

RESEARCH COMMUNICATION

Cytotoxicity, Apoptosis Induction and Anti-Metastatic Potential of *Oroxylum indicum* in Human Breast Cancer Cells

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Abstract

Despite clinical advances in anticancer therapy, there is still a need for novel anticancer metabolites, with higher efficacy and lesser side effects. *Oroxylum indicum* (L.) Vent. is a small tree of the Bignoniaceae family which is well known for its food and medicinal properties. In present study, the chemopreventive properties of *O. indicum* hot and cold non-polar extracts (petroleum ether and chloroform) were investigated with MDA-MB-231 (cancer cells) and WRL-68 (non-tumor cells) by XTT assay. All the extracts, and particularly the petroleum ether hot extract (PHO), exhibited significantly ($P < 0.05$) higher cytotoxicity in MDA-MB-231 when compared to WRL-68 cells. PHO was then tested for apoptosis induction in estrogen receptor (ER)-negative (MDA-MB-231) and ER-positive (MCF-7) breast cancer cells by cellular DNA fragmentation ELISA, where it proved more efficient in the MDA-MB-231 cells. Further, when PHO was tested for anti-metastatic potential in a cell migration inhibition assay, it exhibited beneficial effects. Thus non-polar extracts of *O. indicum* (especially PHO) can effectively target ER-negative breast cancer cells to induce apoptosis, without harming normal cells by cancer-specific cytotoxicity. Hence, it could be considered as an extract with candidate precursors to possibly harness or alleviate ER-negative breast cancer progression even in advanced stages of malignancy.

Keywords: Cancer specific cytotoxicity - apoptosis - *Oroxylum indicum* - ER negative breast cancer - ELISA - anti-

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Introduction

Globally, breast cancer is one of the most common cancers among women and its metastatic malignancy is being a major cause of mortality since years (Venkatesan et al., 2011; Kimman et al., 2012). Resistance to most of the available anticancer agents such as anthracyclines and taxanes, and its increasing incidence are the foremost obstacle in current breast cancer therapy (Valero and Hortobagyi, 2003; Lodha et al., 2011). This ultimately necessitates an unmet need for novel therapies of breast cancer yet in its advanced malignancy (Kwiecinski et al., 2008; Meiyanto et al., 2012).

Medicinal plants as a natural resource have received considerable attention in recent years as potential chemotherapeutic agents (Dwivedi et al., 2011). The use of plants as medicines is probably as old as Human kind itself. More than 150 000 plant species have been studied and many of them contain therapeutic substances (Loc & Kiet, 2011). About 80% of the world population in third world countries relies almost exclusively on plant products for their primary health care (Mans et al., 2000). Several known metabolites possessing anticancer properties, such as flavonoids, terpenoids, alkaloids and phenylpropanoids were isolated from natural sources (Kintzios, 2006; Park et al., 2008).

Cytotoxic phytochemicals such as vinca alkaloids

or paclitaxel (Taxol) are often used in oncology as highly potent drugs and/or serve as model for synthetic compounds (Pandi et al., 2011; Huang et al., 2012). Drugs such as these have been customarily isolated as single plant extracts or fractions thereof or have been mixtures of fractions/extracts from different plants and used subsequent to their evaluation of safety and efficacy in model systems and humans (Dahiru et al., 2006). Tests in experimental systems (*in vitro* and *in vivo*) have demonstrated that most of the phytochemicals act by interfering with several cell signaling pathways and lead to cell cycle arrest and/or differentiation induction (Chathoth et al., 2008) apart from their apoptosis-inducing potential.

Apoptosis is a central event essential to maintain tissue homeostasis for all organ systems in the human body (Thongrakard & Tencomnao, 2010). Suppression of apoptosis in carcinogenesis plays a central role in the development and progression of cancer. Tumor cells use a variety of molecular mechanisms to suppress apoptosis (Elmore, 2007). Hence, induction of apoptosis in tumor cells is a specific therapeutic approach towards cancer chemotherapy.

There have been a plethora of reports in the scientific literature documenting the chemopreventive potential of phytochemicals such as Lupulone, Hesperidin and blueberry phytochemicals in various cancer cell lines like those from tumors in the colon and the breast (Park et al.,

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2008; Lamy et al., 2009; Adams et al., 2010). Further, studies have been done to demonstrate that the common unifying theme involves the activation of caspase-3, despite differences in the relative involvement of the upstream molecular players in the extrinsic and intrinsic pathways of apoptosis with respect to Curcumin treatment (Gao et al., 2005; Takai et al., 2012).

Oroxylum indicum (L.) Vent. (also known as Shivnak, Sonapatha, Shyonaka or Midnight horror) is a deciduous tree belonging to Bignoniaceae family characterized with few branches. Apart from its ability to treat several other ailments, the bark decoction of *O. indicum* has also been reported for its use in treating cancer, despite the lack of mechanistic information about this therapeutic modality (Mao, 2002). *O. indicum* bark extracts were furthermore reported to possess antiproliferative property on human breast cancer cells (Lambertini et al., 2004) and also anti-inflammatory activity (Laupattarakasem et al., 2003). However, such studies were performed using polar extracts unlike our study using non-polar extracts from the same plant. Previous phytochemical studies of *O. indicum* led to the identification of ellagic acid (Maitreyi et al., 2008), 5,7-Dihydroxy flavone (chrysin) (Babu et al., 2006), 5-hydroxy-8-methoxy-7-O- β -Dglucopyranuronosyl flavone (Nair & Joshi, 1979), Stigmast-5-en-3-ol (Rasadah et al., 1998), 5,6,7-trihydroxy flavone (baicalein) (Chen et al., 2003; Chen et al., 2005), 4',5-Dihydroxy-7-methoxy isoflavone (pratensol) (Polya, 2003), 3-(4-hydroxy phenyl)-2-propenoic acid and 3, 4', 5, 7-tetrahydroxy-flavonol (Islam et al., 2010). However, the antineoplastic effects of the crude extract of *O. indicum* have hitherto not been extensively studied and can have significance since there could be a synergy between the different phytochemical constituents, which may have been undetected in other studies. This study, to the best of our knowledge is the first of its kind to test the relative ability of stem bark non-polar extracts of *O. indicum*, to exhibit selective-cytotoxicity, apoptotic and anti-metastatic potentials.

Materials and Methods

Chemicals

Phenazine methosulfate (PMS) (also known as N-methylphenazonium methosulfate), L-15 (Leibovitz) cell culture medium (with L-glutamine) and MEM (minimal essential medium) cell culture medium (with Earle's salt, NEAA and L-glutamine) were purchased from Himedia Laboratories Pvt. Ltd. (India). XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cellular DNA fragmentation ELISA (Cat. No. 11 585 045 001) to determine apoptosis was procured from Roche Diagnostics, Germany. The remaining chemicals and solvents used were of standard analytical grade and HPLC grade, respectively.

Plant Material

O. indicum stem bark was collected in December, 2007, from their natural habitat in the Mundoor forest

range near Kanjikode (10° 47' North, 76° 47' East; 120 m above sea level), Palakkad district, Kerala, India. The plant was identified by Prof. R. V. Nair, Senior Botanist, Centre for Indian Medical Heritage (CIMH), Kanjikode, Palakkad, Kerala, India (Ref: CIMH/MP/2019/2007). The collected specimens were shade dried, powdered and extracted. Voucher specimens are being maintained in our laboratory for future reference.

Extraction

Hot extraction. Stem bark powder was serially extracted with petroleum ether and chloroform using a Soxhlet apparatus in a ratio of 1:6 (g: ml). The extracts obtained were evaporated to dryness at 40 °C under reduced pressure (petroleum ether: 180 mbar, chloroform: 118 mbar in a rotary evaporator (BUchi, Switzerland). The samples were stored in a vacuum desiccator at room temperature until further use.

Cold extraction. Stem bark powder was serially extracted with petroleum ether and chloroform in a ratio of 1:6 (g: ml) at room temperature with the flask shaken at regular intervals. The samples were stored in a vacuum desiccator at room temperature until further use.

Cell lines and maintenance

MDA-MB-231 (human breast carcinoma), MCF-7 (human breast carcinoma) and WRL-68 (human liver embryonic) cell lines were procured from National Centre for Cell Science (Pune, India). MDA-MB-231 cells were maintained in L-15 (Leibovitz's) culture medium, and MCF-7 and WRL-68 were maintained in Minimum essential medium (MEM) (Eagle) with Non-essential amino acids, all with 10% fetal bovine serum in a humidified atmosphere at 37 °C (with 5% CO₂ for MCF-7 and WRL-68 only). The cell lines were maintained in their growing phase at 70% confluency with regular passaging.

Cytotoxicity assessment

Extracts were tested for its cytotoxicity by XTT-formazan dye formation assay (Weislow et al., 1989). MDA-MB-231 and WRL-68 cells were seeded in their respective culture medium (200 μ l, 1 x 10⁴ cells/well and 6 x 10³ cells/well respectively) in a 96-well plate and incubated at 37 °C for 24 h with/without 5% CO₂ supply. After incubation, the control wells were replenished with fresh medium and the test wells were treated with 25, 50, 100 and 200 μ g/ml of extracts. The cells were further incubated for 24 h maintaining the same conditions. After the treatment incubation period, medium in each well was replenished with 200 μ l of fresh medium plus 50 μ l of XTT (0.6 mg/ml containing 25 μ M PMS). The plate was then reincubated for 4 h in the same conditions after which the absorbance was measured at 450 nm (with a 630 nm reference filter) in a Dynex Opsy MRTM Microplate Reader (Dynex Technologies, VA, USA).

Percentage cytotoxicity was calculated by the following formula:

$$\% \text{ Cytotoxicity} = [(Ac-At)/Ac] \times 100$$

Ac is the mean absorbance of the control wells and At is the mean absorbance of test wells with a particular extract dosage.

Apoptosis induction

The cellular DNA fragmentation ELISA is a photometric enzyme-linked immunosorbent assay (ELISA) in culture supernatants. It employs measurement of apoptotic cell death by detection of BrdU-labeled DNA fragments in the cytoplasm of affected cells. The experiment was performed as per the supplier's instructions. Cells (MDA-MB-231 and MCF-7) were labeled with 10 μ M BrdU at 1 x 10⁵ cells/ml density. BrdU-labeled Cells (1 X 10⁴) in 100 μ l were treated with varying concentrations (12.5, 25, 50, 100 and 200 μ g/ml) of the extracts for a period of 4 h. The cells were then lysed with lysing buffer and the supernatant containing apoptotic fragments were obtained after centrifugation at 1500 rpm for 10 min. Obtained sample (100 μ l) was transferred to anti-DNA coated 96-well flat-bottom microplates. The plates were incubated for 90 min at 15-25 °C then washed with washing buffer. The DNA bound to coated microplates was denatured by microwave irradiation (500 W for 5 min), followed by addition of 100 μ l anti-BrdU-POD conjugate. The plates were further incubated for 90 min and were washed again with washing buffer. An amount of 100 μ l substrate (TMB) solution was then added and the plates were shaken until color development is sufficient. The absorbance was read at 450 nm after addition of 25 μ l stop solution.

Cell migration inhibition assay

The extracts were investigated to possess cell migration inhibition efficiency through the method described by Dimmeler et al. (2000) with some modifications. MDA-MB-231 cells (6 x 10⁵ per well) were seeded on 6-well plates and incubated at 37 °C for 24 h to attain a confluent monolayer. *In vitro* 'scratch' wounds were created post-incubation by scrapping the monolayer with sterile cell scraper. Subsequently, wells were washed gently with growth medium to remove dislodged cells and were again added with the fresh medium in control wells and medium containing PHO in treatment wells. The plates were re-incubated at 37 °C to further observe migration of cells at every 4 h intervals (0, 4, 8, 12 and 16 h). The migration of cells was then monitored by a decrease in distance between wounded edges in a computer-attached inverted phase contrast microscope (Hund wetzlar, Germany).

Statistical Analysis

All analyses were carried out in triplicates. Data were presented as mean \pm SD. Statistical analyses were performed by one-way ANOVA. Significant differences

between groups were determined at P<0.05. To evaluate relationships between experimental parameters, results were analyzed for significance by Student's t-test (P<0.05). MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

Results

Hot extraction

Fifty grams of stem bark powder yielded 0.29 g (percentage extract yield: 0.58% of dry weight) of crude petroleum ether extract (PHO) and 0.20 g (percentage extract yield: 0.40% of dry weight) of crude chloroform extract (CHO).

Cold extraction

Fifty grams of stem bark powder yielded 0.13 g (percentage extract yield: 0.26% of dry weight) of crude petroleum ether extract (PCO) and 0.23 g (percentage extract yield: 0.46% of dry weight) of crude chloroform extract (CCO).

Cytotoxicity

Cytotoxicity was estimated by measuring the amount of resultant XTT-formazan as a consequence of XTT reduction by succinate dehydrogenase in living cells (Weislow et al., 1989). All four tested extracts showed a concentration-dependent increase in cytotoxicity with increasing doses in both the cell lines, MDA-MB-231 and WRL-68 (Figure 1).

Apoptosis inductivity

Apoptosis inductivity of the extracts was estimated as a photometric measure of the amount of apoptotic DNA fragments formed. The extract PHO was able to induce apoptosis in both the cell lines tested (MDA-MB-231 and MCF-7) as detected by the concentration-dependent increase in absorbance corresponding to the increase in apoptotic DNA fragments (Figure 2).

Cell migration inhibition efficiency

As also to prove the characteristic of an anti-metastatic agent, PHO was analysed to portray inhibition of cell migration in metastatic breast cancer cells (MDA-MB-231). Consequent to the creation of scratch wound, cells in control wells were observed to exhibit time

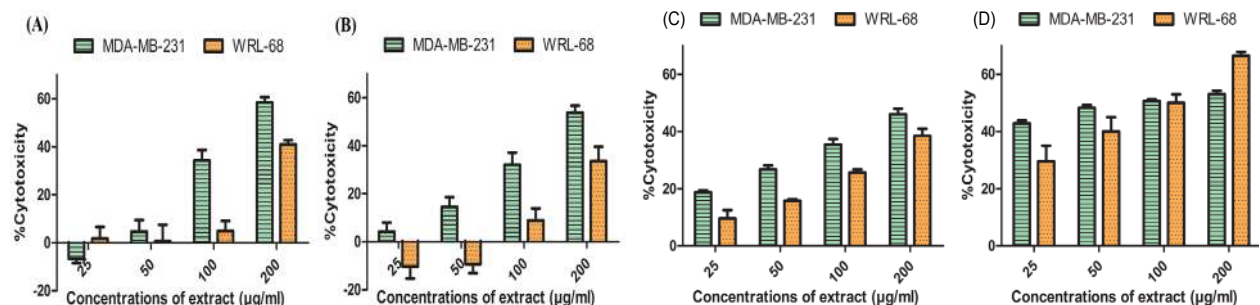


Figure 1. Percentage Cytotoxicity of PHO (A), PCO (B), CHO (C), and CCO (D) in MDA-MB-231 and WRL-68 cells. Data expressed as mean \pm SD (n = 3; P < 0.05)

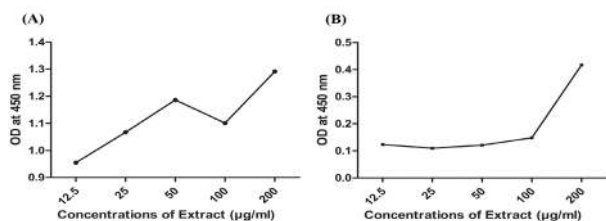


Figure 2. Dose-Dependent Increase of Apoptotic Fragments in MDA-MB-231 (A) and MCF-7 (B) Cells after PHO Treatment as Determined by Cellular DNA Fragmentation ELISA.

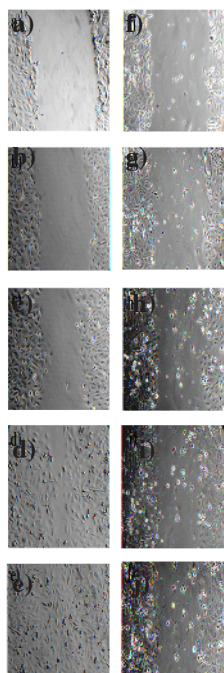


Figure 3. Time-Dependent Migration of MDA-MB-231 Cells from Edges of the Wounds, after 0, 4, 8, 12 and 16 h Upon Creation of Scratch Wound, in Control Well (a, b, c, d, e) and CHR Treated Well (f, g, h, i, j) Respectively

dependent migration (0, 4, 8, 12 and 16 h) in order to occupy free space. Control cells at its 16th h reached a complete confluency. Whereas, cells added with PHO (122.49 µg/ml - IC₅₀ value) displayed migration to a minimum with a complete seize in migration at 16th h (Figure 3).

Discussion

Use of medicinal plants as an approach in prevention and treatment of cancer is being followed since thousands of years. Out of 92 anticancer drugs which were available commercially prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, 60% are of natural origin (Cragg et al., 1997). Concerning to the sales for year 2000, natural products or its derivatives covered 14 of the top 35 drugs that retailed globally (Butlet, 2004). Plant-chemotherapeutics was also recognized by the National Cancer Institute, where it collected about 35,000 plant samples from 20 countries and screened 1,14,000 extracts for their anticancer activity (Shoeb et al., 2005). Some of the well known phytochemicals in use for cancer therapy are the vinca

alkaloids, the taxanes, and the camptothecins.

One of a promising characteristic for a chemotherapeutic drug is the ability of it to exterminate cancer cells (Mooney, 2005; Kumar et al., 2011). *O. indicum* in its methanol and aqueous extracts have previously been reported for its cytotoxicity in MDA-MB-435S and Hep3B cell lines (Kumar et al., 2010). However, the report on its non-polar counterpart was lacking. In the present study, *O. indicum* extracts were primarily tested for its selective-cytotoxicity to cancer cells. For this reason, the evaluation employed two cell lines, a cancer and a normal cell type [MDA-MB-231 (human breast adenocarcinoma) and WRL-68 (normal human liver embryonic)] respectively. Out of all four extracts evaluated, PHO was observed to be the better followed by PCO, CCO and CHO, all exhibiting cytotoxicity, significantly ($P < 0.05$) higher in MDA-MB-231 than in WRL-68 cells. Similar observations were also made by Weisburg et al. (2004), wherein phytochemical-mediated selective destruction of cancer cells was demonstrated. Hence, the results imply that the *O. indicum* extracts were able to selectively target cancer cells in its due course of cell annihilation.

The sole property of cytotoxicity alone may not be an adequate criterion for an extract to have antineoplastic potential. Demonstration of apoptosis inductivity is necessary as a proof-of-concept approach for developing agents for chemoprevention, as this has always been the accepted strategy for specifically eradicating cancer cells. Most of the available anticancer drugs follow this strategic mode of action (Alshatwi et al., 2011; Hasan et al., 2011). Specifically, the bark of *O. indicum*, have been used to demonstrate apoptotic activity, albeit from polar extracts (Brahma et al., 2011; Rajkumar et al., 2011). Nevertheless, in current study, PHO being a cancer specific-cytotoxin was also able to induce significant levels of apoptosis in both MDA-MB-231 and MCF-7 cells. Yet, the amount of apoptotic DNA fragments produced was quantitatively higher in MDA-MB-231 cells when compared to MCF-7. This indicates that the extract more specifically reacts to estrogen receptor (ER)-negative breast cancer cells (MDA-MB-231) than the ER-positive equivalent (MCF-7) and may be due to the reported differences in the bcl-2 levels in the two cell lines (Calcabrini et al., 2006; Zhong et al., 2009). Further, these results provide an impetus for examining the mechanisms of PHO-mediated apoptosis inductivity in MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) cell lines.

Regardless of advancements in local treatments for cancer, there is hitherto an existence of clinical challenge combating systemic metastatic disease (Shevdea and Welcha, 2003). Consequently, PHO was also intended to possess anti-metastatic activity, which was analysed through cell migration inhibition assay. Cell migration is initiated upon a scratch wound, which is followed by protrusion of cells in a direction perpendicular to the scratch wound. As stated, cells in control wells showed a time dependent migration of cells (0, 4, 8, 12, and 16 h) as evident by the decrease in distance between the scratch wound. PHO has demonstrated an obvious inhibition of cell migration in MDA-MB-231 cells along with few morphological changes to indicate arrest in the cell

proliferation and migration. Results similar to this have also been reported by Yodkeeree et al. (2010), through a phytochemical mediated suppression of migration and invasion in MDA-MB-231 cells. PHO thus might be a suitable source for deriving precursors to be used in targeted therapy of malignant breast cancer.

Conclusively, the non-polar extract of *O. indicum* consequently possess effectual cytotoxicity and distinctive apoptosis-inducing abilities, along with evident anti-metastatic potentials and thus could be considered as a hitherto unreported source of bioactive phytochemicals for the development of efficacious and possibly specific anticancer agents against ER-negative breast cancer.

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