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Oldenlandia diffusa (willd.) Roxb. Essential oil from assam, India: GC-MS analysis and anticancer activity

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Abstract. In this study, essential oil from *Oldenlandia diffusa* (aerial parts) was extracted using hydrodistillation method. Characterization of the essential oil was performed using GC-MS analysis and 71 compounds were identified. Pentacosane (13.29 %), hexacosane (11.59 %), tetracosane (11.18 %), heptacosane (9.76 %), tricosane (6.90 %), phytol (5.71 %), hexatriacontane (4.87 %) and isophytol (4.69 %) were the major compounds constituting the oil. Furthermore, cytotoxicity of the extracted oil was observed against PA1 (Ovarian), MIAPaCa-2 (Pancreatic), A549 (Lung), MCF7 (Breast), HeLa (Cervical), HepG2 (Liver), PC-3 (Prostatic), MDA-MB-231 (Breast) and L6 normal (Rat skeletal muscle) cell lines. The oil exhibited dose and time dependent inhibition effects against the cancer cell lines. Best inhibition activity was observed against PA1, HeLa and PC-3 cancer cell lines. The IC_{50} values ranged from 24.19 ± 0.837 to 3.12 ± 0.126 µg/mL in PA1 cells, 51.87 ± 3.104 - 28.95 ± 0.76 µg/mL in HeLa cells and 52.92 ± 1.233 - 14.62 ± 0.465 µg/mL in PC-3 cells at 24, 48 and 72 h. L6 normal cell lines are not affected by the essential oil. Moreover, the plant is an edible herb and is traditionally used by people of Assam, India as vegetables for various Assamese cuisines. From the experiments, it was clear that the essential oil of *Oldenlandia diffusa* should be further explored as an anticancer agent for developing medicinal drugs.

Keywords: Oldenlandia diffusa, essential oil, GC-MS, anticancer activity.

Classification numbers: 1.1.1., 1.2.1., 1.4.6.

1. INTRODUCTION

For thousands of years, medicinal plants have been used for the remedy of different diseases including cancer. In recent times, it has been observed that the traditional use of plants for medicinal purposes has expanded massively. Inadequate drug supplies, side effects of synthetic drugs, treatment costs, and drug resistance have led to increased research into herbal drug technology. According to the World Health Organization (WHO), 80 % of people globally

rely on herbal medicines to meet their healthcare needs [1]. Consuming medicinal plants as drugs has no side-effects, which is their biggest advantage. Moreover, herbal drugs can be used irrespective of a person's health, age and gender. India is considered to have a large archive of medicinal plants. Indian forests provide major raw materials for herbal drug development.

According to the Global Cancer Observatory (GLOBOCAN) in 2020, cancer is the second leading cause of death globally and there were an estimated 10 million deaths in the same year [2]. In India, cancer cases are anticipated to increase by 2.08 million, accounting for a rise of 57.50 % in 2040 from 2020 [3]. As mentioned by Nair *et al*., it is a cluster of diseases where it influences all living cells of both sexes at all ages [4]. At present, hormonal therapy, surgery, chemotherapy and radiotherapy are the main conventional methods being used in the treatment of cancer. The issue of drug resistance and adverse effects on patients has put many chemotherapy treatments into a situation where their therapeutic impact is limited [5]. Hence, there is an intense emphasis on the discovery and development of novel kinds of natural anticancer medicines, using plants and microbes, which exhibit effective and targeted toxicity on tumor cells [6]. A study by Bhattacharya *et al*. signifies the exploitation of natural products in curbing cancer proliferation for the treatment of different cancer types through diverse mechanisms [7]. Consequently, it is of paramount significance to assess the bioactive potential of plants, with the aim of generating more potent, safer, and affordable cancer treatments.

Among the potentially bioactive plants, *Oldenlandia diffusa* (Willd.) Roxb. (Synonym is *Hedyotis diffusa* Willd. and known as "Bonjaluk" in Assamese), an annual herb from Rubiaceae family, is extensively allocated in East and Southeast Asia, such as China, Indonesia, Malaysia and Japan. Traditional Chinese medicines have utilised *Oldenlandia diffusa* for ages in tonsillitis, boils, hepatitis, appendicitis, dysentery, urethritis and snake bites treatment and accounted to encompass various pharmacological effects, primarily antioxidant, antitumor, anti-inflammatory and immunity-enhancing among others [8]. The *Oldenlandia diffusa* can enhance cancer cells to apoptotic cell death, inhibit proliferation and migration of hepatocellular carcinoma cells [9]. A previous study also reported that the compound ursolic acid present in *Oldenlandia diffusa* is a major compound that is responsible for the cytotoxic effect of the plant species against cancer cell lines [10]. In addition to that, it was reported that *Oldenlandia diffusa* (Bonjaluk) is an edible herb and is traditionally used by people of Assam, India as vegetables for various Assamese cuisines [11, 12].

Essential oils are volatile in nature and comprised of many health-beneficial compounds which have been used as an alternative therapy for various medicines in the past. Studies have been conducted to understand the composition of *Oldenlandia diffusa* essential oil [13, 14] but no detailed investigation on bioactivity has been performed so far. This is the very first study to explore the cytotoxic activity of essential oil extracted from *Oldenlandia diffusa* of Assam against PA1 (Ovarian), MIAPaCa-2 (Pancreatic), A549 (Lung), MCF7 (Breast), HeLa (Cervical), HepG2 (Liver), PC-3 (Prostatic), MDA-MB-231 (Breast), and L6 normal (Rat skeletal muscle) cancer cell lines. This study will open up great prospects for herbal formulation research in the near future.

2. MATERIALS AND METHODS

2.1. Plant material

Fresh herbs of *Oldenlandia diffusa* (Willd.) Roxb. were gathered in February 2019 from Sahpuriagaon paddy field area, Jorhat, Assam, India. Voucher specimens [Accession number

(NPC/273)] deposition and identification were performed by Prof. M. Bordoloi at CSIR-NEIST, Jorhat 785006, Assam.

2.2. Chemicals

The chemicals used in this study mainly included anhydrous sodium sulphate, hexane, DMSO, Gentamycin, sodium bicarbonate, doxorubicin, isopropanol and HCl. All the used chemicals were of analytical grade and bought from Sigma-Aldrich. Foetus bovine serum, penstrep, glutamine, DMEM, MEM, F12 Kaighn's modification were from Gibco, Thermo Fisher Scientific. MTT assay kit was purchased from Merck. Milli-Q quality (Merck Millipore Corporation, Merck KGaA, Germany) water was used for preparation of media and stock solutions.

2.3. Extraction of essential oil

The technique by Saikia *et al*. [15] with slight alterations was employed to extract the essential oil. 383 g of collected fresh plant species of *Oldenlandia diffusa* (aerial parts) were cleaned and chopped into small parts. Using a heating mantle, the plant materials were dipped in 1 L of distilled water in a round bottom flask and heated up to 100 °C. Further, hydro-distillation with Clevenger apparatus method was employed to extract the oil from the herbs. The condenser of the Clevenger apparatus was connected to a Buchi F-305 Recirculating Chiller at -10 °C allowing the volatile components to cool down and be collected in a sample tube. The whole process was repeated thrice. The isolated essential oil was then carefully collected in glass bottles. Moreover, to dehydrate the extracted essential oil, anhydrous sodium sulfate was used. The oil was kept in dark sealed containers in the refrigerator at 4°C until used for testing for anticancer activity by GC-MS. The essential oil yield was calculated using the following formula:

$$\text{Yield (\%)} = \text{Dry weight of crude oil (g)/Raw material taken (g)} \times 100\ \%.$$

2.4. GC and GC-MS analysis

The components of the essential oil extracted from *Oldenlandia diffusa* were analyzed *via* the technique mentioned by Nath *et al*. [16] through a combination of capillary GC/FID and GC/MS (Trace DSQ, Thermo Fisher Scientific, Austria) fitted with a TR-5MS (30 m×0.25 mm ID×0.25 µm) capillary column. The carrier gas used was helium at a flow rate of 1.2 mL/min. The initial temperature of the oven was kept at 40 ^{o}C for 2 minutes, programmed to a maximum temperature of 280 °C at a rate of 6 ^{o}C/minute and held constant for 8 minutes. The temperatures of the injector and the flame ionization detector (FID) were programmed at 280 °C. The oil was diluted with *n*-hexane at a ratio of 1:10 (v/v) before injection and then at a split flow ratio of 1:10, the diluted sample (1 µL) was injected. The ion source temperature was kept at 200 °C. GC/MS analysis of the oil was completed using a quadrupole mass analyzer with an electron impact ionization (EI) system. The spectral data was acquired over the mass range of 50- 400 *m/z*, with a total run time of 30 minutes. The relative peak area in the chromatogram which equals to the percentage of each constituent of the oil was measured from electronic integration using FID detection without response factor rectification. The retention indices (experimental RI) of the chemical components were calculated relative to a homologous series of *n*-alkanes (C2–C29) retention times and evaluated using literature data. Each chemical component was identified by Wiley and NIST 17 libraries accessible with the GC–MS instrument, correlating mass spectra of constituents against retention times with the database [17, 18]. To assess retention indices (RI), the Kovat's technique was employed via the equation below.

$$\text{Retention indices (RI)} = 100\ [n + (N - n) \times \frac{\log RT\ (\text{unknown}) - \log RT\ (n)}{\log RT\ (N) - \log RT\ (n)}]$$

wherein, n = carbon no. of smaller *n*-alkane, N = carbon no. of larger *n*-alkane, and RT = retention time.

2.5. Cytotoxic assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for *in vitro* cytotoxicity testing of *Oldenlandia diffusa* essential oil at different concentrations (2- 150 µg/mL) at 24, 48 and 72 h via the method employed by Neipihoi *et al*. [19] with slight modifications. Human cancer cells, *viz*., PA1 (Ovarian), MIAPaCa-2 (Pancreatic), A549 (Lung), MCF7 (Breast), HeLa (Cervical), HepG2 (Liver), PC-3 (Prostatic), MDA-MB-231 (Breast) were the cell lines used for the experiment and they were acquired from National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's modified eagle medium (DMEM) was used to culture MIAPaCa-2, MCF7 and MDA-MB-231 cells; minimal essential medium (MEM) was used to culture PA1, HeLa and HepG2 cells; and Ham's F-12K (Kaighn's) medium was used for A549 and PC-3 cells which were supplemented with 10 % FBS (foetus bovine serum), 10 % pen strep and 1 % gentamycin. These cultures were kept in flasks and incubated at 37 °C with a humidified atmosphere of 5 % CO_2 in a CO_2 incubator. Once the cells grew completely in the flask (1×10^6 per mL), they were inoculated in flat bottom 96-well plates (Nunc™, Thermo Fisher Scientific Inc.) with 100 µL of aliquots of complete medium and incubated overnight. Then the cells were added with FBS-free medium after removing complete medium and again incubated for 24 h. Essential oil was then added in concentrations from 2 - 150 µg/mL to all cell lines in triplicates and treated for different time periods (24, 48 and 72 h). The same course of action was repeated for standard drug doxorubicin and thus the cells in culture media without treatment are considered as control.

According to the manufacturer's guideline (Merck Millipore Corporation, Germany), *in vitro* MTT assay for antiproliferative activity of essential oil was performed. After completing the treatment durations of 24, 48 and 72 h, micrographs were taken under an inverted microscope (Motic AE31) fitted with a Canon digital camera to visualize the changes in structure and morphology of treated cells and were compared with the control cells. Then, 10 µL of MTT (5 mg/mL) was appended to each well. The plates were incubated for another 4 hours. After incubation, the presence of dark formazan crystals was observed under an inverted microscope at the bottom of the wells. Formazan was dissolved by recurrent pipetting with isopropanol and 0.04 N HCl (100 µL). The absorbance was measured on a multi-mode microplate reader (FilterMax F3, Molecular Devices). The wavelength used was 570 nm. The effect of the essential oil on the proliferation of cells was expressed as the % cell viability, using the following formula:

$$\%\ \text{Cell viability} = A_{570}\ \text{of treated cells} / A_{570}\ \text{of control cells} \times 100\ \%$$

IC_{50} of the oil was determined by analysis of dose response curves.

2.6. Statistical analysis

All experiments were performed in triplicate and the estimated parameters were reported as mean ± SD. MS excel 2007 was used to calculate the IC_{50} values and $p < 0.05$ was regarded as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Chemical composition and yield of the essential oil

Identification and quantification of the chemical composition of *Oldenlandia diffusa* oil were performed using GC and GC-MS analysis. The color of the essential oil was light yellow and the yield was 0.44 % (v/w). In this study, a total of 71 compounds were identified from the aerial parts of *Oldenlandia diffusa* essential oil. The major constituents in *Oldenlandia diffusa* oil were pentacosane (13.29 %), hexacosane (11.59 %), tetracosane (11.18 %), heptacosane (9.76 %), tricosane (6.90 %), phytol (5.71 %), hexatriacontane (4.87 %) and isophytol (4.69 %), totaling of 98.63 % of chemical components (Table 1). The structures of the compounds found in *Oldenlandia diffusa* oil are illustrated in Figure 1. It is interesting that the essential oil of *Oldenlandia diffusa* comprises a significant amount of long chain hydrocarbons. The rest of the compounds are found in either adequate or trace amounts. The constituents reported in this study are different from the earlier reports of GC-MS analysis of same herbs found in Malaysia and China [13, 14]. *Hedyotis diffusa* Willd. studied in China showed the oil yield of 0.1 %(v/w) and the main constituents present were hexadecanoic acid (48.89 %), pentadecanoic acid (6.11 %) and D-limonene (5.74 %) [14]. Another research performed by Wong and Tan on the *Hedyotis diffusa* Willd. essential oil collected in Malaysia gave a pale-yellow oil of 0.02 % (w/w), with the most significant compounds being phenolics p-vinylphenol (22.2 %) and p-vinylguaiacol (18.6 %), and terpenoid linalool (13.6 %) [13].

Table 1. Chemical compounds identified by GC-MS from *Oldenlandia diffusa* EO.

Sl. No	Constituent	[a]RT	[b](RI)	[c](RI)[lit]	Content %	Molecular formula	[d]Identification	Molecular weight
1	Ethyl pent-4-enoate	4.02	884	888	0.83	$C_7H_{12}O_2$	RI, MS	128
2	Nonane	4.46	900	900	0.42	C_9H_{20}	RI, MS	128
3	Octane, 2-methyl-	4.62	864	865	0.54	C_9H_{20}	RI, MS	128
4	trans-4-Heptenal	5.64	895	895	0.48	$C_7H_{12}O$	RI, MS	112
5	2-Methylhexanoic acid	6.31	1030	1032	0.25	$C_7H_{14}O_2$	RI, MS	130
6	1,3-Dioxolane, 4-methyl-2-(2-methylpropyl)-	6.65	938	940	0.34	$C_7H_{14}O_2$	RI, MS	144
7	Methyl heptanoate	7.13	1025	1025	0.23	$C_8H_{16}O_2$	RI, MS	144
8	*p*-Cymen-8-ol	7.43	1182	1182	0.31	$C_{10}H_{14}O$	RI, MS	150
9	3-Decanol	7.68	1196	1196	0.27	$C_{10}H_{22}O$	RI, MS	158
10	Terpinen-4-ol	8.18	1175	1177	0.29	$C_{10}H_{14}O_2$	RI, MS	166
11	Dodecene	8.93	1188	1189	0.27	$C_{12}H_{24}$	RI, MS	168
12	1,2-Dihydro-1,5,8-trimethylnaphthalene	9.07	1377	1376	0.11	$C_{13}H_{16}$	RI, MS	172
13	Phenyl-4-methyl-pentan-3-one	9.73	1380	1382	0.31	$C_{12}H_{16}O$	RI, MS	174
14	Hexanoic acid, 3-hexenyl ester, (Z)-	10.91	1387	1387	0.21	$C_{12}H_{22}O_2$	RI, MS	198
15	4-Octanol, 7-methyl-, acetate	12.33	1172	1172	0.39	$C_{11}H_{22}O_2$	RI, MS	186
16	3-Dodecanone	12.06	1389	1390	0.27	$C_{12}H_{24}O$	RI, MS	184
17	Phenyl ethyl isobutanoate	12.33	1393	1393	0.14	$C_{12}H_{16}O_2$	RI, MS	192

18	1-Tetradecene	12.54	1388	1389	0.18	$C_{14}H_{28}$	RI, MS	196
19	Ethyl decanoate	13.61	1395	1395	0.13	$C_{12}H_{24}O_2$	RI, MS	200
20	(Z)-3-hexen-1-yl 2-methyl-2-pentenoate	13.84	1400	1400	0.29	$C_{12}H_{20}O_2$	RI, MS	196
21	9-Decenyl acetate	14.27	1399	1399	0.64	$C_{12}H_{22}O_2$	RI, MS	198
22	Tetradecane	14.51	1400	1400	0.39	$C_{14}H_{30}$	RI, MS	198
23	Ethyl-2-nonynoate	14.80	1382	1382	0.67	$C_{11}H_{18}O_2$	RI, MS	182
24	Decanoic acid, ethyl ester	15.42	1379	1381	0.11	$C_{12}H_{24}O_2$	RI, MS	200
25	Hexyl hexanoate	15.63	1383	1383	0.28	$C_{12}H_{24}O_2$	RI, MS	200
26	α-Cedrene	15.91	1411	1412	0.69	$C_{15}H_{24}$	RI, MS	204
27	Ethyl-(2E)-decenoate	16.05	1418	1418	1.12	$C_{12}H_{22}O_2$	RI, MS	198
28	E-Trimenal	16.25	1420	1421	0.75	$C_{13}H_{22}$	RI, MS	194
29	Methylundecanoate	16.59	1424	1426	0.15	$C_{12}H_{24}O_2$	RI, MS	200
30	Allyl cyclohexyl propanoate	16.76	1423	1436	0.49	$C_{12}H_{20}O_2$	RI, MS	196
31	Dihydro-apofarnesal	17.17	1516	1516	0.26	$C_{14}H_{26}O$	RI, MS	210
32	Isoamyl salicylate	17.28	1535	1536	0.26	$C_{12}H_{16}O_3$	RI, MS	208
33	Trans-Isoelemicin	17.54	1645	1643	0.32	$C_{12}H_{16}O_3$	RI, MS	208
34	(Z)-6-Pentadecen-2-one	17.69	1665	1668	0.18	$C_{15}H_{28}O$	RI, MS	224
35	1-Tetradecanol	17.88	1674	1672	0.13	$C_{14}H_{30}O$	RI, MS	214
36	α-Cyperone	17.96	1713	1713	0.86	$C_{15}H_{22}O$	RI, MS	218
37	1-Hexadecene	18.25	1589	1589	0.16	$C_{16}H_{32}$	RI, MS	224
38	Dodecane, 5,8-diethyl-	18.90	1590	1591	0.51	$C_{12}H_{16}O_3$	RI, MS	226
39	Isobornyl n-butanoate	18.97	1475	1475	0.74	$C_{14}H_{24}O_2$	RI, MS	224
40	Ethyl dodecanoate	19.28	1595	1595	0.47	$C_{14}H_{28}O_2$	RI, MS	228
41	Isoamyl nonanoate	19.46	1545	1545	1.34	$C_{14}H_{28}O_2$	RI, MS	228
42	Hexadecane	19.62	1600	1600	0.24	$C_{16}H_{36}$	RI, MS	226
43	Octanedioic acid, diethyl ester	19.95	1585	1585	0.36	$C_{12}H_{22}O_4$	RI, MS	230
44	7,10-Anhydro-11,12-dihydrochiloscypholone	20.33	1452	1452	0.20	$C_{15}H_{24}O_2$	RI, MS	236
45	α-Bisabololoxide-B	20.66	1651	1655	0.47	$C_{15}H_{26}O_2$	RI, MS	238
46	Cyclobarbital	20.88	1970	1973	0.35	$C_{12}H_{16}N_2O_3$	RI, MS	236
47	2-Pentadecanone, 6,10,14-trimethyl-	21.20	1845	1844	0.32	$C_{18}H_{36}O$	RI, MS	268
48	11-Octadecenal	21.78	1992	1995	0.56	$C_{18}H_{34}O$	RI, MS	266
49	10,13-Octadecadiynoic acid, methyl ester	21.93	2110	2100	0.13	$C_{19}H_{36}O_2$	RI, MS	296
50	Pentadecanoic acid, 14-	22.12	1875	1877	0.36	$C_{17}H_{34}O_2$	RI, MS	270

	methyl-, methyl ester							
51	Phytol	22.36	1942	1943	5.71	$C_{20}H_{40}O$	RI, MS	296
52	Hexadecanoic acid	22.68	1971	1976	1.74	$C_{16}H_{32}O_2$	RI, MS	256
53	1,4-diphenyl-3-buten-2-one	22.99	2039	2038	1.95	$C_{16}H_{14}O$	RI, MS	222
54	2-Nonadecanone	23.98	2085	2090	0.43	$C_{19}H_{38}O$	RI, MS	282
55	Isophytol	24.13	1945	1947	4.69	$C_{20}H_{40}O$	RI, MS	296
56	9,12-Octadecadienoic acid (Z,Z)	24.41	2150	2145	1.82	$C_{18}H_{32}O_2$	RI, MS	280
57	Docosane	24.74	2200	2200	2.15	$C_{22}H_{46}$	RI, MS	310
58	11,14,17-Eicosatrienoic acid, methyl ester	25.06	2240	2241	0.22	$C_{21}H_{36}O_2$	RI, MS	320
59	1-Monopalmitin	25.22	2241	2240	0.36	$C_{19}H_{38}O_4$	RI, MS	330
60	Tricosane	25.38	2300	2300	6.90	$C_{23}H_{48}$	RI, MS	324
61	Tetracosane	25.93	2400	2400	11.18	$C_{24}H_{50}$	RI, MS	338
62	Pentacosane	26.42	2500	2500	13.29	$C_{25}H_{52}$	RI, MS	352
63	Hexacosane	26.88	2600	2600	11.59	$C_{26}H_{54}$	RI, MS	366
64	9,12,15-Octadecatrienoic acid, phenylmethyl ester, (Z,Z,Z)-	21.78	2705	2702	1.21	$C_{25}H_{36}O_2$	RI, MS	368
65	Heptacosane	27.36	2700	2700	9.76	$C_{27}H_{56}$	RI, MS	380
66	Cholesta-8,24-dien-3-ol, (3β,5α)-	27.88	3175	3170	0.14	$C_{27}H_{44}O$	RI, MS	384
67	Nonacosane	28.48	2905	2900	0.17	$C_{29}H_{60}$	RI, MS	408
68	Stigmasta-5,24(28)-dien-3-ol, (3β,24Z)-	28.64	3125	-	0.23	$C_{29}H_{48}O$	RI, MS	412
69	Dotriacontane	28.90	3200	3200	0.34	$C_{32}H_{66}$	RI, MS	450
70	Cholest-5-ene-16,22-dione, 3β,26-dihydroxy-, 3-acetate, (20S,25R)-	29.18	3452	-	0.11	$C_{29}H_{44}O_5$	RI, MS	472
71	Hexatriacontane	28.48	3600	-	4.87	$C_{36}H_{74}$	RI, MS	507
					98.63			

[a]RT = Retention time determined on a Thermo Fisher Scientific TR-5MS 30 m × 0.25 mm ID × 0.25 µm column; [b](RI) = Experimental retention indices calculated relative to a homologous series of *n*-alkanes (C5-C40); [c](RI)^{lit} = Retention indices from the literature, using NIST and Wiley library spectra with literature studies and Ref. [17].

[d]Identification = Identification by GC–mass spectrometry analysis and RI, using NIST and Wiley library spectra and of authentic compounds on specific columns.

This disparity in the qualitative and quantitative structure of the constituents in the present investigation compared with the earlier reports may be attributed to the location's topographical, atmospheric, and edaphic characteristics. Consequently, Boira and Blanquer described that the chemical composition and biological activity of the essential oils diverge from one nation to another [20].

3.2. Cytotoxic activity of *Oldenlandia diffusa* essential oil

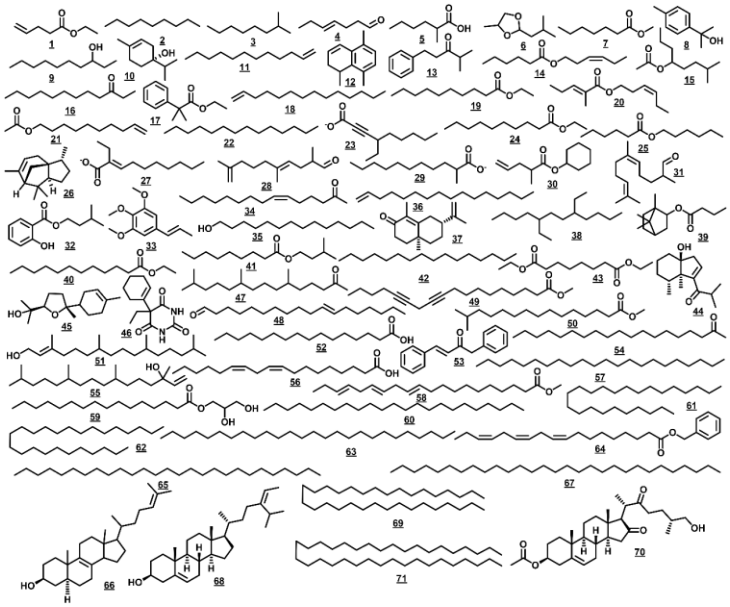


Figure 1. Structures of the compounds identified from *Oldenlandia diffusa* essential oil.

Table 2. In vitro anticancer activity of *Oldenlandia diffusa* essential oil (ODEO) at 24, 48 and 72 h against 8 cancer cell lines.

Cell line	Name of Sample					
	Oldenlandia diffusa essential oil			**Doxorubicin**		
	IC_{50} (µg/mL) ± SD			**IC_{50} (µg/mL) ± SD**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
PA1	24.19 ± 0.837^{a}	13.37 ± 0.093^{a}	3.12 ± 0.126^{a}	0.45 ± 0.024^{a}	0.23 ± 0.003^{a}	0.21 ± 0.002^{a}
MIAPaCa-2	265.63 ± 0.487^{d}	102.13 ± 6.725^{c}	127.08 ± 7.613^{e}	1.61 ± 0.04^{a}	0.7 ± 0.019^{abc}	0.42 ± 0.022^{a}
A549	69.66 ± 1.492^{b}	82.25 ± 0.693^{c}	83.34 ± 1.177^{d}	0.46 ± 0.024^{a}	0.36 ± 0.003^{a}	0.3 ± 0.005^{a}
MCF7	194.01 ± 13.833^{c}	100.12 ± 9.598^{c}	64.28 ± 8.458^{c}	0.82 ± 0.015^{a}	0.39 ± 0.022^{a}	0.38 ± 0.013^{a}
HeLa	51.87 ± 3.104^{b}	34.78 ± 0.713^{b}	28.95 ± 0.76^{b}	1.68 ± 0.019^{a}	1.41 ± 0.08^{bc}	0.73 ± 0.019^{b}
HepG2	189.84 ± 15.337^{c}	184.87 ± 10.991^{d}	313.82 ± 7.814^{f}	3.87 ± 0.08^{a}	1.59 ± 0.091^{c}	0.86 ± 0.052^{b}
PC-3	52.92 ± 1.233^{b}	49.03 ± 3.17^{b}	14.62 ± 0.465^{ab}	1.39 ± 0.082^{a}	0.45 ± 0.021^{ab}	0.39 ± 0.008^{a}
MDA-MB-231	58.7 ± 1.157^{b}	85.64 ± 3.884^{c}	87.55 ± 1.694^{d}	1.42 ± 0.07^{a}	0.63 ± 0.018^{abc}	0.44 ± 0.025^{a}
L6	> 400^{e}	> 400^{e}	> 400^{e}	11.145 ± 3.796^{b}	5.522 ± 0.823^{d}	4.041 ± 0.196^{c}

IC50 values are means of triplicate Standard deviation. PA1 - Human Ovarian carcinoma; MIAPaCa-2 - Human Pancreatic carcinoma; A549 - Human Lung carcinoma; MCF7 - Human Breast adenocarcinoma; HeLa - Human Cervical adenocarcinoma; HepG2 - Human Liver carcinoma; PC-3 - Human Prostatic adenocarcinoma; MDA-MB-231 - Human Breast adenocarcinoma; L6 - Normal Rat skeletal muscle cells. The means, not sharing a common letter within each row, were significantly different at $p < 0.01$.

This is the first report mentioning the anticancer potential of the essential oil of *Oldenlandia diffusa*. MTT assay revealed that *Oldenlandia diffusa* essential oil had outstanding cytotoxic properties on human cancer cell lines. The oil had a dose- and time-dependent impact on the decline of percentage viability of cancer cells. The lowest IC_{50} values given in Table 2 were observed in PA1 (ovarian carcinoma), HeLa (cervical adenocarcinoma) and PC-3 (prostatic adenocarcinoma) at all three different time scales, namely 24, 48 and 72 h, revealing that the essential oil was significantly effective against PA1, HeLa and PC-3. The IC_{50} values ranged from 24.19 ± 0.837 to 3.12 ± 0.126 µg/mL in PA1 cells, 51.87 ± 3.104 - 28.95 ± 0.76 µg/mL in HeLa cells and 52.92 ± 1.233 - 14.62 ± 0.465 µg/mL in PC-3 cells at 24, 48 and 72 h. Moreover, it was seen that the essential oil was toxic against A549 and MDA-MB-231 cells at 24 h. This ascertains that *Oldenlandia diffusa* essential oil can be utilized to treat cancer cells. Further, to check significant scientific implications, we tested the isolated *Oldenlandia diffusa* essential oil on the L6 normal (rat skeletal muscle) cell line and obtained an IC_{50} value greater than 400 µg/mL. The dose- and time-dependent activity for percentage viability with respect to concentration is represented graphically in Figures 2a - 2c, respectively.

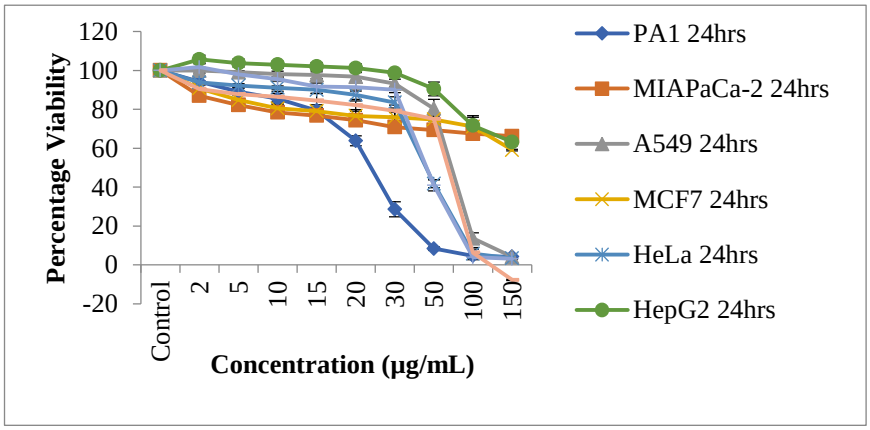


Figure 2a. Percentage viability graph at 24 h treated with *Oldenlandia diffusa* essential oil against 8 cancer cell lines.

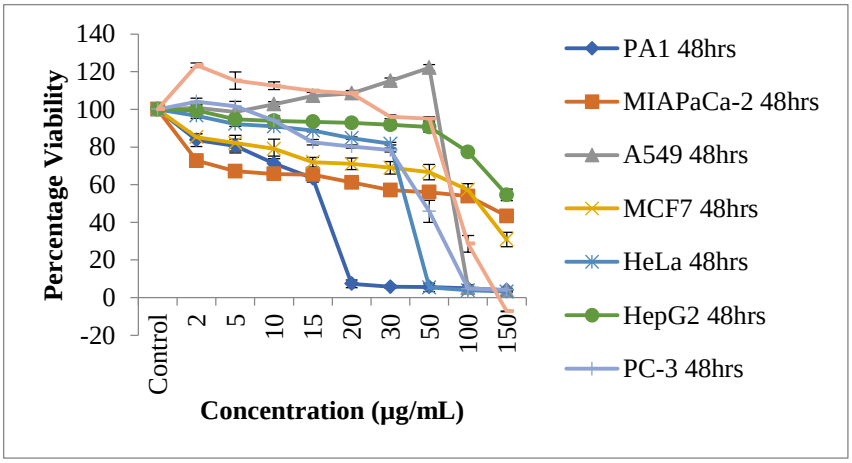


Figure 2b. Percentage viability graph at 48 h treated with *Oldenlandia diffusa* essential oil against 8 cancer cell lines.

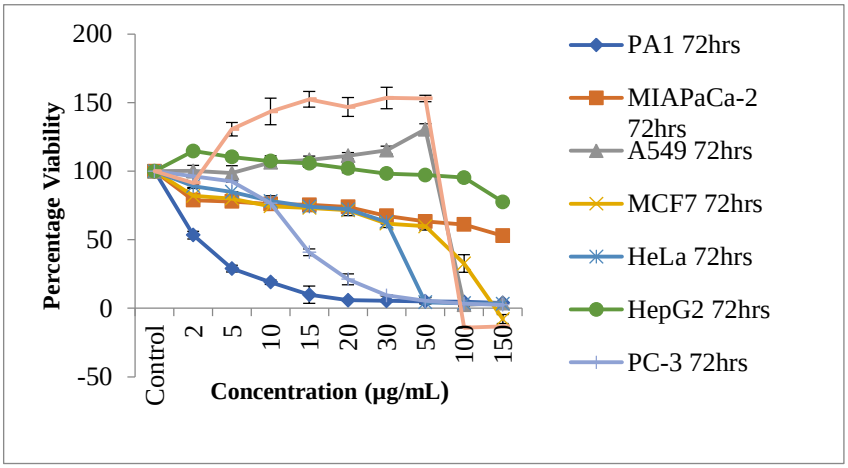


Figure 2c. Percentage viability graph at 72 h treated with *Oldenlandia diffusa* essential oil against 8 cancer cell lines.

Photomicrographs taken with an inverted microscope (Motic AE31) supplement this findings and show that in contrast to control cells, cell lines exposed to *Oldenlandia diffusa* essential oil displayed shrunken, rounded cells and compacted nucleus indicative of a lack of cellular adherence and induction of apoptosis. Additionally, when the oil content increased, the number of cells decreased (Figures 3a - 3c).

Numerous researchers have highlighted the pharmacological actions of volatile oils associated with significant chemicals, including their anticancer effects. In a previous study, Walia *et al*. found that the essential oil from the leaves of *Malus domestica*, which contains phytol, pentacosane, and tricosane among other major components, demonstrated cytotoxic activity against C-6, A549, CHOK1, and THP-1 cells [21]. Tian *et al*. reported their substantial findings about the cytotoxic activity using MTT assay of essential oils from *Zingiber striolatum* flowers, leaves and stems against K562 (IC_{50}: 12.94 - 37.89 µg/mL), PC-3 (IC_{50}: 69.06 - 82.56 µg/mL) and A549 (IC_{50}: 45.73 - 66.12 µg/mL) cell lines. Amongst other major classes of chemical constituents of the essential oils, such as monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and diterpenes, tricosane (3.2 % in the oil from the flowers), phytol (11.9 % in the oil from the leaves), and phytol (7.2 % in the oil from the stems) are the predominant constituents that are thought to be responsible for this activity [22]. Furthermore, in a multidrug resistant *in vitro* model of acute myeloid leukaemia (AML), the main components of *Euphorbia intisy* essential oil, phytol and heptacosane are found to inhibit P-glycoprotein (P-gp). Through the suppression of NF-κB, phytol reduces P-gp expression without affecting the efflux mechanism. Heptacosane exhibits the capacity to retain the substrate doxorubicin inside the cell and enhance its cytotoxic effects by acting as a substrate and strong P-gp inhibitor. Potentially, heptacosane and chemotherapeutic medicines might be utilized in combination therapy [23]. Another research mentions that phytol is an effective alternative for the treatment of liver cancer since it induces apoptosis in hepatocellular carcinoma cells (Huh7 and HepG2) by activating caspase-9/3 and inhibiting the epithelial mesenchymal transition (EMT) [24]. In addition, tetracosane exhibited considerable cytotoxicity against HT-29 colon cancer cells (IC_{50} 128.7 µM), MDA-MB-231 estrogen-dependent breast cancer cells (IC_{50} > 250 µM) and AGS gastric cancer cells (IC_{50} > 250 µM) [25]. The essential oil from the leaves of *Cleidion javanicum* Bl. comprising principal components like ethyl linoleolate (32.12 %), hexadecanoic acid (26.77 %), trans-phytol (24.64 %)

and iso-phytol (4.80 %) demonstrated anticancer activity against three cancerous human cell lines, KB (IC_{50} 47.16 µg/mL), MCF7 (IC_{50} 40.23 µg/mL) and NCI-H187 (IC_{50} 49.95 µg/mL) [26].

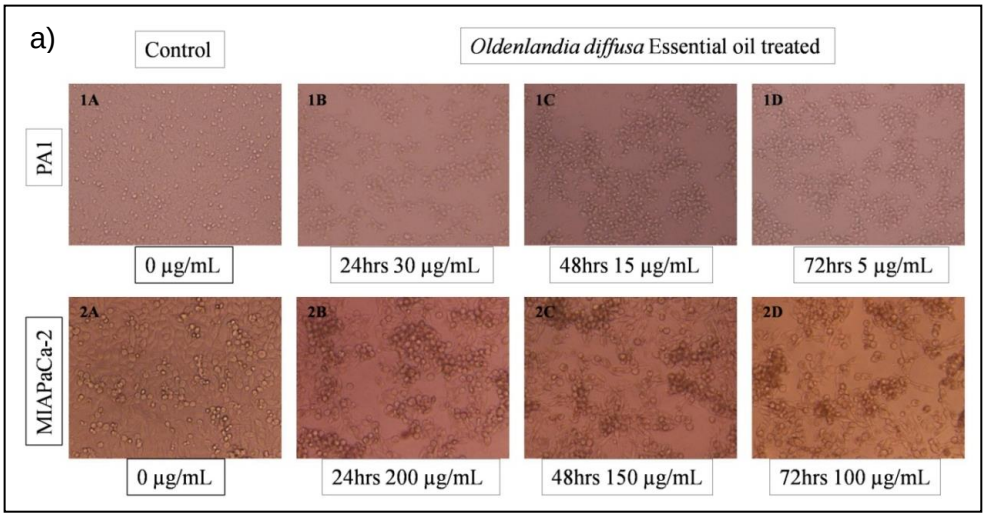


Figure 3a. Photomicrograph of cancer cells treated with *Oldenlandia diffusa* essential oil (ODEO) at 24, 48 and 72 h. (1A) PA1 control, (1B-1D) PA1 cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values; (2A) MIAPaCa-2 control, (2B-2D) MIAPaCa-2 cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values. In contrast to control cells, cell lines treated with *Oldenlandia diffusa* essential oil exhibited shrunken and rounded cells.

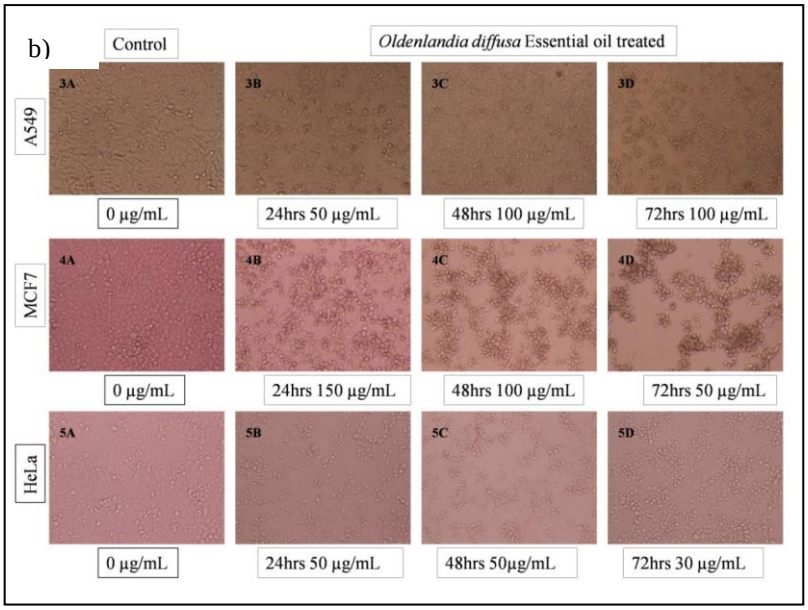
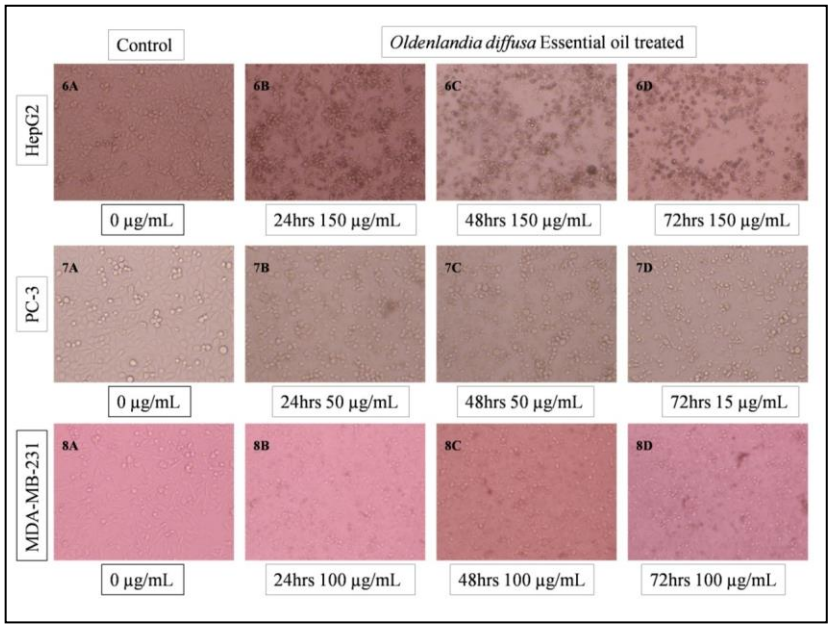


Figure 3b. Photomicrograph of cancer cells treated with *Oldenlandia diffusa* essential oil (ODEO) at 24, 48 and 72 h. (3A) A549 control, (3B-3D) A549 cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values; (4A) MCF7 control, (4B-4D) MCF7 cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values; (5A) HeLa control, (5B-5D) HeLa cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values. In contrast to control cells, cell lines treated with *Oldenlandia diffusa* essential oil exhibited shrunken and rounded cells.



c)

Figure 3c. Photomicrograph of cancer cells treated with *Oldenlandia diffusa* essential oil (ODEO) at 24, 48 and 72 h. (6A) HepG2 control, (6B-6D) HepG2 cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values; (7A) PC-3 control, (7B-7D) PC-3 cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values; (8A) MDA-MB-231 control, (8B-8D) MDA-MB-231 cells treated with ODEO at 24, 48 and 72 h, respectively, at concentrations near IC_{50} values. In contrast to control cells, cell lines treated with *Oldenlandia diffusa* essential oil exhibited shrunken and rounded cells.

Next, the hydrocarbons triacontane, pentacosane, tetracosane, hexacosane and tetratetracontane found in *Phoradendron mucronatum* leaf extracts, among other important phytochemical classes of chemicals, had an inhibiting impact on cell proliferation towards NCI-H292, MCF-7, and HEp-2 [27]. Alkhalaf *et al*. found that as opposed to a lipid extract of the fruit, a *Persea americana* or avocado seed extract demonstrated impressively substantial anti-cancer activity against the cell lines HCT116 and HePG2 in a dose-dependent manner. High levels of hydrocarbon, sterols, and unsaturated fatty acids may be attributed to the activity [28]. In light of the prior research, The remarkable anticancer potential of *Oldenlandia diffusa* essential oil, especially against PA1, HeLa, and PC-3 cells, can be explained by the presence of the key components and their individual or synergistic effects among several compounds.

4. CONCLUSIONS

This is the first report that illustrates the anticancer potential of the essential oil of *Oldenlandia diffusa* from Assam, India. Through GC-MS analysis, 71 constituents were identified in the aerial parts of *Oldenlandia diffusa* essential oil. The principal constituents in the *Oldenlandia diffusa* oil were pentacosane (13.29 %), hexacosane (11.59 %), tetracosane (11.18 %), heptacosane (9.76 %), tricosane (6.90 %), phytol (5.71 %), hexatriacontane (4.87 %) and isophytol (4.69 %). *Oldenlandia diffusa* essential oil exhibited outstanding cytotoxic properties on human cancer cell lines. The oil had dose- and time-dependent impact on the decline of percentage viability of cancer cells. The lowest IC_{50} values were observed in PA1 (ovarian carcinoma), HeLa (cervical adenocarcinoma) and PC-3 (prostatic adenocarcinoma) at all three

different time scales 24, 48 and 72 h, and L6 normal (rat skeletal muscle) cell lines were found to be non-toxic revealing that the essential oil was significantly effective against these cancer cell lines. The IC_{50} values ranged from 24.19 ± 0.837 to 3.12 ± 0.126 µg/mL in PA1 cells, 51.87 ± 3.104 - 28.95 ± 0.76 µg/mL in HeLa cells and 52.92±1.233 - 14.62 ± 0.465 µg/mL in PC-3 cells at 24, 48 and 72 h. Photomicrographs supplementing these findings showed morphological changes in cancer cell lines when exposed to the oil indicating a lack of cellular adherence and induction of apoptosis. We speculate that the remarkable anticancer potential of *Oldenlandia diffusa* essential oil could be related to the key components in high percentages such as hydrocarbons and diterpene alcohols and their individual or synergic effects among several compounds. The plant is an edible herb and is traditionally used by people of Assam as vegetables for various Assamese cuisines. Therefore, it may be considered that the herb as well as its essential oil are non-toxic to humans. Thus, this interesting outcome suggests the prospect of *O. diffusa* (Willd.) Roxb. essential oil to be utilized as a promising resource for cancer therapeutics and formulation of herbal drugs.

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CRediT authorship contribution statement. Bardwi Narzary: Investigation, Data Curation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Funding acquisition. Kashyap J. Tamuli: Investigation, Data Curation, Writing - Review & Editing. Partha P. Dutta: Statistical analyses, writing and editing. Manobjyoti Bordoloi: Conceptualization, Methodology, Validation, Formal analysis, Resources, Data Curation, Writing - Review & Editing, Supervision, Funding acquisition. Dipsikha Bora: Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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