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Research Article

Sesquiterpene Lactones Isolated from *Elephantopus scaber* L. Inhibits Human Lymphocyte Proliferation and the Growth of Tumour Cell Lines and Induces Apoptosis *In Vitro*

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This study was designed to isolate the compounds responsible for the cytotoxic properties of South Indian *Elephantopus scaber* L. and further investigate their effects on quiescent and proliferating cells. Bioassay-guided isolation of the whole plant of chloroform extract of South Indian *Elephantopus scaber* afforded the known sesquiterpene lactone, deoxyelephantopin, and isodeoxyelephantopin whose structures were determined by spectroscopic methods. These compounds caused a dose dependent reduction in the viability of L-929 tumour cells in 72 h culture (IC_{50} value of 2.7 μg/mL and 3.3 μg/mL) by the cell viability assay. Both the compounds act selectively on quiescent and PHA-stimulated proliferating human lymphocytes and inhibited tritiated thymidine incorporation into cellular DNA of DLA tumour cells. The compound deoxyelephantopin at a concentration of 3 μg/mL caused maximum apoptotic cells. It also exhibited significant *in vivo* antitumour efficacy against DLA tumour cells. The results, therefore, indicate that the antiproliferative property of deoxyelephantopin and isodeoxyelephantopin could be used in regimens for treating tumors with extensive proliferative potencies.

1. Introduction

Plants have served as the major source of medication, for the treatment of human ailments since time immemorial. Plants have a long history of use in the treatment of cancer. The goal for the search for new anticancer drugs is to find drugs that act via a specific mode of action. In this manner, it is hoped that cancer cells can be targeted with little or no damage to noncancerous cells. Natural products are perfectly suited to the current molecular approach of drug development as they produce few adverse side effects, each inhibits multiple aspects of cancer cell proliferation and as a group show propensity for synergistic interaction [1].

Elephantopus scaber L. (Asteraceae) is a genus of herb, distributed all over the world, especially in America. Of the 32 species, only one species, namely, *Elephantopus scaber* is known to grow in India. Studies have shown that several species of the genus *Elephantopus* contain a large number of cytotoxic and antitumoural sesquiterpene lactones [2–4] and there is an interest in this plant over the world. Sesquiterpene lactones are characteristic constituents of the family Asteraceae and their principal characteristics are the presence of α, β-unsaturated γ-lactone [5].

Previous studies from our laboratory have shown that chloroform extract and the partially purified fraction of South Indian *E. scaber* exhibited cytotoxic as well as antitumour properties [6]. Inspired by the antitumour properties exhibited by *E. scaber*, the c hloroform extract was chosen to isolate the compounds responsible for the cytotoxic properties and further investigate their effects on quiescent and proliferating cells.

2. Methods

2.1. Experimental. All melting points are uncorrected and were determined on a Meltemp II hot stage melting point apparatus. The IR spectra were recorded on Bomem MB Series FT IR spectrometer. NMR spectra were recorded on Bruker 300 MHz FT NMR spectrometer, using $CDCl_3$ or $CDCl_3$-CCl_4 mixture (7 : 3) as solvent. TMS was used as the internal standard and chemical shifts are in δ-scale. Abbreviations used in ^{1}H NMR are s-singlet, d-doublet, dd-doublet of a doublet, and m-multiple. Analytical thin layer chromatography was performed on glass plates coated with silica gel (Merck) containing 13% calcium sulphate as binder. Column chromatography was done using 100–200 mesh silica gel. The solvents were removed from the fractions under reduced pressure using a Buchi rotary evaporator.

2.2. Animals. Swiss albino mice (males) in the age group of 8–10 weeks, weighing 20–30 g, were used for the experiments. The animals were housed under conventional laboratory conditions and fed with standard pellet diet (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. All experiments involving animals were done, strictly adhering to the guidelines for animal experimentation and handling, issued by the Government of India.

2.3. Chemicals. RPMI-1640 medium was obtained from Gibco BRL, USA, and the antibiotics streptomycin, penicillin, and gentamycin sulphate from Hi-Media Laboratories, Mumbai. Foetal calf serum, Trypsin, Ethidium bromide, Acridine Orange, Histopaque, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and vincristine were obtained from Sigma Chemical Company, USA. Phytohaemagglutinin (PHA-M) and Heparin were obtained from DIFCO, USA. The solvents used for the phytochemical analyses were LR grade, but except for final purification and spectroscopic studies, AR or spectroscopic grade solvents were used. Silica gel was obtained from Sisco Research Laboratories and Merck Laboratories, Mumbai. Tritiated thymidine was obtained from the Board of Radiation and Isotopes Technology (BRIT), Mumbai.

2.4. Tumour Cells. Murine fibroblast cell line, L-929 and Human colon carcinoma cell line, HCT 116 were grown in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamycin sulphate, in a humidified CO_2 incubator at 37°C. Daltons Lymphoma Ascitis (DLA) tumours were maintained as ascites by serial transplantation in mice, by intraperitoneal (i.p.) injection of 1×10^6 cells/mouse. The tumor cells were aspirated from the tumor-bearing mice aseptically and washed thrice in phosphate-buffered saline before transplantation.

2.5. Extraction. Fresh *E. scaber* plants, collected from TBGRI campus, Thiruvananthapuram in October 2002, after authentication by the plant taxonomist of the Institute was deposited in the Herbarium of the institute. The plants were cleaned and dried under shade for two days and 400 g of the powdered plant material was extracted with hexane (bp.60°C) using Soxhlet extractor for 12 h. The plant material was dried and again extracted with chloroform for 12 h. The extracts were concentrated under reduced pressure using rotary evaporator and afforded 9.1 g of hexane extract and 5.8 g of chloroform extract, respectively.

2.6. Preliminary Phytochemical Analysis. Several reactions for the identification of the chemical constituents present in the chloroform extract were carried out by the standard methods of Harborne [7].

2.7. Fractionation of $CHCl_3$ Extract. The $CHCl_3$ extract (5.8 g) was subjected to column chromatography on silica gel (60–120 mesh; 200 g). The column was eluted with hexane and gradient of hexane-EtOAc mixture and 50 fractions each of 100 mL were collected. The fractions were pooled according to similarities in thin-layer Chromatography (TLC).

2.8. Isolation of Isodeoxyelephantopin. Fraction 16–19 (473 mg), eluted with 15% EtOAc in hexane from the abovementioned column, was further purified by column chromatography using silica gel (60–120 mesh, 1.5 g). The column was eluted with hexane and hexane-EtOAc gradient mixture and 50 mL fractions were collected. The fractions eluted with 10% EtOAc in hexane afforded the pure compound.

2.9. Isolation of Deoxyelephantopin. Fraction 23–26 (425 mg) was purified by column chromatography using silica gel (60–120 mesh, 1.5 g). Low polar impurities were removed by eluting the column with 25% EtOAc in hexane and further elution with 40% EtOAc in hexane afforded a UV active compound.

2.10. Cytotoxicity Assay Using MTT Method. The *in vitro* response of deoxyelephantopin and isodeoxyelephantopin of *E. scaber* against L-929 cell line was studied using MTT assay [8]. L-929 cell line was maintained in RPMI-1640 medium, supplemented with 10% FCS. Briefly, cells harvested in the log phase of growth were harvested, counted, and seeded (4×10^4 cells/well in 500 μL volume) in 24-well titre plates. After 24 h of incubation at 37°C in 5% CO_2, to allow cell attachment, cultures were treated with varying concentrations of drugs (2–10 μg/mL) diluted with medium. Vehicle controls were also kept, that is, DMSO at concentrations not exceeding 1%. The plates were further incubated for 48 h. On completion of incubation, with the drugs, the plates were removed and to each well 20 μL of a 5 mg/mL stock solution of MTT were added and the plates were further incubated for 4 h. Then, the supernatant from each well was carefully removed and the MTT-formazan crystals were dissolved in 100 μL 100% DMSO and the absorbance at 570 nm was measured, using a spectrophotometer. Three replicate wells were set up for each experiment. For each drug, the concentration required to reduce absorbance by 50% (IC_{50}) in comparison to control cells was determined. IC_{50} values were derived by substituting the percentage inhibition values of

the drugs against the doses used. % growth inhibition = 100 − [(absorbance of the drug treated cells/absorbance of the untreated control cells) × 100%].

2.11. Effect of Deoxyelephantopin and Isodeoxyelephantopin on Quiescent and PHA-Stimulated Proliferating Human Lymphocytes

2.11.1. Lymphocyte Isolation. For culturing human peripheral blood lymphocytes, venous blood from healthy human volunteers was collected in heparinized tubes. Lymphocytes were separated from polymorphonuclear leukocytes and erythrocytes by Histopaque gradient method. The blood was diluted with 1 : 3 volume of PBS and layered onto 1 mL of Histopaque and centrifuged at 2,000 rpm for 20 min at room temperature. Lymphocytes were aspirated from the gradient plasma interfaces and washed twice in PBS and the final cell pellets were resuspended in RPMI-1640 medium containing 10% FCS, 100 μL penicillin, 100 μL streptomycin, and 100 μL fungicide (pH 7.4). Viability of the cells was checked by staining with 0.1% trypan blue.

2.11.2. Lymphocyte Proliferation Assay. Briefly, 200 μL of isolated lymphocytes (1×10^6/well) were seeded on 24-well titre plates and grown in 10% FCS containing RPMI-1640 medium at 37°C in 5% CO_2 in a humidified chamber. The cells were stimulated with 10 μL PHA (5 μg/mL) and wells without PHA served as positive control. To study the lymphocyte proliferation, in the absence of mitogen PHA, 10 μL of PHA was replaced by medium and served as negative control. Both PHA-stimulated and unstimulated lymphocytes were treated with varying concentrations of deoxyelephantopin and isodeoxyelephantopin (2–10 μg/mL) for 72 h. 18 h prior to termination, 2 μCi/mL [^{3}H] thymidine was added. The cells were then harvested using cell harvester and the radioactivity was measured using liquid scintillation counter. From the counts obtained (CPM), the percentage inhibition in thymidine incorporation was calculated. The percentage of incorporation was calculated as follows: % incorporation = [(CPM of treated group/CPM of control group)] × 100 % inhibition incorporation = 100 − % incorporation.

2.12. Effect of Deoxyelephantopin and Isodeoxyelephantopin on DNA Synthesis.

Thymidine labelling technique was used to evaluate DNA synthesis of Dalton's ascitic tumour cells (DLA) following drug treatment. Incorporation of tritiated thymidine into DNA was done by incubating DLA cells (1×10^6) with 2 μCi/mL of [^{3}H] labelled thymidine in the presence of various concentrations of drugs (2–10 μg/mL) in 2 mL RPMI-1640 medium containing 10% FCS at 37°C and 5% CO_2 atmosphere for 4 h. Control tubes were maintained without drugs. After incubation, the DNA was precipitated with 0.8 N perchloric acid and redissolved in 0.5 mL of NaOH (0.5 M) and cells harvested onto glass fiber discs, using a PHD cell harvester (Cambridge technology, Cambridge, MA). The glass fiber discs were counted in vials containing 3 mL scintillation-counting mixture, in a Wallac1410 β-liquid scintillation counter. From the counts obtained (CPM), the percentage inhibition in thymidine incorporation was calculated.

2.13. Morphological Analysis of Apoptotic Cells

2.13.1. Analysis of DLA Cell Death (Apoptosis) Using Fluorescence Microscopy. Cells were cultured in 24-well titre plates. 1×10^6 DLA cells were incubated in RPMI 1640 medium with 5% FCS containing various concentrations (1–10 μg/mL) of deoxyelephantopin in a CO_2 incubator at 37°C for 6 h and 18 h. For assessment of apoptosis, 6 h and 18 h after exposure to different concentrations of drug treatment, acridine orange-ethidium bromide dual staining of unfixed DLA cells was used. These dyes stain the DNA and allow visualization of the condensed chromatin of apoptotic cells. The medium was removed, cells pelleted gently, and 1 μL of acridine orange (100 μg/mL) + ethidium bromide (100 μg/mL) in 1 mL PBS was added to cells and immediately washed once with phosphate buffer saline (PBS) and resuspended in 10 μL of 10% glycerol in PBS and the slides analyzed by fluorescence microscopy (Nikon Diaphot, UV 410). The number of cells manifesting morphologic features of apoptosis such as chromatin condensation and loss of nuclear envelope [9] was counted as a function of the total number of cells present in the field. For cell death evaluation, three samples were prepared for each experimental point and the experiment was repeated three times.

2.13.2. Analysis of HCT 116 Cell Death (Apoptosis) Using Fluorescence Microscopy. 2×10^4/well HCT 116 human colon cancer cell line was seeded into each well of 24 titre-well plastic culture plate containing 12 mm cover slip. After 24 h, the cells were incubated with varying concentrations of deoxyelephantopin (1–10 μg/mL). After 48 h, the cover slip was carefully removed from the medium and washed twice with PBS and 1 μL of acridine orange (100 μg/mL) + ethidium bromide (100 μg/mL) in 1 mL PBS was added to cells. The cells were immediately washed once with PBS and suspended in 10 μL of 10% glycerol in PBS and viewed under fluorescence microscopy (Nikon Diaphot, UV 410) and apoptosis evaluated.

2.14. Effect of Deoxyelephantopin on the Ascitic Tumour Reduction in Mice.

The antitumour activity of deoxyelephantopin was determined, as described previously [10]. Briefly, Swiss albino mice (20–25 mg) were challenged with DLA cells (1×10^6 cells) and divided into 5 groups of 10 each. After 24 h, animals of group I serving as control were given 0.5 mL of 10% DMSO. The group II, III, and IV received the deoxyelephantopin 25 mg, 50 mg, and 100 mg/kg, b.w., i.p, respectively, and group V received vincristine (1 mg/kg b.w, i.p). Vincristine was used as a standard anticancer agent for comparison. The administration of deoxyelephantopin and vincristine was continued intraperitoneally for 15 days. Growth inhibitory effect of DLA tumour cells implanted into the peritoneal cavity was evaluated by determination of animal survival, recorded and expressed as mean survival time (MST)

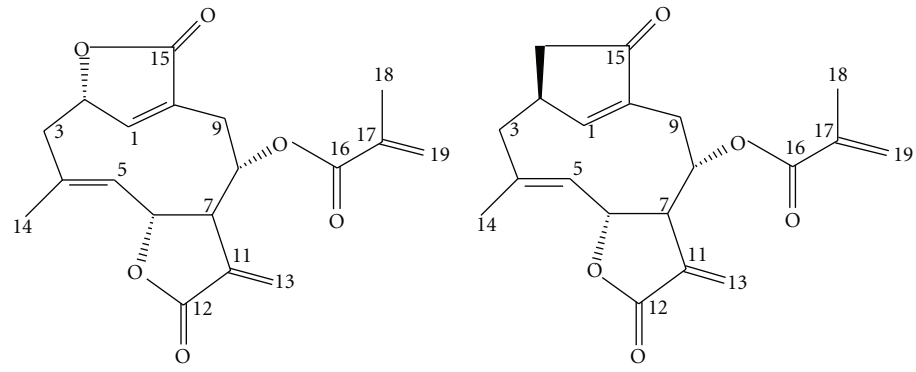

Figure 1: (a) Structure of isodeoxyelephantopin, (b) structure of deoxyelephantopin.

in days, and the percentage increase in life span % ILS) of drug treated group was determined.

2.15. Statistical Analysis. Experimental data were expressed as mean ± S.D. Student's "*t*" test was applied for expressing the significance and *P* value less than 0.05 was considered as significant.

3. Result

The preliminary phytochemical analysis of the chloroform extract revealed the presence of terpenes, flavonoids, coumarins, and alkaloids.

The extract upon fractionation using column chromatography and further purification by rechromatography yielded two UV active sesquiterpene dilactones viz: isodeoxyelephantopin and deoxyelephantopin**.** Isodeoxyelephantopin (90 mg) was crystallized from petroleum ether- EtOAc mixture as colourless needles (mp. 151–153°C, Lit.150–153°C).

3.1. Isodeoxyelephantopin. IR (cm^{-1}): 3075, 2968, 2935, 1765, 1750, 1714, 1638, 1444, 1320, 1155, 983, 951, 886, 815.

^{1}H NMR: 7.16 (s, 1H), 6.20 (d, 1H, J = 3.7 Hz), 6.15 (s, 1H), 5.68 (s, 1H), 5.65 (d, 1H, J = 3.3 Hz), 5.38 (d, 1H, J = 3.9 Hz), 5.21–5.10 (m, 2H), 4.55–4.49 (m, 1H), 3.17–2. (m, 3H), 2.75 (dd, 1H, J = 3.6 Hz, 12.6 Hz), 1.93 (s, 3H), 1.79 (s, 3H).

^{13}C NMR: 174.4, 169.5, 166.6, 149.5, 135.5, 135.5, 134.1, 131.5, 126.9, 125.4, 123.2, 79.5, 78.8, 74.1, 49.9, 40.1, 30.1, 21.6, 18.2.

From the data, the structure of the compound was assigned as isodeoxyelephantopin (Figure 1(a)). The structure was confirmed by the spectral data and melting point of the compound [11, 12].

Purification of another pool of chloroform fractions by repeated chromatography afforded another UV active sesquiterpene dilactone, deoxyelephantopin (80 mg). This compound was crystallized from petroleum ether-EtOAc mixture as colourless needles (mp. 199–200°C, Lit. 196–199°C).

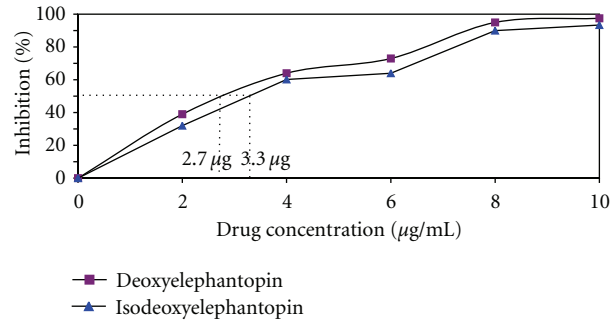

Figure 2: Percentage inhibition of deoxyelephantopin and isodeoxyelephantopin against L-929 tumour cell line using MTT assay.

3.2. Deoxyelephantopin. IR (cm^{-1}): 3100, 2962, 1751, 1770, 1707, 1632, 1432, 1295, 1201 1170, 927.

^{1}H NMR: 7.11 (s, 1H), 6.22 (d, 1H, J = 3.5 Hz), 6.14 (s, 1H), 5.65 (m, 2H), 5.46 (d, 1H, J =1.7 Hz), 5.14 (dd, 1H, J = 8.2 Hz, 10.2 Hz), 4.78 (d, 1H, J = 10.3 Hz), 4.65 (m, 1H), 3.03–2.68 (m, 5H), 1.93 (s, 3H), 1.84 (s, 3H).

^{13}CNMR: 172.4, 169.3, 166.3, 153.3, 135.9, 135.6, 134.1, 133.7, 128.5, 81.3, 77.9, 71.5, 52.3, 41.3, 33.5, 20.1, 18.2.

From the data, the structure was assigned as deoxyelephantopin (Figure 1(b)). The structure was confirmed by comparing the spectral values and melting point of the compound (13, 12).

3.3. Cytotoxicity Using L-929 Tumour Cell Line. The deoxyelephantopin and isodeoxyelephantopin showed a dose-dependent cytotoxicity to L-929 cell line in culture. The sesquiterpene lactones, deoxyelephantopin, and isodeoxyelephantopin exhibited maximum cytotoxicity, having an IC_{50} value of 2.7 μg/mL and 3.3 μg/mL, respectively (Figure 2).

3.4. Lymphocyte Proliferation. To ascertain whether deoxyelephantopin and isodeoxyelephantopin specifically inhibit the proliferation of dividing cells, their effect was assessed on unstimulated and PHA-stimulated human lymphocytes

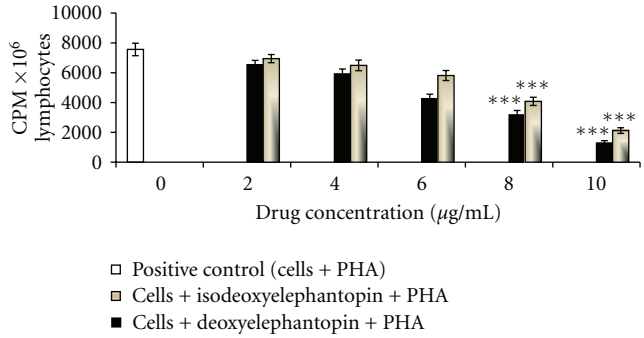

Figure 3: Influence of deoxyelephantopin and isodeoxyelephantopin on proliferative responses to the PHA-stimulation of normal lymphocytes. Values are ± SD of three replicates of three independent experiments, $^{***}P < 0.001$.

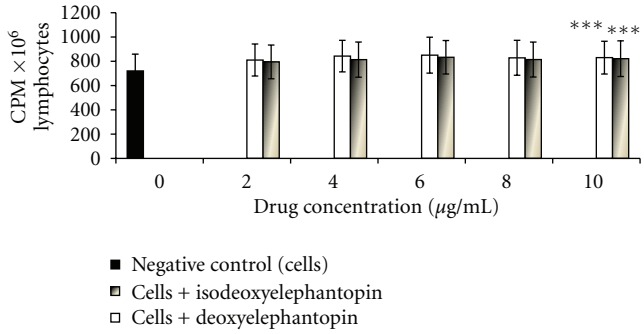

Figure 4: Influence of the deoxyelephantopin and isodeoxyelephantopin on proliferative responses in absence of mitogen PHA. Values are ± SD of three replicates of three independent experiments, $^{***}P < 0.001$.

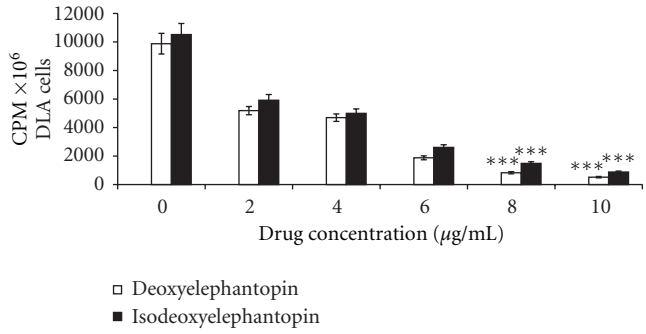

Figure 5: Effect of deoxyelephantopin and isodeoxyelephantopin on DNA synthesis of DLA tumour cells. Values are ± SD of three replicates of three independent experiments, $^{***}P < 0.001$.

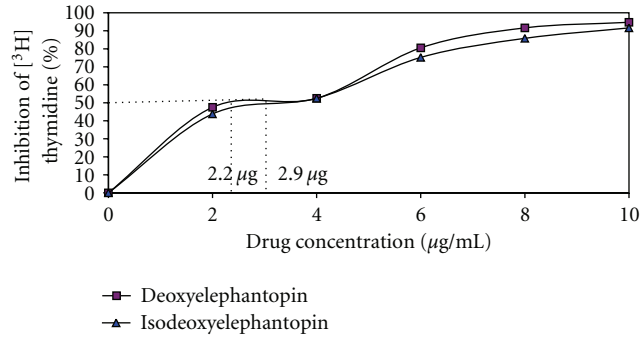

Figure 6: Effect of deoxyelephantopin and isodeoxyelephantopin on percentage inhibition of DNA synthesis of DLA tumour cells.

in culture. Deoxyelephantopin and isodeoxyelephantopin significantly decreased the [^{3}H] thymidine incorporation of proliferating (PHA-stimulated) lymphocytes in a dose-dependent manner, when compared to the positive control (Figure 3). However, there was no significant change in the proliferation of unstimulated lymphocytes exposed to deoxyelephantopin and isodeoxyelephantopin (Figure 4).

3.5. Inhibition of DNA Synthesis. DLA cells treated both with deoxyelephantopin and isodeoxyelephantopin showed decreased [^{3}H] thymidine incorporation into the nuclear DNA, suggesting that they inhibited DNA synthesis. The results on DNA synthesis also indicated a concentration-dependent decrease in [^{3}H] thymidine incorporation (Figure 5). The concentration of deoxyelephantopin and isodeoxyelephantopin required to produce 50% in-incorporation was 2.2 μg/mL and 2.9 μg/mL, respectively (Figure 6).

3.6. Morphological Analysis of Apoptotic Cells. The percentages of viable and nonviable (apoptotic) DLA cells after *in vitro* exposure to various concentrations of deoxyelephantopin with acridine orange and ethidium bromide staining during 6 h and 18 h treatment period are presented in Table 1. DLA cells exposed to 2 μg/mL deoxyelephantopin showed reduction in the percentage of viable cells, but the cells exposed to 3 μg/mL of deoxyelephantopin exhibited a significant reduction in the percentage of viable cells (52.63% and 70.65%), compared to the control group during 6 h and 18 h treatment period, respectively. The higher concentrations of drug such as 8 μg/mL and 10 μg/mL showed necrosis, which is characterized by swelling of cytoplasm and organelles, which are associated with membrane lysis, instead of apoptosis. The proportion of apoptotic cells was higher during 18 h treatment period.

During 48 h treatment period, HCT-116 colon cancer cell line stained with ethidium bromide and acridine orange exhibited a significant reduction in the percentage of viable cells (77.08%) and increase in percentage of apoptotic cells when exposed to 3 μg/mL deoxyelephantopin compared to the control group (Table 2). Deoxyelephantopin caused shrinkage after 24 h in HCT-116 cells and the shrinkage was prominent after 48 h duration of exposure due to cytoplasmic condensation, where most of the cells rounded up (dead cells).

3.7. Effect of Deoxyelephantopin on the Ascitic Tumour Reduction in Mice. The deoxyelephantopin exhibited remarkable antitumour activity. Administration of deoxyelephantopin (50 mg/kg, b.w and 100 mg/kg b.w) significantly reduced murine ascitic tumour growth and increased the life span of DLA tumour-bearing mice by 67.64% and 72.51%,

Table 1: Apoptosis (percentage of cells) of DLA cells treated with deoxyelephantopin as determined by microscopic morphological observation, stained with acridine orange and ethidium bromide.

Con. of deoxyelephantopin (μg/mL)	Treatment time (6 h) Percentage of cells[x] (%)			Treatment time (18 h) Percentage of cells[x] (%)		
	Viable cells	Non-viable cells		Viable cells	Non-viable cells	
		Apoptotic	Necrotic		Apoptotic	Necrotic
0 (control)	95 ± 4.2	—	5 ± 0.25	92 ± 4.5	2 ± 0.28	6 ± 0.49
1	68 ± 3.8	26 ± 1.71	6 ± 0.40	61 ± 4.02	30 ± 2.15	9 ± 0.56
3	45 ± 2.15	39 ± 2.79***	16 ± 1.72	27 ± 1.93	46 ± 3.29***	27 ± 1.85
6	29 ± 1.78	30 ± 1.68	41 ± 2.56	20 ± 1.37	35 ± 2.4	45 ± 4.04
8	15 ± 0.87	9 ± 0.52	76 ± 5.22	13 ± 1.17	5 ± 0.45	82 ± 5.12
10	12 ± 0.67	9 ± 0.55	79 ± 4.86	10 ± 0.90	6 ± 0.37	84 ± 7.55

[x] cells (viable + nonviable) = 100%, *** $P < 0.001$ compared to control.

Table 2: Apoptosis (percentage of cells) of HCT-116 colon cancer cells treated with deoxyelephantopin as determined by microscopic morphological observation, stained with acridine orange and ethidium bromide.

Con.of deoxy elephantopin (μg/mL)	Treatment time (24 h) Percentage of cells[x] (%)			Treatment time (48 h) Percentage of cells[x] (%)		
	Viable cells	Non-viable cells		Viable cells	Non-viable cells	
		Apoptotic	Necrotic		Apoptotic	Necrotic
0 (control)	97 ± 4.4	—	3 ± 0.25	96 ± 4.6	2 ± 0.15	1 ± 0.10
1	59 ± 4.33	28 ± 2.11	13 ± 0.87	55 ± 2.79	31 ± 2.1	14 ± 0.97
3	30 ± 1.6	39 ± 2.6***	31 ± 2.79	22 ± 1.52	42 ± 2.5***	36 ± 1.98
6	20 ± 1.51	32 ± 2.41	48 ± 3.25	18 ± 1.24	30 ± 1.9	52 ± 3.59
8	13 ± 0.35	6 ± 0.44	81 ± 4.11	12 ± 0.81	4 ± 0.28	84 ± 4.27
10	9 ± 0.60	7 ± 0.47	84 ± 5.8***	9 ± 0.46	4 ± 0.26	87 ± 5.5

[x] cells (viable + nonviable) = 100%, *** $P < 0.001$ compared to control.

respectively (Table 3). The higher dose of deoxyelephantopin (100 mg/kg, b.w, i.p) was found to be very effective when compared to the other two doses and afforded the protection of 6 animals out of 10 DLA-challenged mice. In the case of control animals treated with DMSO, the tumour grew progressively and the survival time was significantly reduced. The results were comparable with those of standard anticancer drug, vincristine.

4. Discussion

The MTT cell viability assay is widely used in determining drug sensitivity in primary screening of potential chemotherapeutic drugs. The isolated pure compounds belonging to the class of sesquiterpene lactones, deoxyelephantopin, and isodeoxyelephantopin caused a significant dose-dependent reduction in the viability of L-929 tumour cells in 72 h culture. The structural difference between deoxyelephantopin and isodeoxyelephantopin is that γ-lactone ring oxygen atom at C-2 is β-oriented in deoxyelephantopin whereas is α-oriented in deoxyelephantopin. Kupchan et al. [13] have shown that the presence of C_{11}-C_{13} exocyclic double bond conjugated to the γ-lactone function which can undergo a Michael addition is essential for cytotoxicity. Sesquiterpene which incorporated a cyclopentenone or a methylene lactone (in addition to the α, β methylene-γ lactone) appeared to produce enhanced cytotoxicity. The success of deoxyelephantopin and isodeoxyelephantopin that possess C_{11}-C_{13} exocyclic methylene conjugated to γ-lactone, to exhibit significant cytotoxicity to tumour cell line, is in agreement with these suggestions. These findings are in agreement with the studies of sesquiterpene lactones, helenain possessing both the reactive α-methylene-γ-lactone moiety, and a reactive α, β-unsubstituted cyclopentenone ring, displaying the strongest cytotoxicity against tumour cell lines using MTT assay [14].

Lymphocyte proliferation *in vitro* is usually determined by measuring [^{3}H] thymidine uptake by nuclear DNA after 72 h of culture. Mitogen stimulated cells commence DNA synthesis approximately 24 h after mitogen addition. Maximum thymidine incorporation occurs during 60–72 h time period and thus 72 h culture has been selected in the present study. The compounds, deoxyelephantopin and isodeoxyelephantopin, failed to augment human T-lymphocyte proliferation, induced by T-mitogen PHA as evidenced by decreased [^{3}H] thymidine uptake by nuclear DNA. Quiescent lymphocytes were refractory to the action of deoxyelephantopin and isodeoxyelephantopin.

It has been reported that cleistanthin A, a diphyllin glycoside from *Cleistanthus collinus* possessing cytotoxic and tumour regressing properties, was cytotoxic to PHA-stimulated proliferating human lymphocytes [15]. The effects of

Table 3: Effect of intraperitoneal administration of deoxyelephantopin on the life span of DLA-tumour-bearing mice.

Treatments	Number of mice survived after tumour inoculation (days)			Mean survival time (days)	Life span (%)	Increase in life span (%)
	15	25	35			
Group I DLA (Control)	10/10	5/10	0/10	22.56 ± 2.57	100	—
Group II DLA + Deoxy-elephantopin (25 mg/kg, i.p)	10/10	6/10	3/10	30.90 ± 3.36	136.96	36.96
Group III DLA + Deoxy-elephantopin (50 mg/kg, i.p)	10/10	8/10	5/10	37.82 ± 3.70	167.64	67.64
Group IV DLA + Deoxy-elephantopin (100 mg/kg, i.p)	10/10	8/10	6/10	38.92 ± 3.5***	172.51	72.51
Group V DLA + Vincristine (1 mg/kg, i.p)	10/10	10/10	6/10	43.54 ± 3.9	192.99	92.99

Values are mean ± SD of three experiments, $n = 10$, $^{***}P < 0.001$ compared to control (the time period required for the death of all the animals in the control group was 22).

resveratrol on the G_0-G_1 transition and cell cycle progression of mitogenically stimulated human lymphocytes have cancer-preventive properties [16]. A compound is judged as a good anticancer drug, when it is more specific towards tumour cells in comparison to normal cells. The results of the present study indicate that the sesquiterpene lactones, deoxyelephantopin, and isodeoxyelephantopin are not cytotoxic to normal human lymphocytes and only the proliferating cells were affected.

The site of action of a drug can be evaluated by [^{3}H] thymidine incorporation studies. The [^{3}H] thymidine incorporation studies revealed that the deoxyelephantopin and isodeoxyelephantopin inhibited DNA synthesis as seen by decreased incorporation of thymidine. The incorporation of [^{3}H] thymidine into DNA was high in the control than the drug-treated cultures. The growth of DLA tumour cells is due to uncontrolled cell division and the basic fact underlying this phenomenon should be enhanced DNA synthesis. Anticancer drugs exercise their effects through a variety of mechanism of action. It may inhibit stages/stages in the multiplication of DNA and/or in the synthesis of proteins. Since DNA synthesis was inhibited, it meant the compounds exerted cytotoxic effect on tumour cells. The radioactivity incorporated is inversely proportional to the concentration of the drug used. The rate of incorporation depends on the type of plant drug, the concentration of radioactive compound, and the type of cells used. Thus, [^{3}H] thymidine incorporation studies indicate the possible mechanism of action of deoxyelephantopin and isodeoxyelephantopin used at the DNA level. These findings are in agreement with the studies of sesquiterpene lactones with the exocyclic methylene groups from *Helianthus annuus* that play an important role in triggering the inhibitory effect of DNA and RNA synthesis as evident by measuring ^{14}C-labelled thymidine, uridine, and leucine incorporated into murine cells of the ascitic Ehrlich carcinoma (EAC) [17].

Analysis of cell death (apoptosis) using fluorescence microscopy indicates that maximum apoptotic cells were obtained at a concentration of 3 μg/mL of deoxyelephantopin that can be considered as optimum dose for inducing apoptosis both in DLA cells and HCT-116 cancer cells. The cytotoxic sesquiterpene lactones mediate their death-inducing effect in leukaemia T cells by triggering apoptosis [18]. The involvement of apoptosis is one of the mechanisms of cell death, induced by clinically relevant chemotherapeutic drugs in human leukemic T-lymphocytes [19]. Hence, in the present study, it is possible to confirm apoptosis as one of the methods of antitumour activity, since results of the study indicate that deoxyelephantopin, a sesquiterpene lactone isolated from *E. scaber* induces apoptosis in DLA tumour cells and HCT-116 colon cancer cells.

The deoxyelephantopin administered after DLA transplantation reduced the ascitic tumour growth as evidenced from percentage increase of life span of treated mice. This indicated the cytotoxic effect of deoxyelephantopin on tumour cells and its *in vivo* efficacy against DLA tumour cells is equal to vincristine (1 mg/kg, b.w). Although deoxyelephantopin (25 mg/kg, i.p.) evoked antitumour response *in vivo*, significant antitumoureffect was noted with higher doses of deoxyelepantopin (50 mg/kg, i.p and 100 mg/kg, i.p) exhibiting no toxic effects. Increase in life span is a reliable criterion for judging the value of any anticancer drug. Hence, the increase in life span of DLA tumour-bearing mice by treatment with deoxyelephantopin is a positive finding and supports the antitumour effect of the drug.

Our results, therefore, indicate that the antiproliferative property of deoxyelephantopin and isodeoxyelephantopin could be used in regimens for treating tumors with extensive proliferative potencies.

Declaration of Interest

The authors declare no conflict of interest.

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