

The enriched fraction of *Elephantopus scaber* Triggers apoptosis and inhibits multi-drug resistance transporters in human epithelial cancer cells

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ABSTRACT

Background: Medicinal plants have played an important role in the development of clinically useful anticancer agents. *Elephantopus scaber* (Asteraceae) (ES) is widely used in Indian traditional system of medicine for the treatment of various ailments including cancer. **Objective:** To investigate anticancer effects of ES in human epithelial cancer cells. **Materials and Methods:** Cytotoxicity of ethanolic extract of ES (ES-ET) and its fractions, such as ES Petroleum ether fraction (ES-PET), ES Dichloromethane fraction (ES DCM), n Butyl alcohol fraction (ES-BT), and ES-Rest (ES-R) were assessed in human epithelial cancer cell lines using sulforhodamine B (SRB) assay. Acridine orange/ethidium bromide assay and Hoechst 33342 assays were used to gauge induction of apoptosis. Cell cycle analysis and micronuclei assay were used to assess cell cycle specific pharmacological effects and drug induced genotoxicity. Further, the ability of ES to inhibit multi drug resistant (MDR) transporters (ABC-B1 and ABC-G2) was determined by Rhodamine (Rho) and Mitoxantrone (MXR) efflux assays. **Results:** The enriched fraction of ES (ES DCM) possessed dose-dependent potent cytotoxicity in human epithelial cancer cells. Further, treatment of cancer cells (HeLa, A549, MCF-7, and Caco-2) with ES DCM showed hall mark properties of apoptosis (membrane blebbing, nuclear condensation etc.). Similarly, ES DCM caused enhanced sub G0 content and micronuclei formation indicating the induction of apoptosis and drug induced genotoxicity in cancer cells, respectively. Interestingly, ES DCM inhibited MDR transporters (ABC B1 and ABC G2) in cancer cells. **Conclusion:** The enriched fraction of ES imparted cytotoxic effects, triggered apoptosis, induced genotoxicity, and inhibited MDR transporters in human epithelial cancer cells. Thus, ES appears to be potential anticancer agent.

Key words: Anti-cancer, apoptosis, cytotoxicity, *Elephantopus scaber* L, multi-drug resistance

INTRODUCTION

Cancer is the second leading cause of mortality following heart diseases, accounting for 23% of all deaths.^[1] Chemotherapy, surgery, and radiotherapy have remained the mainstay of cancer treatment. Recent advances in diagnosis, chemotherapy, surgical techniques, and radiotherapy have led to increased survival time for several cancer patients. Recently, a greater emphasis has been given toward the use of medicinal plants for the effective treatment of cancer. Ayurveda, a traditional sect of Indian medicinal plants describes the significance

of medicinal plant extracts for cancer prevention and treatment.^[2] Natural product secondary metabolites of plant origin play a very important role in current cancer chemotherapy. Indeed, natural products derived from medicinal plant extracts lead to the development of several clinically useful anti-cancer agents (Paclitaxel, vincristine, podophyllotoxins, etc.). Interestingly, numerous bio-active compounds have been isolated from plant sources and were currently in preclinical or clinical trials undergoing extensive laboratory investigation (Flavopiridol, roscovitine, bruceantin, betulinic acid, etc.).^[3] Therefore, there is a significant scientific interest in screening and development of for anti-cancer agents from plant sources.

Chemotherapy involves the use of drugs to eliminate uncontrolled proliferation of cancer cells and typically show cytotoxicity and induce apoptosis in cancer

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cells.^[4] Apoptosis is a highly organized cell death process characterized by loss of plasma membrane phospholipid asymmetry, enzymatic cleavage of the DNA into oligonucleosomal fragments, segmentation of the cells into membrane-bound apoptotic bodies, and activation of evolutionary conserved intracellular pathways.^[5] Chemotherapeutic drugs typically exert their anti-cancer effects by triggering apoptosis in cancer cells. Induction of apoptosis in cancer cells is a valuable tool for an effective anti-cancer therapy.^[6] The major obstacle associated with current chemotherapy is the development of drug resistance. Some of the cancer types are intrinsically resistant to various anticancer drugs while the others develop multi-drug resistant (MDR) upon treatment.^[7] One of the immediate measures the cell utilizes toward drug resistance is the active efflux of the drug so as to minimize the intracellular concentration leading to poor prognosis.^[7] Among the various cell membrane based mechanism, the involvement of adenosine triphosphate (ATP)-binding cassette (ABC) transporters (a family of plasma membrane efflux transporters) have received extensive attention.^[8] These trans-membrane proteins, particularly ABC-B1 (MDR-1 or p-glycoprotein), ABC-C1 (MRP-1), and ABC-G2 (breast cancer resistance protein [BCRP]) actively extrude the cytotoxic drugs by utilizing ATP, thus leading to MDR in cancer.^[7,8]

Elephantopus scaber Linn. (ES) of the family Asteraceae is an evergreen shrub and widely grows in many parts of India.^[9] ES is used as a diuretic, antifebrile, antiviral, antibacterial agent, and for the treatment of hepatitis, bronchitis, the cough associated with pneumonia, and arthralgia. Further, ES (applied topically) also employed as an antipyretic, for the treatment of erysipelas, skin infections, and measles.^[10] The chemical investigations on ES indicated that it contained sesquiterpene lactones (STLs), flavonoids, triterpenoids, and flavonoid esters.^[11] Interestingly, the alcoholic and chloroform extracts of ES contain unique germacranolide type STLs (elephantopin and deoxy-elephantopin), which may contribute to the potential cytotoxic effects.^[11] However, detailed investigations on anti-cancer effects of ES and the mechanism of cytotoxic effects in cancer cells (induction of apoptosis) remains unknown. The current study investigated the cytotoxic, apoptotic, and genotoxic effects of the enriched fraction of ES against a panel of human epithelial cancer cells (cervical, lung, breast, and colon). A bio-activity (anti-cancer) guided fractionation strategy was used to identify the most-active enriched fraction of ES (ES-DCM). Further, agents that are proficient to reverse MDR in cancer cells have drawn considerable attention for the development of novel anticancer drugs. Hence, the current study also investigated the ability of ES-DCM to inhibit MDR

transporters (ABC-B1 or MDR-1 and ABCG-2 or BCRP) in human epithelial cancer cell lines.

MATERIALS AND METHODS

Plant material

The whole plant of *Elephantopus scaber* Linn. (ES) of the family Asteraceae was collected from the Udupi district, Karnataka (India). The taxonomic identification was carried out by Dr. Gopala Krishna Bhatt (Professor and Head, Department of Botany, Poorna Prajna College, Udupi, Karnataka). A voucher specimen was deposited in the herbarium of the institute.

Preparation of plant extract and fractions

Dried and coarsely powdered plant material of ES (2 kg) was extracted in a soxhlet apparatus for 72 h using absolute ethanol as solvent. The extract was filtered, concentrated in a rotary evaporator *in vacuum*, and completely dried to yield the total ethanolic extract of ES (ES-ET). The practical yield of ES-ET was 5.60%.

The ES-ET (100 g) dispersed in methanol: Water (1:4) was fractionated successively with solvents of increasing polarity index, such as petroleum ether (PET), dichloromethane (DCM), and *n*-butyl alcohol (BT) to get ES-PET (ES-Petroleum ether fraction), ES-DCM (ES-Dichloromethane fraction), and ES-*n*-Butyl alcohol fraction (ES-BT), respectively. The remaining hydro-alcoholic fraction was considered as rest fraction (ES-R). Each fraction was concentrated using a rotary evaporator *in vacuum*, and completely dried. The yield of ES-PET, ES-DCM, ES-BT, and ES-R was 13.50%, 18.60%, 22.50%, and 42.00%, respectively. For anti-cancer studies, ES-ET, ES-PET, ES-DCM, ES-BT, and ES-R were dissolved in dimethyl sulfoxide (DMSO).

High-performance thin layer chromatography (HPTLC) finger printing

HPTLC fingerprinting of ES-DCM was carried out. Briefly, samples (10 μ L) were separately applied on Silica gel 60 F₂₅₄ precoated HPTLC plates, 10 cm \times 10 cm (Merck, Germany) with the help of Camag Linomat-V applicator and eluted the plate to a distance of 8.5 cm at room temperature (25°C) in solvent system, toluene: Ethyl acetate: Formic acid (6:3:1). Sample solutions were applied on 6 \times 0.45 mm wide band using Camag Linomat-V automated TLC applicator. For the detection and quantitation; after sample application, plates were developed in a Camag twin through glass tank presaturated with the mobile phase; toluene: Ethyl acetate: Formic acid (6:3:1) for 20 min, the plate was developed horizontally in Camag horizontal developing chamber

at the room temperature. The plate was observed under ultraviolet (UV)-366 nm in Camag UV cabinet and the HPTLC fluorescence image was documented. The corresponding digital scanning profiling was carried out with a Camag TLC scanner III, and the documentation of chromatograms was carried out with digital camera.

The HPTLC fingerprinting of the bio-active ES-DCM revealed sharp, symmetrical, and well-resolved peaks in the solvent system of toluene: Ethyl acetate: Formic acid (6:3:1) [Supplementary Figure 1]. ES-DCM show presence of six major peaks including the standard phytochemical, lupeol (Peak No. 4, R_f 0.60, 10.37%). UV spectra measured for the bands showed maximum absorbance at 366 nm, hence, this was chosen as the wavelength for the UV densitometry [Supplementary Figure 1].

Drugs and chemicals

Dulbecco's modified eagle medium (DMEM), Trypsin-ethylenediaminetetraacetic acid, Hank's balanced salt solution (HBSS), Sulforhodamine B (SRB), Doxorubicin (Dox), acridine orange (AO), ethidium bromide (EB), Hoechst 33342 (bisbenzimidazole trihydrochloride), propidium iodide (PI), RNase A, Rhodamine 123 (Rho-123), verapamil (Ver), mitoxantrone (MXR), and Fumitremorgin C (FTC) were purchased Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco, Invitrogen, USA. DMSO was purchased from calbiochem.

Cell culture

Human cervical carcinoma (HeLa), A549 (human lung adenocarcinoma), MCF-7, BT-474, MDA-MB-231 (human

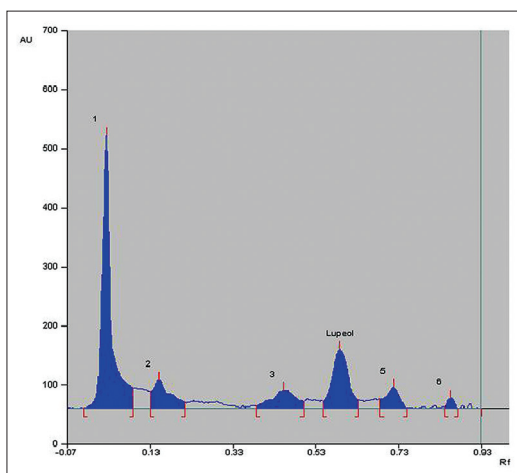
breast carcinoma), and Caco-2 (human colon carcinoma) were cultured in DMEM supplemented with FBS (10%), penicillin (100 I.U./mL), and streptomycin (100 µg/mL) in a humidified 5% CO₂ incubator at 37°C. Cells were cultured in healthy condition and exponentially growing cells (~70% confluency) were used for experiments.

Cytotoxicity assay

Cytotoxic effects of ES-ET, ES-PET, ES-DCM, ES-BT, and ES-R were evaluated using SRB assay as described before.^[12] Briefly, HeLa (6,000/100 µL), A549 (10,000/100 µL), MCF-7, BT-474, MDA-MB-231 (14,000/100 µL), and Caco-2 (12,000/100 µL) cells were seeded in 96 well microtiter tissue culture plates and cultured for 24 h. Cells were then treated with ES-ET, ES-PET, ES-DCM, ES-BT, and ES-R (25, 50, 100, and 200 µg/ml) for 48 h in triplicates (defined concentrations of ES-ET, ES-PET, ES-DCM, ES-BT, and ES-R were freshly prepared in culture media by serial dilution). Doxorubicin (anticancer drug) and DMSO were used as a positive control and vehicle control, respectively. At the end of the treatment, cells were fixed with 10% w/v of trichloroacetic acid (100 µL) for 1 h at 4°C. The plates were then washed with deionized water and air-dried. Samples were stained with 100 µL of SRB solution (in 0.4% w/v in acetic acid) for 30 min at RT. The plate was then washed with acetic acid (1.00%) and air dried. Tris-base (10 mM, 100 µL, pH 10) was added to each well for solubilization. Optical density (O.D) values were measured at 540 nm with a reference wavelength of 630 nm using microtiter plate reader (Biotek EL ×800-MS). Dose response curve was generated by plotting percentage cell viability (O.D of test/O.D of vehicle control ×100) in y-axis against the concentration (µg/mL) in the x-axis. Inhibitory concentration (IC₅₀) values for ES-ET, ES-PET, ES-DCM, ES-BT, and ES-R were determined by nonlinear regression (curve fit) analysis.

Acridine orange-ethidium bromide assay and Hoechst 33342 assay

The induction of apoptosis after treatment with the enriched fraction of ES (ES-DCM) was detected by AO-EB and Hoechst 33342 staining as described earlier.^[13,14] Briefly, HeLa, A549, MCF-7, and Caco-2 cells (4×10^5) seeded in a six-well tissue culture plate for 24 h were treated with ES-DCM and doxorubicin (positive control) corresponding to their IC₅₀ values [Table 1] for 48 h. DMSO was used as vehicle control. At the end of the treatment, adherent cells were harvested by trypsinization and pooled with floating cells. Thereafter, cells were centrifuged at 2000 rpm for 5 min at RT, washed with HBSS twice. Cells were then stained with a mixture of AO-EB (2 µg/mL) for 10 min at 37°C. Both AO and EB intercalate with DNA, and preferentially stain the cell nuclei. AO is cell-permeant, allowing for visualization



Supplementary Figure 1: High-performance thin layer chromatography (HPTLC) densitogram of enriched fraction of *Elephantopus scaber* dichloromethane fraction (ES-DCM). ES-DCM was dissolved in methanol (1 mg/mL) and analyzed (10 µL) using HPTLC (Camag Linomat-V). Mobile phase used was toluene: Ethyl acetate: Formic acid (6:3:1); detection wavelength at 366 nm; band length at 6 mm; and the solvent front at 85 mm

Table 1: Cytotoxic effects of ES-ET, ES-PET, ES-DCM, ES-BT, and ES-R in human epithelial cancer cells

Groups	IC ₅₀ (μg/mL)					
	HeLa	A549	MCF-7	BT-474	MDA-MB-231	Caco ₂
ES-ET	89.85±3.35	83.79±4.70	78.22±2.36	83.54±1.82	66.02±4.19	69.55±2.24
ES-PET	176.10±9.91	107.30±4.07	138.8±6.00	117±5.28	104.83±5.66	106.10±3.84
ES-DCM	18.01±0.73	45.08±1.99	18.33±1.18	32.76±0.69	36.69±1.24	40.28±1.15
ES-BT	411.70±30.94	594.30±29.40	359.70±28.66	591.50±22.46	324.90±19.63	1069±27.34
ES-R	802.90±29.90	1176±82.20	1545±131.72	1073±35.04	733.70±40.54	1038±48.11
Doxorubicin	0.72±0.02	0.89±0.03	0.50±0.04	0.55±0.03	0.41±0.02	0.75±0.03

IC₅₀ (concentration of the drug required to reduce the percentage cell viability to 50) were obtained from the graph [Figure 1] by nonlinear regression analysis as best curve-fit values. Numerical data represent mean±S.E.M of three independent experiments. ES-ET: *Elephantopus scaber* ethanolic extract; ES-PET: *Elephantopus scaber* petroleum ether fraction; ES-DCM: *Elephantopus scaber* dichloromethane fraction; ES-BT: *Elephantopus scaber* n-butyl alcohol fraction; ES-R: *Elephantopus scaber* rest fraction; SEM: Standard error of mean; IC₅₀: Inhibitory concentration 50%

of nuclear structure in living cells. EB is excluded from living cells. When cells die and their plasma membrane ruptures, EB reaches the nucleus and stains orange-red. Therefore, in this assay, uniformly stained green nuclei indicate live cells and uniformly stained orange-red nuclei indicate necrosis. Whereas, green or orange-red nuclei with condensed chromatin/fragmented DNA indicates the apoptotic cells.

Similarly, cells treated with ES-DCM were also stained with Hoechst 33342 (2 μg/ml) for 10 min at 37°C. At the end of staining, cells were washed with ice-cold HBSS twice, and the pellet was re-suspended in 100 μl of HBSS. The cells were mounted on a glass slide with a cover slip and viewed under a fluorescent microscope (Leica, Germany). Live cells show uniform faint blue staining while the condensed nuclei of apoptotic cells show bright fluorescence.

Cell cycle analysis

Cell cycle analysis was performed by PI based measurements of the DNA content of the cells by flow cytometry.^[15] Briefly, HeLa, A549, MCF-7, and Caco-2 cells (4×10^5) seeded in a six-well tissue culture plate for 24 h were treated with ES-DCM and doxorubicin (positive control) corresponding to their IC₅₀ values [Table 1] for 48 h. DMSO was used as vehicle control. At the end of treatment, adherent cells were harvested by trypsinization and pooled with floating cells. Thereafter, cells were centrifuged at 2000 rpm for 5 min at RT, washed with HBSS twice and fixed with 70% ice cold ethanol and stored at -20°C overnight. The cells were then centrifuged at 3000 rpm for 5 min at RT, washed with HBSS twice and re-suspended in HBSS, and treated with RNase A (100 μg/mL, at 56°C for 3 h). PI (20 μg/ml) was then added and incubated at RT for 15 min. DNA content was analyzed by flow cytometry (Accuri C6, Becton Dickinson, USA).

Micronuclei (genotoxicity) assay

Micronuclei formation assay was carried out as described earlier.^[16] Briefly, HeLa, A549, MCF-7, and

Caco-2 cells (4×10^5) seeded in a six-well tissue culture plate for 24 h were treated with ES-DCM and doxorubicin (positive control) corresponding to their IC₅₀ values [Table 1] for 48 h. DMSO was used as vehicle control. At the end of treatment, adherent cells were harvested by trypsinization and pooled with floating dead cells. Cells were centrifuged at 2000 rpm for 5 min at RT, followed by a mild hypotonic (0.56% potassium chloride) treatment for 2 min, fixed in Carnoy's fixative (methanol: Acetic acid, 3:1), and stored at -20°C overnight. The cells were then centrifuged at 2000 rpm for 5 min at 4°C, re-suspended fixative, mounted on the slides, stained with AO in Sorensen's buffer (0.01%), and washed twice with HBSS. Micronuclei (bi/tri-nucleated) was detected using a fluorescent microscope (Leica, Germany).

Rhodamine 123 efflux assay

Multi-Drug Resistant-1 (MDR-1 or ABCB-1) inhibitory activity of ES-DCM was determined by Rhodamine (Rho) efflux assay using flow cytometry as described earlier.^[17] Briefly, HeLa and Caco-2 cells (1×10^6 cells/mL) in DMEM containing 2% FBS were incubated with Rho 123 (0.50 μM) at 37°C, CO₂ incubator for 30 min (accumulation phase). After centrifugation and removal of supernatant, cells were re-incubated in media with or without DMSO (vehicle control), Ver (50 μM), and ES-DCM (10 μg/mL) at 37°C, CO₂ incubator for an additional 60 min (efflux phase). At the end of incubation, cells were washed twice with ice-cold HBSS and analyzed by flow cytometry (Accuri C6, Becton Dickinson, USA). Mean fluorescence intensity (MFI) was determined for vehicle control (DMSO), Ver, and ES-DCM treated samples.

Mitoxantrone efflux assay

Breast Cancer Resistance Protein (BCRP or ABC-G2) inhibitory activity of ES-DCM was determined by MXR efflux assay using flow cytometry as described before.^[18] Briefly, A549 and MCF-7 cells (1×10^6 cells/mL) in media (DMEM with 2% FBS) were incubated with MXR (10 μM) at 37°C, CO₂ incubator for 30 min (accumulation phase). Cells were then washed with HBSS and re-incubated with DMSO (vehicle control),

FTC (10 μ M), and ES-DCM (10 μ g/mL) at 37°C, CO₂ incubator for an additional 60 min (efflux phase). At the end of incubation, cells were washed twice with ice-cold HBSS and analyzed by flow cytometer (Accuri C6, Becton Dickinson, USA). MFI was determined for vehicle control (DMSO), FTC, and ES-DCM treated samples.

Statistical analysis

All values are expressed as the mean \pm standard error of the mean. Data were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett's *post-hoc* multiple comparisons test or two way ANOVA using Bonferroni posttests. A value of $P < 0.05$ was considered to be statistically significant compared to the vehicle control treated group.

RESULTS

The enriched fraction of *Elephantopus scaber* possesses cytotoxicity against human epithelial cancer cells

Cytotoxic effects of ES-ET, ES-PET, ES-DCM, ES-BT, and ES-R were evaluated on cervical (HeLa), lung (A549) breast (MCF-7, BT-474, MDA-231), and colon (Caco-2) cancer cells using SRB assay. Doxorubicin (positive control), caused a significant cytotoxic effects in the cancer cells with an IC₅₀ values of 0.72 ± 0.02 μ g/mL,

0.89 ± 0.03 μ g/mL, 0.50 ± 0.04 μ g/mL, 0.55 ± 0.03 μ g/mL, 0.41 ± 0.02 μ g/mL, and 0.75 ± 0.03 μ g/mL against HeLa, A549, MCF-7, BT-474, MDA-MB-231, and Caco-2, respectively [Figure 1 and Table 1]. Similar to doxorubicin, the enriched fraction of ES (ES-DCM) possessed significant cytotoxic effects with dose-dependent reduction in the cell viability [Figure 1]. The IC₅₀ values for ES-DCM in HeLa, A549, MCF-7, BT-474, MDA-MB-231, and Caco-2 were found to be 18.01 ± 0.73 μ g/mL, 45.08 ± 1.99 μ g/mL, 18.33 ± 1.18 μ g/mL, 32.76 ± 0.69 μ g/mL, 36.69 ± 1.24 μ g/mL, and 40.28 ± 1.15 μ g/mL, respectively [Table 1]. ES-ET also possessed dose-dependent cytotoxic effects in the cancer cells [Figure 1]. However, ES-PET, ES-BT, and ES-R did not show any significant cytotoxic effects in the cancer cells (IC₅₀ > 100 μ g/mL) [Figure 1 and Table 1]. Thus, these data revealed that the enriched fraction of ES (ES-DCM), but not other fractions (ES-PET, ES-BT, and ES-R), show dose-dependent cytotoxic effects against human epithelial cancer cells. Hence, further studies unraveling the anti-cancer mechanism were undertaken with the most-active enriched fraction of ES (ES-DCM).

The enriched fraction of *Elephantopus scaber* induce apoptosis in human epithelial cancer cells

In order to assess the cytotoxic effects of ES-DCM was due to apoptosis or necrosis, cervical (HeLa), lung (A549) breast (MCF-7), and colon (Caco-2) cells were treated

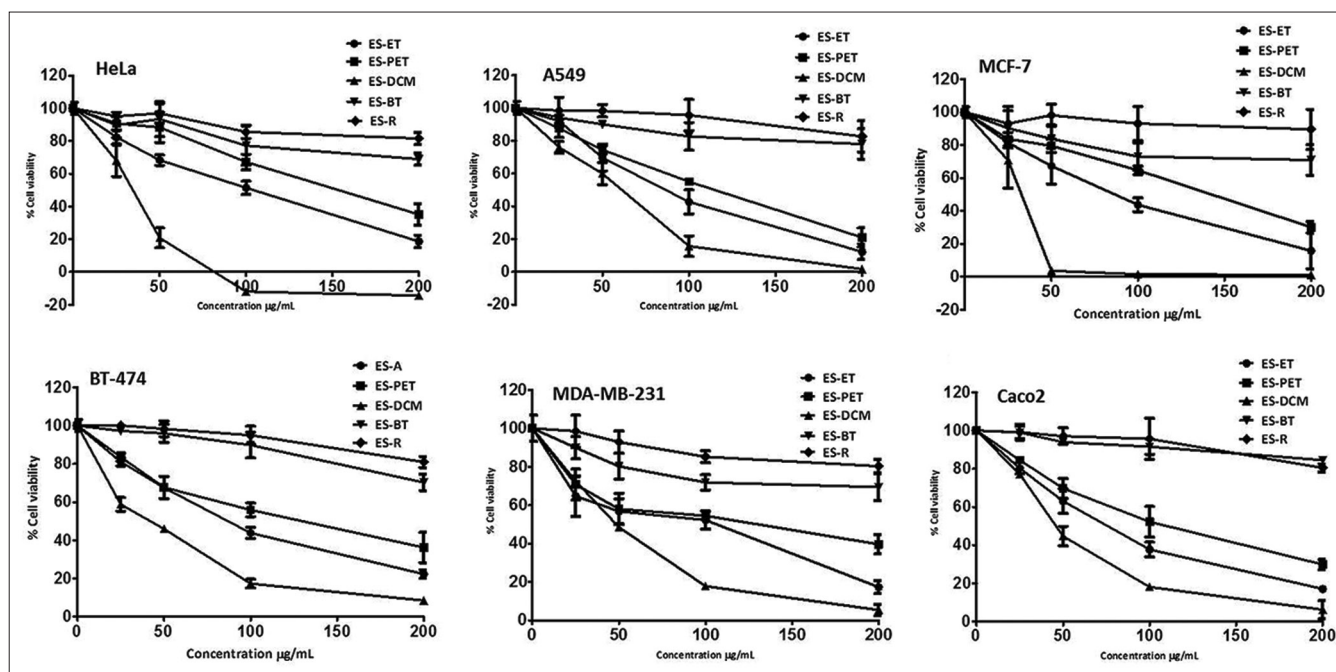


Figure 1: Dose-dependent cytotoxic effects of fractions of *Elephantopus scaber* (ES) in human epithelial cancer cells. Cervical carcinoma (HeLa), lung adenocarcinoma (A549), breast cancer cells (MCF-7, BT-474 and MDA-MB-231), and colon carcinoma (Caco₂) cells were treated with ES Ethanolic extract (ES-ET); ES Petroleum ether fraction (ES-PET), ES Dichloromethane fraction (ES-DCM), ES *n*-butyl alcohol fraction and ES Rest fraction for 48 h. At the end of the treatment, percentage cell viability was determined by sulforhodamine B assay. Each point represents the mean \pm standard error of the mean of three independent experiments performed in triplicates

with ES-DCM and doxorubicin (positive control) at their determined IC_{50} concentration [Table 1] for 48 h. At the end of the treatment, cells were subjected to AO-EB staining to score for apoptotic and necrotic cells using fluorescent microscopy. Homogeneously stained green nuclei with normal morphology in vehicle control (DMSO) treated cells indicates viable cells [Figure 2a]. However, treatment with doxorubicin show both green and orange-red colored condensed/fragmented nuclei indicating apoptosis [Figure 2a]. Similarly, treatments with ES-DCM caused the condensed nuclei (bright green), fragmented chromatin, and apoptotic bodies (orange-red) [Figure 2a]. We failed to detect any necrosis phenotype (uniformly stained orange-red nuclei). Thus, these data indicated that ES-DCM induced apoptosis, but not necrosis in cancer cells. Further, Hoechst 33342 assay using fluorescent microscopy corroborated the hall mark properties of apoptosis, such as membrane blebbing, condensed and fragmented nuclei upon treatment with ES-DCM [Figure 2b].

Cell cycle inhibitory effects of enriched fraction of *Elephantopus scaber* in human epithelial cancer cells

In order to evaluate the effects of ES-DCM on cancer cell cycle, cervical (HeLa), lung (A549) breast (MCF-7), and colon (Caco-2) cells were treated with ES-DCM

and doxorubicin (positive control) at their determined IC_{50} concentration [Table 1] for 48 h. At the end of the treatment, DNA content in G_0/G_1 phase, Synthetic (S) phase, G_2/M phase, and sub- G_0 (indicates apoptosis) were determined based PI staining using flow cytometry. Vehicle control (DMSO) treated cells showed a normal cell cycle profile [Figure 3]. However, treatment with doxorubicin imparted G_2/M arrest (blockade of mitosis) and increased sub- G_0 DNA content [Figure 3]. Similarly, treatment with ES-DCM caused a significant increase in the sub- G_0 phase, indicating apoptosis [Figure 3]. Thus, these results revealed that ES-DCM induced apoptosis, but no any cell cycle specific arrest (G_2/M or G_0/G_1 arrest) in cancer cells [Table 2].

The enriched fraction of *Elephantopus scaber* triggers micronuclei formation (genotoxicity) in human epithelial cancer cells

Formation of micronuclei is a hallmark of drug-induced genotoxicity.^[16] The genotoxic effects of ES-DCM on cancer cells were evaluated using micronuclei formation assay. Cervical (HeLa), lung (A549) breast (MCF-7), and colon (Caco-2) cells were treated with ES-DCM and doxorubicin (positive control) at their determined IC_{50} concentration [Table 1] for 48 h. At the end of the treatment, cells were harvested and analyzed for micronuclei formation using fluorescent microscopy. Vehicle control (DMSO)

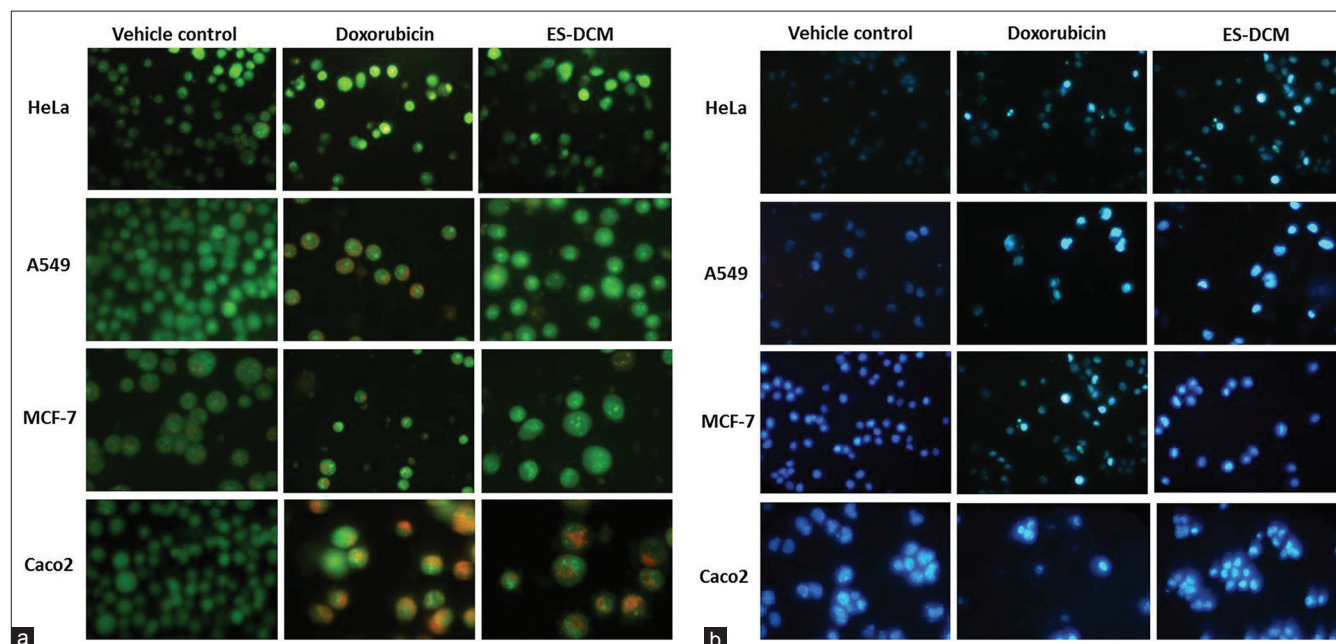


Figure 2: Apoptotic effects of enriched fraction of *Elephantopus scaber* (ES-DCM) in human epithelial cancer cells. Cervical carcinoma (HeLa), lung adenocarcinoma (A549), breast carcinoma (MCF-7), and colon carcinoma (Caco2) cells were treated with the enriched fraction of ES (ES-DCM) for 48 h. Dimethyl sulfoxide and doxorubicin were used as vehicle control and positive control, respectively. At the end of the treatment, cells were harvested and stained to detect apoptosis using fluorescence microscopy. (a) Acridine orange/ethidium bromide staining to distinguish between necrosis and apoptosis. Uniformly stained green colored nuclei indicate live cells; condensed or fragmented nucleus with green or orange-red color indicates the apoptosis. (b) Hoechst 33342 staining to detect apoptosis. Condensed and fragmented nuclei indicate the hall mark properties of apoptosis. The data are representative of three independent experiments

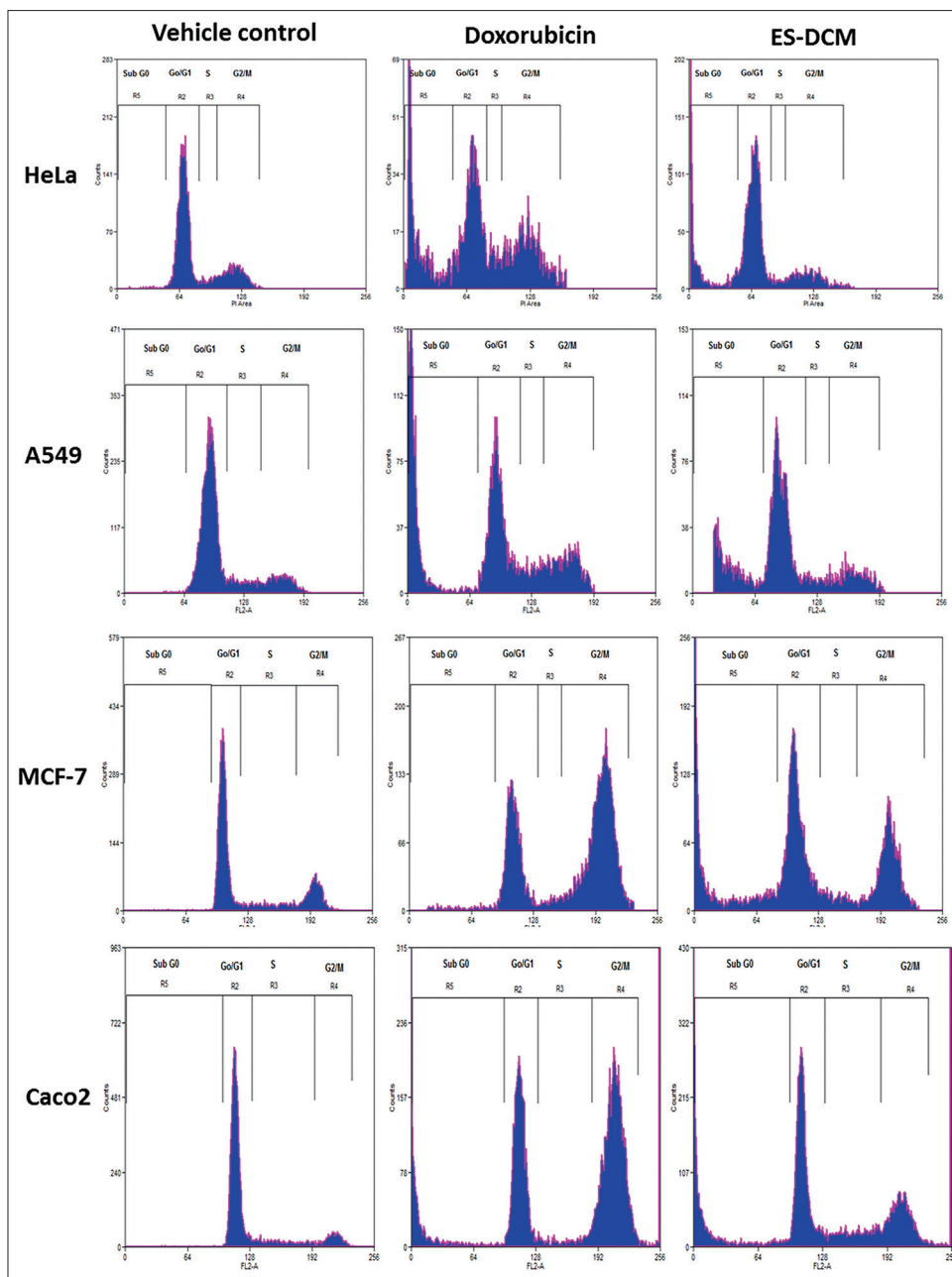


Figure 3: Cell cycle inhibitory effects of enriched fraction of *Elephantopus scaber* (ES-DCM) in human epithelial cancer cells. Cervical carcinoma (HeLa), lung adenocarcinoma (A549), breast carcinoma (MCF-7), and colon carcinoma (Caco₂) cells were treated with the enriched fraction of ES (ES-DCM) for 48 h. Dimethyl sulfoxide and doxorubicin were used as vehicle control and positive control, respectively. At the end of the treatment, cells were harvested, stained with propidium iodide, and DNA content was analyzed using flow cytometry. Percentage DNA content in G₀/G₁, S, G₂M, and sub-G₀ were determined. The data represents mean \pm standard error of the mean of three independent experiments

treated cells showed normal morphology without any multi-nucleated phenotype [Figure 4]. However, treatment with doxorubicin revealed micronuclei formation, especially, bi-tri or multinucleated interphase cells. Similarly, treatment with ES-DCM led to the formation of bi-tri or multinucleated phenotypes indicating drug induced genotoxic effects in cancer cells [Figure 4]. Thus, the data revealed that ES-DCM triggered chromosomal damage and genome instability in cancer cells.

The enriched fraction of *Elephantopus scaber* inhibits multi-drug resistant transporters

Next, we have investigated the inhibitory effects of ES-DCM against MDR transporters, particularly, MDR-1 (ABC-B1) and BCRP (ABC-G2) using a functional assay (based on drug efflux activity) using flow cytometry.^[17,18] Rhodamine 123 (Rho-123) and MXR (fluorescent substrates of MDR-1 and BCRP, respectively) efflux assays have been used to

Table 2: Cell cycle inhibitory effects of the enriched fraction of ES-DCM in human epithelial cancer cells

Groups	HeLa				A549			
	Sub G ₀ phase	G ₀ /G ₁ phase	S phase	G ₂ /M phase	Sub G ₀ phase	G ₀ /G ₁ phase	S phase	G ₂ /M phase
Vehicle control	1.26±0.05	68.99±6.20	6.32±0.25	23.91±2.50	0.60±0.02	73.90±8.50	11.14±0.90	14.88±1.22
Doxorubicin	27.63±2.22***	35.92±4.20	6.71±0.19	29.92±3.22	29.47±3.10**	35.21±2.90	7.53±0.50	18.53±2.60
ES-DCM	21.69±1.90***	58.54±5.50	3.27±0.50	16.71±1.80	22.82±0.90***	54.43±6.20	6.13±0.30	16.59±2.20
	MCF-7				Caco-2			
	Sub G ₀ phase	G ₀ /G ₁ phase	S phase	G ₂ /M phase	Sub G ₀ phase	G ₀ /G ₁ phase	S phase	G ₂ /M phase
Vehicle control	0.60±0.03	64.20±3.50	13.15±1.10	22.39±2.70	0.59±0.02	74.99±8.00	11.38±0.80	13.36±2.10
Doxorubicin	2.81±0.07	30.60±3.70	3.33±0.70	62.89±5.50***	9.83±0.10**	27.69±2.40	4.09±0.20	45.85±3.12***
ES-DCM	22.50±2.30***	37.13±3.80	6.36±0.80	26.05±3.50	17.63±0.90***	33.92±5.40	11.80±0.85	16.69±2.70

DNA content in G₀/G₁, S, G₂/M, and sub-G₀ were determined based on PI staining using flow cytometry. Numerical data are means±S.E.M. of three independent experiments (figure 3). The *P* value indicates statistical significance for treated groups compared to the vehicle control (****P*<0.001; ***P*<0.01) determined by two-way ANOVA using Bonferroni posttests. PI: Propidium iodide; SEM: Standard error of mean; ES-DCM: *Elephantopus scaber* dichloromethane fraction

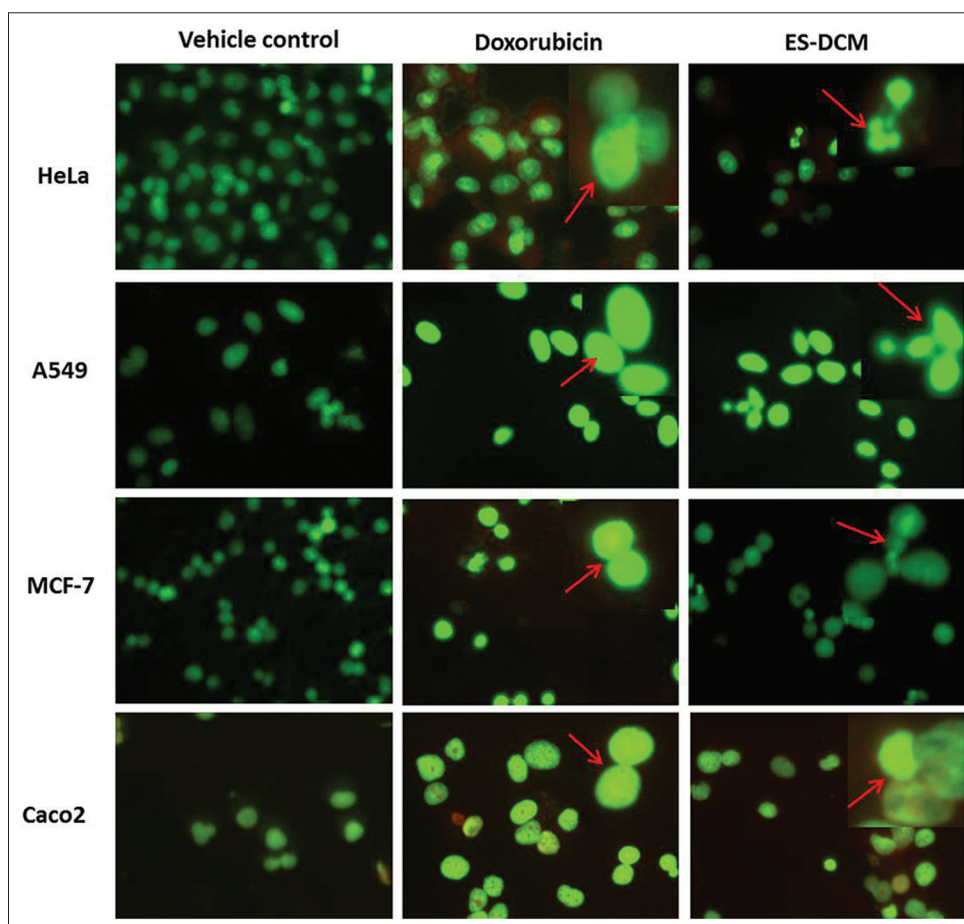


Figure 4: Genotoxicity of enriched fraction of *Elephantopus scaber* (ES-DCM) in human epithelial cancer cells. Cervical carcinoma (HeLa), lung adenocarcinoma (A549), breast carcinoma (MCF-7), and colon carcinoma (Caco₂) cells were treated with enriched fraction of *Elephantopus scaber* (ES-DCM) for 48 h. Dimethyl sulfoxide and doxorubicin were used as vehicle control and positive control, respectively. At the end of the treatment, cells were harvested and assessed for the frequency of micronuclei formation (micronucleated bi/tri-nucleate cells). The data are representative of three independent experiments

quantitatively assess the MDR transporters inhibitory potential of ES-DCM. First, Rho-123 and MXR efflux assays were carried out in cervical (HeLa), lung (A549) breast (MCF7), and colon (Caco-2) cells to assess the functional activity of MDR transporters. Data revealed that HeLa and Caco-2 cells express MDR-1, whereas, A549 and MCF-7 harbor expression of BCRP (data not

shown). Accordingly, we set up Rho-123 efflux assay (in HeLa and Caco-2) and MXR efflux assay (in A549 and MCF-7) to quantitatively assess MDR-1 and BCRP inhibitory activity of ES-DCM. Hence, HeLa, A549, MCF-7, and Caco-2 cells were treated with ES-DCM in the efflux phase. Ver and FTC were used positive control for MDR-1 and BCRP, respectively.

Rho-123 efflux assay revealed that, the MFI of Rho-123 in the efflux phase (with vehicle control, DMSO) was 177.60 ± 7.93 (in HeLa cells). However, in the presence of Ver, the MFI increased to 833.90 ± 17.10 , indicating an increased intracellular accumulation of Rho-123 owing to the inhibition of MDR-1 mediated drug efflux [Figure 5a]. Similar to Ver, the treatment with ES-DCM also increased the MFI to 497.60 ± 13.65 [Figure 5a] suggesting that it may function to inhibit MDR-1 mediated drug efflux. Similar result was obtained with Caco-2 cells [Figure 5b]. Compared to Ver (Relative inhibition), ES-DCM contained ~60% and ~67%, inhibition of MDR-1 (ABC-B1) activity in HeLa and Caco-2, respectively [Figure 5a and b]. Similarly, MXR efflux assay revealed that the MFI of MXR in the efflux phase (with vehicle control) was 46.29 ± 1.88 (in A549 cells). However, in the presence of FTC, the MFI enhanced to 91.52 ± 3.38 , indicating an enhanced intracellular accumulation of MXR due to the inhibition of BCRP mediated drug efflux [Figure 6a]. Similar to FTC, treatment with ES-DCM enhanced the MFI to 75.76 ± 3.29 suggesting that it may also function to inhibit BCRP activity [Figure 6a]. Similar data was obtained with MCF-7 cells [Figure 6b]. Compared to FTC (Relative inhibition), ES-DCM possessed ~82%

and ~78% inhibition of BCRP (ABC-G2) in A549 and MCF-7, respectively [Figure 6a and b]. Thus, these data suggested that the enriched fraction of ES possessed MDR modulating properties, particularly by inhibiting MDR-1 (ABC-B1) and BCRP (ABC-G2) transporters.

DISCUSSION

Medicinal plant extracts played an important role in the drug discovery and formed the basis of most early medicines. Natural products drug discovery has led to the isolation of highly active anti-cancer agents. The current study investigated the anti-cancer properties of indigenous medicinal plant, *Elephantopus scaber*, against various human epithelial cancer cells, such as cervical (HeLa), lung (A549), breast (MCF-7), and colon (Caco-2). A methodical screening for cytotoxicity revealed that the ES-ET possessed dose-dependent decrease in the cell viability in cancer cells. The IC_{50} (concentration of the drug required to reduce the percentage cell viability to 50) of ES-ET was found to be 66–89 $\mu\text{g/mL}$. An $IC_{50} < 100 \mu\text{g/mL}$ for medicinal plant extracts are considered as potential anti-cancer agents and recommended for further study.^[19] Hence,

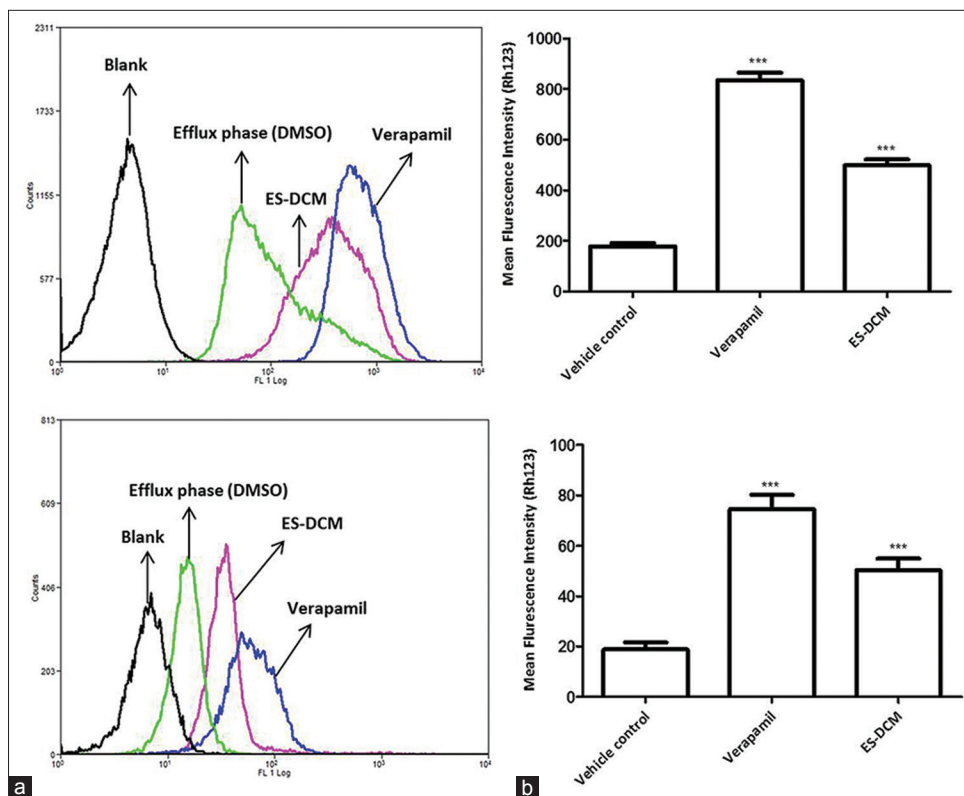


Figure 5: MDR-1 (ABC-B1) inhibitory effects of enriched fraction of *Elephantopus scaber* (ES-DCM) in human epithelial cancer cells. HeLa (a) and Caco-2 (b) cells were incubated with rhodamine 123 (Rho-123) in Dulbecco's modified eagle medium with 2% fetal bovine serum for 30 min at 37°C (accumulation phase), after which excess dye was washed and cells were re-incubated with vehicle control (dimethyl sulfoxide), verapamil, and ES-DCM for 60 min at 37°C (efflux phase). The mean cellular Rho-123 fluorescence in the efflux phase was analyzed by flow cytometry. Error bars represent \pm standard error of the mean; $n = 3$, *** $P < 0.001$

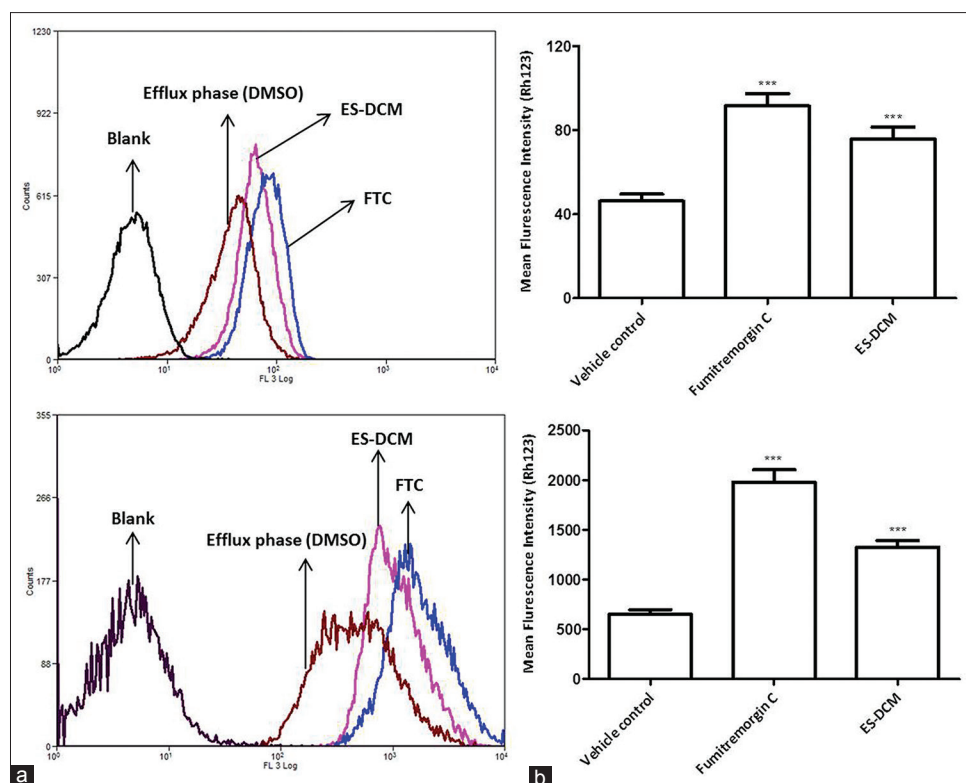


Figure 6: ABC-G2 (BCRP) inhibitory effects of enriched fraction of *Elephantopus scaber* (ES-DCM) in human epithelial cancer cells. A549 (a) and MCF-7 (b) cells were incubated with mitoxantrone (MXR) in Dulbecco's modified eagle medium with 2% Fetal bovine serum for 30 min at 37°C (accumulation phase), after which excess dye was washed and cells were re-incubated with vehicle control (dimethyl sulfoxide), fumitremorgin C (FTC), and ES-DCM for 60 min at 37°C (efflux phase). The mean cellular MXR fluorescence in the efflux phase was analyzed by flow cytometry. Error bars represent \pm standard error of the mean; $n = 3$, *** $P < 0.001$

according to the bio-activity guided fractionation protocol, the bio-active ES-ET was further fractionated successively with solvents of increasing order of polarity index, such as petroleum ether (PET), dichloromethane (DCM), *n*-butyl alcohol (BT), and methanol-water to yield ES-PET, ES-DCM, ES-BT, and ES-R, respectively. Subsequently, the cytotoxic effects of these fractions were evaluated using standard cytotoxicity assay (SRB assay). Interestingly the data revealed that the ES-DCM possessed significant cytotoxic effects in cancer cells with an IC_{50} of 18–45 $\mu\text{g/mL}$. However, the fractions such as, ES-PET, ES-BT, ES-R did not show any significant cytotoxicity. These results indicate that ES-DCM may harbor the bio-active phytochemicals responsible for the anti-cancer activity. STLs are a large and diverse group of natural compounds present in the medicinal plants, especially Asteraceae family.^[20] Pharmacological activity described for STLs mainly include anti-microbial, anti-viral, anti-inflammatory, and anti-tumor.^[21] In recent years, the anti-cancer potential of STLs has attracted a great deal of interest. Several groups have reported that ES contain STLs, particularly, germacranolide type (elephantopin and deoxy-elephantopin).^[10] Therefore the bio-active

ES-DCM was considered as the enriched fraction of ES (bearing STLs) and accordingly it was further selected to elucidate the mechanism of anti-cancer activity. Anti-cancer drugs typically induce apoptosis in cancer cells, and agents that are capable of inducing apoptosis in cancer cells have received considerable interest in the development of novel cancer chemotherapeutic drugs.^[14] Hence, the current study also addressed the question of whether ES-DCM mediated cytotoxic effects were due to apoptosis. AO-EB assay and Hoechst 33342 assay by fluorescent microscopy revealed that ES-DCM induced apoptosis, but not necrosis in cancer cells. Similarly, cell cycle analysis using flow cytometry revealed the hall mark properties of apoptosis, particularly increased sub- G_0 content. Genotoxicity assay indicated the micronuclei formation, particularly, bi/tri nucleated morphology confirming drug-induced genotoxicity in cancer cells. Thus, this study corroborated that the enriched fraction of ES induces apoptosis in human epithelial cancer cells.

Cancer cells can often develop resistant to a single drug or a class of drugs with a similar mechanism of action. After selection for resistance to a single drug, cells might also show cross-resistance to other

structurally and mechanistically unrelated drugs known as multi-drug resistance (MDR) and is often associated with the over-expression of ABC transporters, a family of trans-membrane protein pumps, particularly, ABC-B1 (MDR-1) and ABC-G2 (BCRP) which can enhance efflux of a various chemicals structurally unrelated at the expense of ATP depletion, resulting in decrease of the intracellular cytotoxic drug accumulation.^[17] MDR is a major obstacle in successful cancer therapy and reversal of MDR by inhibiting the ABC transporters is one of the key strategies to eliminate drug resistance.^[22] Typically, clinical trials have attempted to overcome MDR through combination therapies in which an anticancer was given along with ABC-transporter inhibitors. However, such studies involving the use of MDR transporters inhibitors (Ver, fumitremorgin C, MK571 etc.) was mainly unsuccessful owing to drug interactions, systemic toxicity, and nonspecificity, etc.^[8,22] Natural products show great promise as anti-MDR agents. Recently, several studies reported the use of plant extracts in modulating MDR and identifying new plant-derived molecules. Indeed, various plant-derived natural products such as, curcumin, epigallocatechin gallate, quercetin, silymarin, resveratrol, naringenin, daidzein, tetrahydrocurcumin, and hesperetin reported for its inhibitory functions against MDR transporters (ABC-B1 and ABC-G2).^[23] We have investigated the MDR-reversal properties of bio-active ES-DCM. Flow cytometry based functional assay have been used for quantitative measurements of MDR transporters in cancer cells.^[17] Fluorescent substrates (Rho-123 for ABC-B1 and MXR for ABC-G2) and inhibitors of ABC transporters (Ver for ABC-B1 and FTC for ABC-G2) were used to quantitatively assess the MDR transporter activity.^[17,18] When a fluorescence substrate diffuses into the cell, ABC-B1 or ABC-G2 actively pumps out the fluorochrome (efflux phase). If an inhibitor of ABC-B1 or ABCG2 is present at the same time along with the fluorescence substrate, the fluorescence marker accumulates in the cell, thus resulting in a higher intensity of fluorescence. Therefore, increased MFI in the presence of a specific fluorescent substrate and inhibitor pair for a specific ABC transporter measures the activity of that particular ABC transporter.^[17] The preliminary studies indicated that HeLa and Caco-2 cells contain ABC-B1, whereas, A549 and MCF-7 harbor ABC-G2. The potency of ES-DCM to inhibit ABC-B1 and ABC-G2 activity was assessed using Rho-123 efflux assay and MXR efflux assay, respectively. Our results revealed that ES-DCM significantly enhanced the intracellular accumulation of Rho-123 (in HeLa and Caco-2 cells) and MXR (in A549 and MCF-7 cells) indicating the inhibition of ABC-B1 and ABC-G2 mediated drug transport, respectively.

CONCLUSION

The current study revealed that indigenous medicinal plant, *Elephantopus scaber* Less. possessed cytotoxicity, triggered apoptosis, induced genotoxicity, and MDR-reversal properties in various human epithelial cancer cell lines. The enriched fraction of ES (ES-DCM) might be most beneficial as an anti-cancer agent for the effective treatment of several drug-resistant cancers. Further, purification of ES-DCM using modern analytical methods (Preparative HPLC or flash chromatography) may lead to the new anti-cancer molecules for an effective treatment of various epithelial cancers.

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