Compounds 1 and 2

Table 5: Spectroscopic Data of Compound 1 (CDCl₃)

Position	Compound 1		Stigmasterol (Literature)	
	δ _H (ppm)	δ _C (ppm)	δ _H (ppm)	δ _C (ppm)
1		37.25		37.6
2		31.89		32.1
3	3.45, 1H, m,	71.81	3.51, 1H, tdd	72.1
4		42.46		42.4
5		140.75		141.1
6	5.34, 1H, s,	121.71	5.31, 1H, t	121.8
7		31.66		31.8
8		30.85		31.8

9		50.15		50.2
10		36.50		36.6
11		19.65		21.5
12		39.67		39.9
13		42.48		42.4
14		56.85		56.8
15		24.30		24.4
16		28.91		29.3
17		56.76		56.2
18	1.13, 3H, s	12.40	1.03, 3H, s	12.2
19	0.68, 3H, s	18.91	0.71, 3H, s	18.9
20		39.79		40.6
21		21.30	0.91, 3H, d, 6.2 Hz	21.7
22	5.17, 1H, m, 8.2 Hz	138.31	5.14, 1H, m	138.7
23	5.00, 1H, m, 8.5 Hz	129.26	4.98, 1H, m	129.6
24	0.84, 1H, m, 6.7 Hz	45.83		46.1
25		25.70		25.4
26	0.89, 3H, m	12.25	0.83, 3H, t	12.1
27		29.14		29.6
28	0.81, 3H, m	19.90	0.82, 3H, d, 6.6 Hz	20.2
29	0.80, 3H, m	19.13	0.80, 3H, d, 6.6 Hz	19.8

s,m,d,t,ddd means singlet, multiplets, doublets, triplets and triplets of doublets of doublets

respectively

257

258 **Table 6: Spectroscopic Data of Compound 2 (CDCl₃+CD₃OD)**

Position	Compound 2		Ergosterol (Literature)	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1		38.43		38.4
2		31.69		31.9
3	3.27, 1H, m,	69.94	3.64, 1H, m	70.5
4		39.50		42.8
5		138.7		139.9
6	5.27, 1H, s,	121.91	5.58, 1H, dd, 5.5, 3.0 Hz	119.6
7		100.85	5.38, 1H, dd, 5.4, 2.9 Hz	116.3
8		140.05		141.4
9		45.61		46.2
10		37.00		37.1
11		19.47		21.1
12		40.01		39.1
13		39.50		42.9
14		55.81		54.6
15		22.27		22.9

16		28.89		28.3
17		56.51		55.7
18		11.65	0.95, 3H, s	12.1
19		15.75	0.65, 3H, s	16.3
20		40.41		40.3
21	1.01, 3H, s	20.80	1.04, 3H, d, 6.6 Hz	21.1
22	5.10, 1H, m, 8.1 Hz	130.31	5.20, 1H, m	135.6
23	4.97, 1H, m	129.4	5.21, 1H, m	131.9
24	0.84, 1H, m, 6.7 Hz	43.18		42.9
25		33.69		33.1
26	0.88, 3H, m	19.01	0.84, 3H, d, 6.7 Hz	19.9
27	0.86, 3H, m	18.68	0.82, 3H, d, 6.7 Hz	19.7
28	0.93, 3H, m	18.46	0.82, 3H, d, 6.6 Hz	17.6

s,m,d,t,ddd,dd means singlet, multiplets, doublets, triplets and triplets of doublets of doublets, doublets of doublet respectively

Spectroscopic identification of isolated compounds revealed that compounds **1** and **2** have a steroidal moiety. The mass spectrum of compound **1** showed a molecular ion peak $[M]^+$ at m/z 421.2 which is also the base peak, followed by other fragment ions at m/z 396.2 $[M-CH_4]^+$ which due to loss of methane and m/z 381.2 $[M-C_2H_7]^+$. The molecular mass of 421.2 is consisted with molecular formula, $C_{29}H_{48}O$. The 1H -NMR spectrum of compound **1** revealed 3 downfield proton signals in the olefinic region at δ_H 5.34 (1H, s, 1.6Hz, H-6); 5.17 (1H, m, 8.2Hz, H-22) and 5.00 (1H, m, 8.5Hz, H-23). The 8.2Hz coupling constant indicating the *cis* nature of the olefinic double bond between C-22 and C-23, which was confirmed by the NOESY spectrum. The signal at δ_H 3.45 (1H, m, H-3) was confirmed by DEPT-90 spectrum to be a methine carbon (CH) bonded to a highly electronegative oxygen. The spectrum showed many alkyl signals in the upfield region between δ_H 0.5-2.5ppm which is due to stigmasterane nucleus of Compound **1**. The ^{13}C -NMR spectrum and DEPT-90 spectra of Compound **1** showed signals due to 4 non-equivalent sp^2 hybridized olefinic carbons at δ_c 140.75 (C-5), 138.31 (CH-23), 129.26 (C-22) and 121.71 (CH-6). Also, a sp^3 hybridized oxygenated methylene carbon at δ_c 71.81 (CH-3). The comparison of DEPT-135 with

¹³C spectra further confirmed that carbon signal at δ_c 140.75 is a quaternary olefinic carbon. The stigmasterane steroidal nucleus of Compound **1** is indicated by signals in the upfield region of the spectrum at δ_c 10-60 which is characteristic of the steroidal family. The COSY and NOESY spectra information agree with the literature data on Stigmasterol [19].

The mass spectrum of Compound **2** showed a molecular ion peak $[M]^+$ at m/z 396.4 (100) which is also the base peak. Other fragment ions included $[M-CH_2]^+$ m/z 382 (23) loss of CH_2 unit, 255 (21), 147 (19). The molecular mass of 396.4 is consistent with molecular formula $C_{28}H_{44}O$. The molecular mass of compound **2** therefore showed a striking difference from compound **1**. The ¹H-NMR of Compound **2** showed similar spectra information as obtained in **1** with olefinic protons at δ_H 5.27 (1H, s, 1.6Hz, H-6), 5.13 (1H, m, H-7), 5.00 (1H, m, H-22), 4.99 (1H, m, H-23). The signal at δ_H 3.27 (1H, m, H-3) is confirmed by the DEPT-90 experiment to be a methylene carbon (CH) bonded to a highly deshielding oxygen. The spectrum further showed many signals in the upfield region at δ_H 0.5-2.5 which is due to the steroidal hump of ergostane nucleus of Compound **2**. The ¹³C NMR spectrum and DEPT-90 spectra of Compound **2** showed signals due to 6 non-equivalent sp^2 hybridized olefinic carbons at δ_c 140.05 (C-8), 138.71 (C-5), 130.33 (CH-22), 129.41 (CH-23), 121.91 (CH-6) and 100.89 (CH-7). Furthermore, a sp^3 hybridized oxygenated methylene carbon at δ_c 69.97 (CH-3). The comparison of ¹³C and DEPT-135 spectra further indicated that carbon signal at δ_c 140.05 and 138.71 are quaternary olefinic carbon signals, which further differentiate compound **2** from **1**. The steroidal hump nucleus of **2** is indicated by many signals in the upfield region of the spectrum at δ_c 10-60 typically common to most steroids. The spectral information thus obtained on **2** is in agreement with literature data on Ergosterol [20].

4. DISCUSSION

299 Brine shrimp lethality assay (BSLA) is a straightforward and inexpensive bioassay used for testing
300 the cytotoxic effect of phytochemical present in the plant extracts [21]. The result on the lethality
301 of methanolic extract and fractions of *S. bialfrae* plant on brine shrimps showed that the plant extract
302 and fractions exhibited a good toxicity with $LC_{50} < 1000 \mu\text{g mL}$. Based on the results of this study,
303 it can be reported that the hexane and dichloromethane fractions of the plant demonstrated relatively
304 lower toxicity compared to the other fractions or extract tested, as well as the standard drug
305 (Doxorubicin). This suggests that these particular fractions may have safer properties and could
306 potentially be explored further for their therapeutic applications with reduced risk of adverse effects.
307 However, it is important to note that further research and evaluation are necessary to fully
308 understand the safety profile and potential benefits of these fractions in the context of drug
309 development and cancer management. According to Meyer *et al.* (1982), substances with LC_{50}
310 value $< 1000 \mu\text{g mL}$ are toxic while ones with values $> 1000 \mu\text{g mL}$ are non-toxic. The observed
311 toxic property of the n-hexane fraction against brine shrimp larvae provides additional support for
312 its cytotoxic activity against the PC-3 cell line. The cytotoxicity exhibited by the n-hexane fraction
313 against the brine shrimp larvae suggests its potential as a bioactive compound with cytotoxic effects.
314 This finding supports the potential of the n-hexane fraction as a candidate for further investigation
315 and development as an anticancer agent against the PC-3 cell line. The cytotoxic activity revealed
316 that n-hexane fraction of the methanolic extract *S. bialfrae* leaf exhibited strong inhibitory
317 activity on PC-3 cell line, followed by the standard drug doxorubicin, dichloromethane, compound
318 1 and 2. Compound 1 was found to exhibit moderate % inhibition on the proliferation of HeLa and
319 PC-3 cell lines respectively compared to the standard drug used. Compound 1 exhibited a better
320 cytotoxic activity than compound 2. Thus, the higher cytotoxic activity exhibited by the *n*-hexane
321 fraction could be attributed to the bioactive compounds that are present in the fraction and their

synergistic interaction [23]. This strong inhibitory activity observed in hexane fraction against PC-3 cell line is in line with the previous studies on other *Senecio* species. According to Loizzo *et al.* [24], the n-hexane extract of *S. ambiguus* exhibited a strong inhibitory activity against prostate carcinoma cell line, a similar result to the n-hexane fraction of *S. bialfrae* against prostate cancer cell line indicating that all *Senecio* species might have some activities in common.

The chemical profiling of hexane fraction from *S. bialfrae* plant revealed the presence of seventeen (17) while DMF had six (6) saturated and unsaturated fatty acids, phytol and benzene dicarboxylate esters. The most abundant saturated fatty acid identified is n-hexadecanoic acid (palmitic acid) known for exerting anti-cancerous, anti-inflammatory, antioxidant and anti-androgenic pharmacological effects [25]. This was followed by unsaturated fatty acid cis, cis, cis-9,12,15-octadecatrienoic acid and phytol, an acyclic diterpene alcohol. Phytol is a known precursor of phytanic acid, vitamins E and K and has been found to possess strong cytotoxic, antioxidant and anti-inflammatory activities [26-27]. These compounds were characterized as white amorphous solids and exhibited a positive reaction with Salkowski reagents, indicating the presence of steroidal structures. Phytosterols are plant-derived compounds that have been widely studied for their various biological activities, including anticancer properties. The identification of these phytosterols in the plant extract suggests their potential contribution to the observed cytotoxic activity. The molecular formula of the isolated molecules found to be stigmasterol and ergosterol are $C_{29}H_{48}O$ and $C_{28}H_{44}O$ respectively which was confirmed by mass spectral and nuclear magnetic resonance of 1D and 2D structure. Phytosterols being a steroidal alcohol play important roles in membrane stability and in structural component in the cell membrane. Since pyhtosterol cannot be synthesized by human being or animal, they are derived through the exogeneous sources, especially the diet [28].

Phytosterols are very abundant in plants, they share many structural similarities with cholesterol

which has been implicated in the pathophysiology of many oxidative stress-induced conditions. Phytosterols have been speculated to possess anti-cancer activities in many organs of the body such as ovary, lungs and stomach [29]. The high amount of phytosterol intake is from vegetarian diet; that are consistent with a reduction of breast cancer risk [30]. Increasing the concentration of phytosterols in the blood can indeed be achieved through the extraction of phytosterols from plant foods and their incorporation into various other food products or dietary supplements. Phytosterols are naturally occurring compounds found in plant cell membranes, and they have a similar structure to cholesterol [31, 32]. By consuming foods or supplements enriched with phytosterols, individuals can potentially reduce cholesterol absorption in the intestine, leading to lower LDL (bad) cholesterol levels in the blood [33]. Vegetables like *S. bialfrae* can be a potential source of phytosterols, and their extraction and incorporation into different food products can provide a convenient way for individuals to increase their phytosterol intake. These enriched products may include fortified spreads, margarines, yogurts, beverages, and dietary supplements. Phytosterol-enriched products are commonly available in the market and are specifically marketed for their cholesterol-lowering benefits. Additionally, incorporating a variety of plant-based foods rich in phytosterols, along with a balanced diet and a healthy lifestyle, is essential for overall well-being.

CONCLUSION

The results of the study showed that the methanolic extract fractions of *S. bialfrae* leaf exhibited good toxicity when tested against brine shrimp larvae. The hexane fraction of *S. bialfrae* leaf exhibited a significant cytotoxic activity on PC-3 cell than other fractions and isolated compounds. The cytotoxic activity observed by hexane fraction may be due to the presence of chemical constituents and their synergistic interaction. Also, the use of mass spectral, ¹H NMR, ¹³C NMR

including 2D-NMR analyses enabled the identification of two phytosterol compounds: stigmasterol and ergosterol in dichloromethane fraction of *S. bialfrae*. Compound 1 (stigmasterol) showed a moderate activity on HeLa cell while other fractions and compound 2 (ergosterol) exhibited a weak activity.

ACKNOWLEDGEMENTS

Akinola F.T. acknowledges the World Academy of Sciences for the Advancement of Science in Developing Countries (TWAS), Trieste, Italy for ICCBS-TWAS Fellowship at the H.E.J. Research Institute of Chemistry, ICCBS, University of Karachi, Karachi, Pakistan. The authors are also grateful to the MS and NMR Unit of H.E.J Research Institute of Chemistry, ICCBS, University of Karachi, Karachi, Pakistan for recording the GC-MS, NMR and MS spectrum of hexane fraction and isolated compounds respectively.

Conflict of interest

The authors declare no conflicts of interest regarding this article

REFERENCES

1. Cragg GM and Newman DJ. Biodiversity: A Continuing Source of Novel Drug Leads. Pure and Applied Chemistry 2005 77:7-24.
2. Firenzuoli F and Gori L. Herbal Medicine Today: Clinical and Research Issues. Evidenced-Based Complementary and Alternative Medicine 2007. 4:37-40
3. Heinrich M, Barnes J, Gibbons S and Williamson EM. *Fundamentals of Pharmacognosy and Phototherapy*. Churchill Livingstone: 2004.Elsevier Science Ltd., UK.

- 390 4. Nasreen S and Radha R. Assessment of Quality of *Withania somnifera* Dunal (Solanaceae)
391 Pharmacognostical and Phyto-Physicochemical Profile. International Journal of Pharmacy
392 Pharmaceutical Sciences 2011. 3: 152-155.
- 393 5. Panda SK, Thatoi HN and Dutta SK. Antibacterial Activity and Phytochemical Screening of Leaf
394 and Bark Extracts of *Vitex negundo* from Similipal Biosphere Reserve Orissa. Journal of
395 Medicinal Plant Research. 2009. 3:294-300.
- 396 6. Okigbo RN, Eme UE and Ogbogu S. Biodiversity and Conservation of Medical and Aromatic
397 Plants in African. Biotechnology and Molecular Biology Reviews. 2008. 3:127-134.
- 398 7. UNESCO. *Culture and Health*, Orientation Texts – World Decade for Cultural Development
399 1988– 1997, Document CLT/DEC/PRO – 1996, Paris, France, 1996, pp 129.
- 400 8. Nascimento GGF, Locatelli J, Freitas PC and Silva GL. Antibacterial activity of plant extracts
401 and phytochemicals on antibiotic-resistant bacteria. *Brazilian Journal of Microbiology*
402 2000.31:247-256.
- 403 9. Saikia B. Ethnomedical Plants from Gohpur of Sonitpur District, Assam. Indian Journal
404 Traditional Knowledge 2006.5: 529-530.
- 405 10. Bello OA, Ayanda OI, Aworunse OS, Olukanmi BI, Soladoye MO, Esan EB *et al.* *Solanecio*
406 *biafrae*: An underutilized nutraceutically-important African indigenous vegetable.
407 Pharmacognosy Review. 2018.12:128-32.
- 408 11. Ogundele SB, Oriola AO, Oyedeji AO, Olorunmola FO, Agbedahunsi JM. Flavonoids from
409 Stem Barks of *Artocarpus altilis* (Parkinson ex F. A. Zorn) Fosberg. Chemistry Africa,
410 2022.1-15

12. Amir AM, Omid AK, Amir HS. Phytochemical Analysis and Cytotoxicity Evaluation of
Kelussia odoratissima Mozaff. Journal of Acupuncture and Meridian Studies. 2017.
10(3):180e-186.
13. Olasupo AD, Olagoke OV and Aborisade AB. Qualitative Phytochemical Screening of Bologi
(*Senecio biafrae*) and Bitter Leaf (*Vernonia amygdalina*) Leaves. Chemical Science
International Journal. 2017. 20(3): 1-6.
14. Ajiboye BO, Ojo OA, Okesola MA, Akinyemi AJ, Talabi JY, Idowu OT *et al.*. *In vitro*
antioxidant activities and inhibitory effects of phenolic extract of *Senecio biafrae* (Oliv and
Hiern) against key enzymes linked with type II diabetes mellitus and Alzheimer's disease.
Food Science and Nutrition.2018. 1-8.
15. Adelakun S, Omotoso O, Aniah J and Oyewo O. *Senecio biafrae* defeated Tetracycline-Induced
Testicular Toxicity in Adult Male Sprague Dawley Rats. Jornal Brasileiro de Reproducao
Assistida, 2018a. 1-22(4):314-322.
16. Adelakun S.A, Ogunlade B, Omotoso O.D. and Oyewo, O.O. Role of Aqueous Crude Leaf
Extract of *Senecio biafrae* Combined with Zinc on Testicular Function of Adult Male
Sprague Dawley Rats. Journal of Family and Reproductive Health .2018b. 12(1): 8-17.
17. Somaida A, Tariq I, Ambreen G, Abdelsalam AM, Ayoub AM. Potent Cytotoxicity of four
Cameroonian Plant Extract on different Cancer Cell Line. Pharmaceuticals. 2020. 13:1-19.
19. Charturvedula VSP and Prakash I. Isolation of Stigmasterol and Sitosterol from the
dichloromethane extract of *Rubus suavissimus*. International Current Pharmaceutical
Journal. 2017. 1(9):239-242.

- 432 20. Alexandre TR, Lima ML, Galuppo MK, Mesquita JT, Nascimento MA, *et al.* Ergosterol isolated
433 from the Basidiomycete *Pleurotus salmoneostramineus* affects *Trypanosoma cruzi* plasma
434 membrane and mitochondria. *Journal of Venomous Animals and Toxins including Tropical*
435 *Diseases*. 2017. 23(30):1-10.
- 436 21. Sandeep W, Mohan KK and Vijay RP. Brine Shrimp Lethality Assay of the Aqueous and
437 Ethanolic Extracts of the Selected Species of Medicinal Plants. *Proceedings* 2019. 41: 1-12.
- 438 22. Meyer BN, Ferrigini RN, Putnam JE, Jacobsen LB, Nichols DE and McLaughlin JL.
439 Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Med*. 1982.
440 45:31 35.
- 441 23. Alcaraz MJ and Jimenez MJ. Flavonoids as anti-inflammatory agents. *Fitoterapia*
442 1988. 59: 25–38.
- 443 24. Loizzo MR, Tundis R, Statti GA and Menichini F. Jacaranone: A Cytotoxic Constituent from
444 *Senecio ambiguus subsp. ambiguus* (Biv.) DC. against Renal Adenocarcinoma ACHN and
445 Prostate Carcinoma LNCaP Cells. *Arch Pharm Res*. 2007;6:701-707
- 446 25. Choudhary D, Shekhawat JK and Kataria V. GC-MS Analysis of Bioactive Phytochemicals in
447 Methanol Extract of Aerial Part and Callus of *Dipterygium glaucum Decne*. *Pharmacogn J*.
448 2019; 11(5):1055-1063
- 449 26. Islam MT, Ali ES, Uddin SJ, Shaw S, Islam MA, Ahmed MI, *et al.* *Phytol*: A review of
450 biomedical activities. *Food Chemical Toxicology*. 2018. 121:82-94.

- 27 Islam MT, de Alencar MV, da Conceição Machado K, da Conceição Machado K, deCarvalho
Melo-Cavalcante AA, de Sousa DP, *et al.* Phytol in a pharma-medico-stance. Chem Biol
Interact. 2015. 240:60-73.
28. Kaur G, Gupta V, Singhai RGand Parveen B. Isolation and Characterization of Stigmasterol
from *Fritillaria roylei*. Biology Medicine and Natural Product Chemistry. 2020. 9: 77-80
29. Bhagyashri V and Vinay S. Impact of different phytochemical classes and ayurvedic plants in
battle against cancer. International Journal of Pharma Sciences and Research. 2016. 7(10):1-
13
30. Cui X, Dai Q, Tseng M, Shu XO, Guo YT, Zheng W. Dietary pattern and breast cancer risk in
the Shanghai breast cancer study. Cancer Epidemiology, Biomarkers and prevention.
2007.16:1443-1448.
31. Awad, A. B., Fink, C. S., and Williams, H. Phytosterols as anticancer dietary components:
Evidence and mechanism of action. Journal of Nutrition, 2000. 130(9), 2127-2130.
32. Salman, H., Bergman, M., Bessler, H., & Punia, R. (2006). Effects of a plant sterol-enriched
spread on biomarkers of endothelial dysfunction and low-grade inflammation in
hypercholesterolaemic subjects. Scandinavian Journal of Clinical & Laboratory
Investigation, 2006. 66(5), 415-424.
32. Panawan S, Watcharapong C, Sugunya M, Suwaporn L, Somsuda T. and Vijittra, L. Structures
of Phytosterols and Triterpenoids with Potential Anti-Cancer Activity in Bran of Black Non-
Glutinous Rice. Nutrients. 2015. 7: 1672-1687.

bioRxiv preprint doi: <https://doi.org/10.1101/2023.05.21.541662>; this version posted May 22, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).

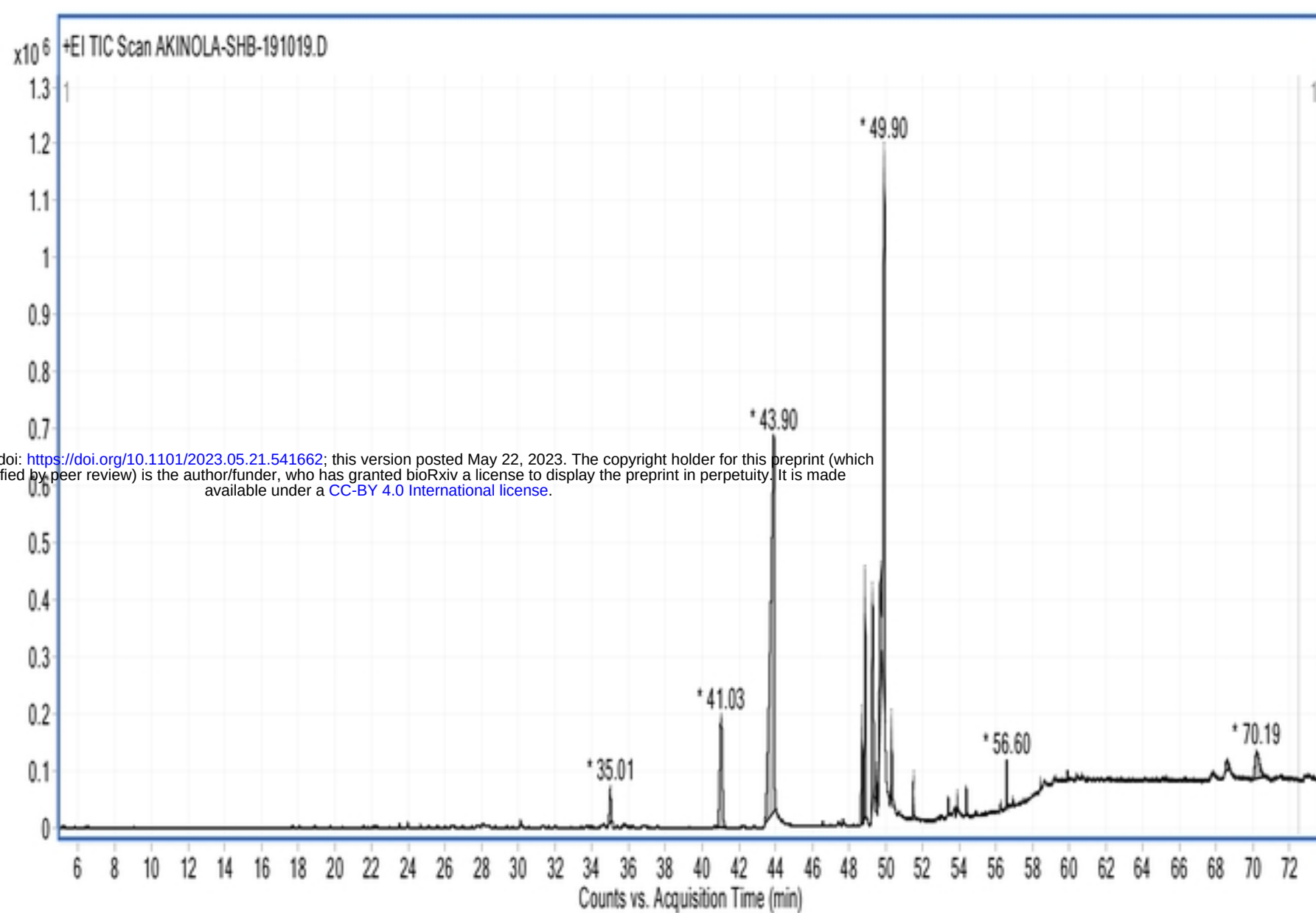
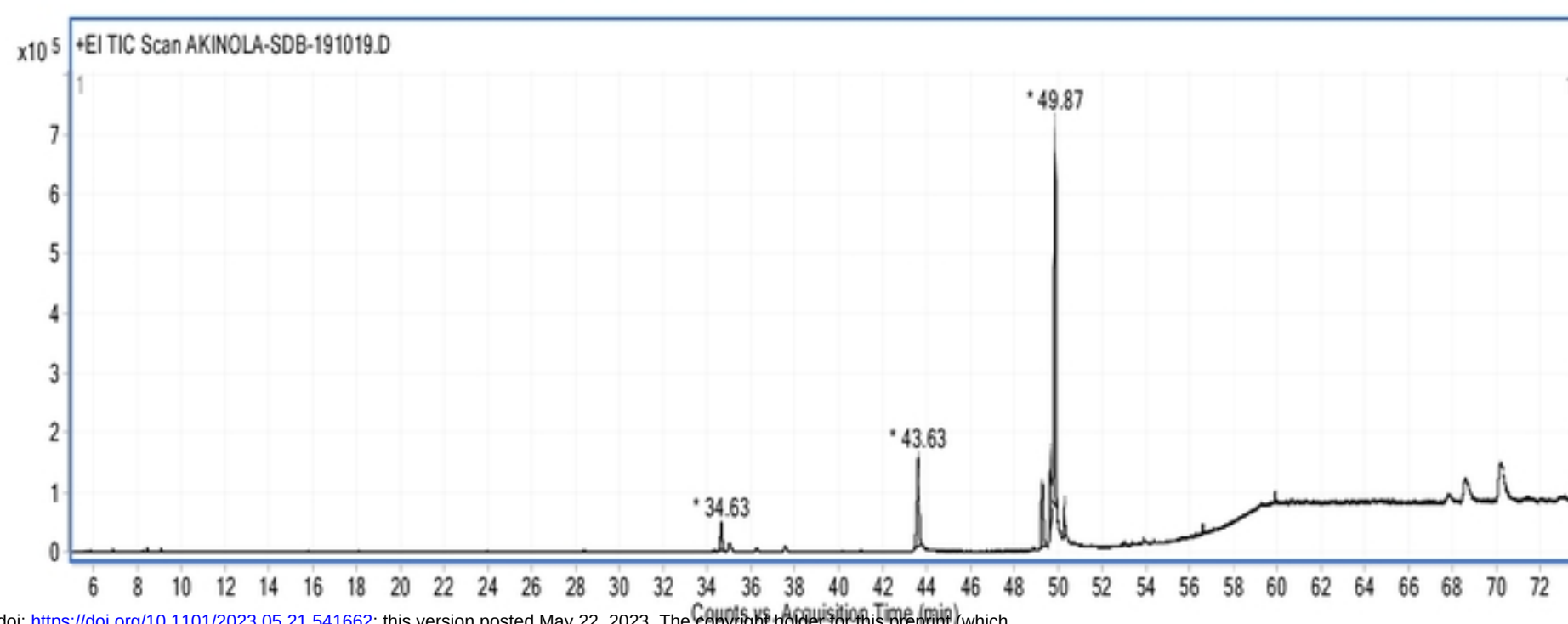
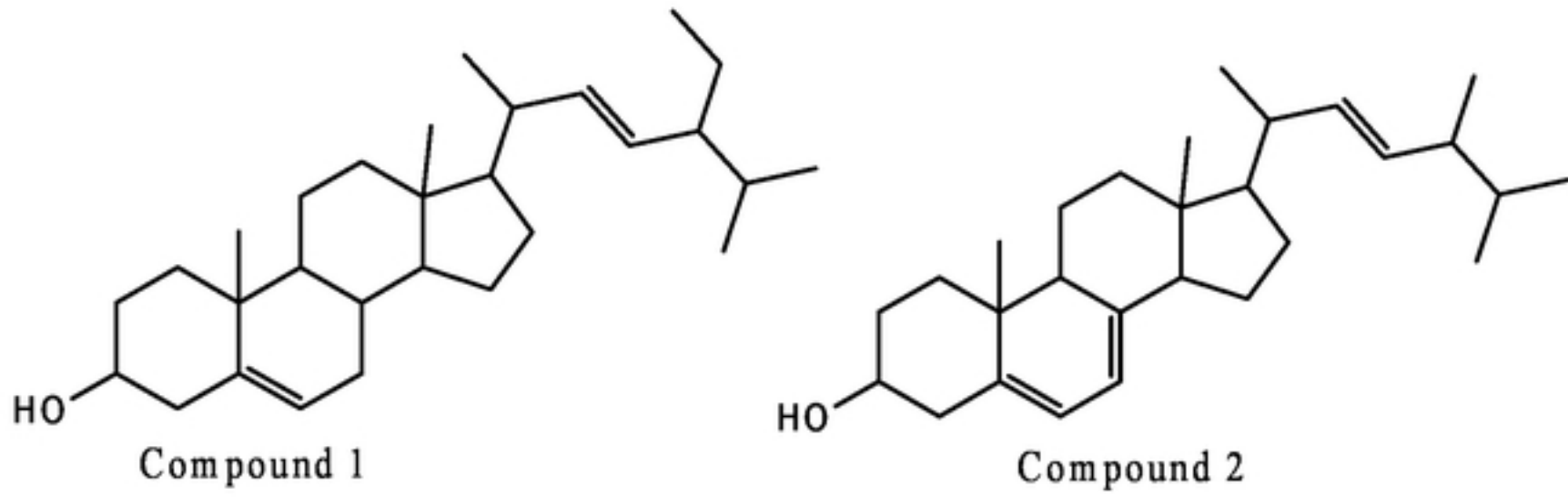


Figure 1: GC-MS Chromatogram of Hexane Fraction



bioRxiv preprint doi: <https://doi.org/10.1101/2023.05.21.541662>; this version posted May 22, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).

Figure 2: GC-MS Chromatogram of Dichloromethane Fraction



Figures 3 and 4. Structures of Compounds 1 and 2

bioRxiv preprint doi: <https://doi.org/10.1101/2023.05.21.541662>; this version posted May 22, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).