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Research Paper

The enriched fraction of *Vernonia cinerea* L. induces apoptosis and inhibits multi-drug resistance transporters in human epithelial cancer cells

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ABSTRACT

Ethnopharmacological relevance: *Vernonia cinerea* Less. (VC) of the family Asteraceaes is considered as the sacred plant; '*Dasapushpam*' which is ethnopharmacologically significant to the people of *Kerala* in India. In fact, VC has been used in the traditional system of medicine (Ayurveda) for the treatment of various ailments including cancer.

Materials and methods: Cytotoxicity of the ethanolic extract of VC (VC-ET), petroleum ether fraction (VC-PET), dichloromethane fraction (VC-DCM), n-butyl alcohol fraction (VC-BT), and rest fraction (VC-R) was evaluated in cervical carcinoma (HeLa), lung adenocarcinoma (A549), breast cancer (MCF-7), and colon carcinoma (Caco-2) cells using Sulforhodamine B (SRB) assay. The apoptotic effects of VC-DCM were assessed in cancer cells using Annexin V assay. The effects of VC-DCM on multi-drug resistance (MDR) transporters in HeLa, A549, MCF-7, and Caco-2 cells were evaluated using flow cytometry based functional assays. Similarly, drug uptake in cancer cells and sensitization of cancer cells towards chemotherapeutic drugs in the presence of VC-DCM were studied using Daunorubicin (DNR) accumulation assay and SRB assay, respectively.

Results: Cytotoxicity assay revealed that the enriched fraction of VC (VC-DCM) possessed dose-dependent cytotoxic effects in human epithelial cancer cells (HeLa, A549, MCF-7, and Caco-2). Further, treatment of cancer cells (HeLa, A549, MCF-7, and Caco-2) with VC-DCM led to a significant increase in both early and late apoptosis, indicating the induction of apoptosis. Interestingly, VC-DCM significantly inhibited functional activity of MDR transporters (ABC-B1 and ABC-G2), enhanced DNR-uptake in cancer cells, and sensitized cancer cells towards chemotherapeutic drug-mediated cytotoxicity, thus indicating the ability of VC-DCM to reverse MDR in cancer and enhance the cytotoxic effects of anticancer drugs.

Conclusions: A methodological investigation on the anti-cancer properties of *Vernonia cinerea* Less. (VC) revealed that an enriched fraction of VC (VC-DCM) possessed cytotoxic effects, triggered apoptosis, inhibited MDR transporters, enhanced drug uptake, and sensitized cancer cells towards anticancer drug-mediated cytotoxicity in human epithelial cancer cells. Thus, VC appears to be promising for an effective treatment of various drug-resistant human epithelial cancers.

1. Introduction

Cancer is one the leading causes of death world-wide (Boffetta et al., 2014). Recent advances in cancer treatment led to the increased survival rate; however, a convincing cure remains elusive. Medicinal plants have been used for the treatment of cancer since time immemorial (Gurib-Fakim, 2006). Ayurveda, a traditional sect of Indian medicinal plants describes the use of medicinal plants for the prevention and treatment of cancer (Balachandran and Govindarajan, 2005). Recently, a greater significance has been attributed to ethnomedicine, involving the use of medicinal plants for effective treatment of cancer (Cordell, 2012). *Vernonia cinerea* Less. (VC) of the family Asteraceae, widely

Abbreviations: ATP, Adenosine triphosphate; ABC, ATP binding cassette; DMSO, Dimethyl Sulfoxide; DMEM, Dulbecco's modified eagle medium; Dox, Doxorubicin; DNR, Daunorubicin; FBS, Fetal bovine serum; FITC, Fluorescein Isothiocyanate; FTC, Fumitremorgin C; HBSS, Hank's Balanced Salt Solution; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); SRB, Sulforhodamine B; MDR, multi-drug resistance; O.D., Optical Density; PI, Propidium Iodide; RT, Room temperature; VC, *Vernonia cinerea* Less.; VC-ET, VC ethanolic extract; VC-PET, VC petroleum ether fraction; VC-DCM, VC dichloromethane fraction; VC-BT, VC n-butyl alcohol fraction; VC-R, rest fraction; HPTLC, High Performance Thin Layer Chromatography

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grows as a weed, and considered as a sacred plant, known as '*Dasapushpam*' is medicinally significant to the people of *Kerala* in India (Varghese et al., 2010; Arun Raj et al., 2013b). In a traditional system of medicine, VC has been used for the treatment of various diseases including cancer (Bajpai et al., 1995; Kala, 2005; Varghese et al., 2010; Rajamurugan et al., 2011; Pratheeshkumar and Kuttan, 2012; Arun Raj et al., 2013a; Toyang and Verpoorte, 2013; Nataru et al., 2014). The chemical investigations suggest that VC contains triterpenes, sterols, phenolic resin, and sesquiterpene lactones (Pratheeshkumar and Kuttan, 2010; Varghese et al., 2010). The preliminary studies suggest that the ethanolic extract of VC and isolated sesquiterpene lactones (Vernolide A and B) possess cytotoxicity in cancer cells (Kuo et al., 2003). However, a detailed investigation on anti-cancer effects of VC remains unknown.

Induction of apoptosis in cancer cells is an important strategy in the development of anti-cancer agents (Wong, 2011). Chemotherapy involves the use of anti-cancer drugs for the treatment of cancer. However, the major limitation of chemotherapy is the development of multi-drug resistance (MDR) upon treatment (Gottesman et al., 2002). ABC transporters (ATP-dependent trans-membrane efflux pumps) particularly, ABC-B1 (MDR-1 or p-glycoprotein) and ABC-G2 (BCRP) actively extrude the cytotoxic drugs (drug efflux) by utilizing ATP leading to the MDR in cancer (Gillet and Gottesman, 2011). Natural products with the potency to target MDR in cancer have drawn considerable attention in anti-cancer drug development (Szakács et al., 2006). The current study investigated the cytotoxic effects of various fractions of VC in cervical (HeLa), lung (A549), breast (MCF-7), and colon (Caco-2) cancer cells using bio-activity guided fractionation. The most bio-active enriched fraction (VC-DCM) was evaluated for induction of apoptosis in human epithelial cancer cells (HeLa, A549, MCF-7, and Caco-2). Further, we have investigated the potency of VC-DCM to inhibit MDR transporters (ABC-B1 and ABCG-2) and the effects of VC-DCM on drug uptake (DNR accumulation) in human epithelial cancer cells. Similarly, the ability of VC-DCM to enhance the cytotoxic effects of anticancer drugs was studied.

2. Materials and methods

2.1. Plant material

The whole plant of *Vernonia cinerea* Less. (VC) of the family Asteraceae was collected from Manipal, 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.

2.2. Preparation of plant extract and fractions

Dried and coarsely powdered plant material of VC (2 kg) was extracted with absolute ethanol for 72 h using soxhlet apparatus. The extract was filtered, concentrated in a rotary evaporator *in vacuum*, and completely dried to yield VC ethanolic extract (VC-ET). The practical yield of VC-ET was 7.20%. The VC-ET (100 g) dispersed in methanol:water (1:4) was fractionated successively with solvents of increasing polarity index to yield VC-PET (VC-petroleum ether fraction), VC-DCM (VC-dichloromethane fraction), VC-BT (VC-n-butyl alcohol fraction), and rest fraction (VC-R). Each fraction was concentrated using rotary evaporator *in vacuum*, and completely dried. The yield of VC-PET, VC-DCM, VC-BT, and VC-R was 12.20%, 21.50%, 26.30%, and 38.00%, respectively. For biological assays, VC-ET, VC-PET, VC-DCM, VC-BT, and VC-R were dissolved in DMSO.

2.3. HPTLC finger printing

VC-DCM (10 μL) was applied on Silica gel 60 F_{254} pre-coated HPTLC plates (10 cm × 10 cm) with Camag Linomat-V applicator (6 × 0.45 mm^2 wide band) and eluted the plate to a distance of 8.5 cm at 25 °C in a solvent system of toluene:ethyl acetate:formic acid (6:3:1). Plates were developed in a Camag twin through glass tank pre-saturated with the mobile phase; toluene:ethyl acetate: formic acid (6:3:1) for 20 min at 25 °C. For the detection and quantitation, plate was observed under 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 using a digital camera.

2.4. Drugs and chemicals

Dulbecco's modified eagle medium (DMEM), Trypsin-EDTA, Hank's Balanced Salt Solution (HBSS), Sulforhodamine B (SRB), Doxorubicin (Dox), Daunorubicin (DNR), Cisplatin, Annexin V-FITC, Propidium Iodide (PI), Rhodamine 123 (Rho-123), verapamil, Mitoxantrone (MXR), and Fumitremorgin C (FTC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco, Invitrogen, USA. Dimethyl Sulfoxide (DMSO) was purchased from Calbiochem.

2.5. Cell culture

HeLa (human cervical carcinoma), A549 (human lung adenocarcinoma), MCF-7 (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% Carbon dioxide (CO_2) incubator at 37 °C.

2.6. Cytotoxicity assay

Cytotoxicity of VC-ET, VC-PET, VC-DCM, VC-BT, and VC-R was evaluated using Sulforhodamine B (SRB) assay (Skehan et al., 1990). Briefly, HeLa (6000/100 μL), A549 (10,000/100 μL), MCF-7 (12,000/100 μL), and Caco-2 (10,000/100 μL) cells were cultured in 96 well microtiter tissue culture plates for 24 h. Cells were treated with VC-ET, VC-PET, VC-DCM, VC-BT, and VC-R (25, 50, 100, and 200 μg/ml, freshly prepared in culture media by serial dilution) for 48 h. Doxorubicin (Dox) 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 Room temperature (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 (nm) with a reference wavelength of 630 (nm) using microtiter plate reader (Biotek ELx800 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 concentration (μg/mL) in the *x*-axis. Cell viability in vehicle control was normalized to 100. IC_{50} values for VC-ET, VC-PET, VC-DCM, VC-BT, and VC-R were obtained from the graph as the concentration which decreases percentage cell viability to 50% using non-linear regression (curve fit) analysis.

In order to assess the ability of VC-DCM to enhance the effects of anticancer drugs, HeLa cells were treated with varying concentrations (0.25 μM, 0.50 μM, and 1.00 μM) of Dox with or without VC-DCM (20 μg/mL) in 96 well microtiter tissue culture plates for 48 h. Similarly, A549 cells were treated with varying concentrations (0.25 μM, 0.50 μM, and 1.00 μM) of MXR with or without VC-