



Journal of Biological Sciences

ISSN 1727-3048

science
alert

ANSI*net*
an open access publisher
<http://ansinet.com>

Demonstration of Differential Anti-Neoplastic Potentials of *Oroxylum indicum* Ethyl Acetate Extracts in Human Breast Cancer Cells

¹D.R. Naveen Kumar, ¹V. Cijo George, ¹P.K. Suresh and ²R. Ashok Kumar
¹School of Bio Sciences and Technology, VIT University, Vellore-632 014, India
²Department of Zoology, Government Arts College, Dharmapuri-636 705, India

Abstract: In the current scenario, medicinal plant research focuses on developing cancer-specific chemotherapeutic drugs which simultaneously render protection to normal cells. Hence, our study aimed to investigate the differential anti-neoplastic potentials of *Oroxylum indicum* ethyl acetate extracts (hot-EHO and cold-ECO). Cancer-cell-specific cytotoxicity of extracts was studied on MDA-MB-231 (cancer cells) and WRL-68 (normal cells). ECO showed significantly ($p < 0.05$) better cytotoxicity in MDA-MB-231 cells ($IC_{50} = 95.15 \pm 2.41 \mu\text{g mL}^{-1}$) than in WRL-68 cells ($IC_{50} = 203.54 \pm 2.96 \mu\text{g mL}^{-1}$), thus substantiating cancer-cell-specific cytotoxicity. The mechanism behind cytotoxicity was further corroborated through apoptosis induction in MDA-MB-231 and MCF-7 cells. ECO has exhibited dose-dependent apoptotic induction in both the cells. ECO also showed evident anti-metastatic potential by significantly ($p < 0.05$) inhibiting migration of metastatic breast cancer cells (MDA-MB-231). Over all, ECO exhibited efficient anti-breast cancer potentials and thus could be considered as a source harboring phytochemicals to possibly treat malignant breast cancer.

Key words: Cancer-cell-specific cytotoxicity, apoptosis, anti-metastasis, breast cancer, *Oroxylum indicum*

INTRODUCTION

Cancer is a dreadful disease among human race and breast cancer is the most common cancer amongst women that stands second in terms of cancer-related mortality (CDC, 2012). Cancer progression is a multi-step and sequential process that involves initiation, promotion, invasion and metastasis in parallel (Hanahan and Weinberg, 2000). Chemoprevention is considered as an imperative curing therapy to cancer and it has seen several improvisations through the growth of modern medicine (Sporn and Suh, 2000; Karikas, 2011). However, cytotoxic chemotherapy is of golden standard and has been proven to be effective even in advanced malignancies (Chonghaile *et al.*, 2011). The major problem in treating breast cancer is because of its malignant stage, where the metastatic cancer cells gains gradual resistance in due course of chemotherapeutic drug administration (Lirdprapamongkol *et al.*, 2003). Plant metabolites are well known to overcome this situation with its higher efficacy and lesser side effects.

The phytochemicals have been successful in cytotoxic chemotherapy against several cancer types. One of the common mechanisms behind this activity is induction of apoptosis (Chathoth *et al.*, 2008), which is being considered as most effective in chemotherapy. The

mechanism of apoptosis induction provides specificity to attack cancer cells and to spare other normal cells within the body (Kaufmann and Earnshaw, 2000). To combat cancer cells in its malignant state, a chemotherapeutic agent is also demanded for its anti-metastatic activity, to prevent migration of cancer cells which cause secondary malignant tumours (Fidler, 2003). Several phytochemicals as a crude extract and as pure components have been reported for these above said activities (Dahiru *et al.*, 2006). However, the need for more effective chemopreventive agents still persists (Kwiecinski *et al.*, 2008).

Oroxylum indicum (L.) Vent. (Bignoniaceae) is a deciduous tree characterized with few branches. The plant has been noted for its action against cancer but lacks specific chemopreventive records (Mao, 2002). Previous records on bark extracts of *O. indicum* explicates its antiproliferative/cytotoxic and apoptotic properties (Lambertini *et al.*, 2003; Kumar *et al.*, 2010; Rajkumar *et al.*, 2011; Naveen Kumar *et al.*, 2012a). Nevertheless, the specific-chemopreventive potentials for aprotic polar extracts along with anti-metastatic activity have hitherto not been cited. Thus, the current study focused to analyse cancer-cell-specific cytotoxicity of *O. indicum* ethyl acetate stem bark extracts, and was

intended to provide a mechanistic basis for its cytotoxicity by analyzing its apoptosis induction potentials, along with its anti-metastatic property.

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-sulphophenyl)-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: Stem bark of *O. indicum* 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 maintained in our laboratory for future reference.

Cell lines for the study: MDA-MB-231 (human breast carcinoma), MCF-7 (human breast carcinoma) and WRL-68 (normal 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% 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 by regular passaging.

Extraction: Powdered stem bark was extracted with ethyl acetate [1:6 (g: mL)] using a Soxhlet apparatus for hot extraction. For maceration, powdered stem bark was extracted with ethyl acetate in a ratio of 1:6 (g: mL) at room temperature with the flask shaken at regular intervals. The extracts obtained were evaporated to dryness at 40°C under reduced pressure [ethyl acetate: 240 mbar in a rotary evaporator (BUchi, Switzerland)]. Until further use, the samples were stored in a vacuum desiccator at room temperature.

BIOACTIVITIES IN VITRO

Determination of cytotoxicity-XTT assay: The cytotoxicity of the extracts were analysed by XTT-formazan dye formation assay (Weislow *et al.*, 1989). Cells (MDA-MB-231 and WRL-68) were seeded (200 µL growth medium containing 1×10⁴ cells/well and 6×10³ cells well⁻¹, respectively) in a 96 well plate and incubated at 37°C for 24 h with/without 5% CO₂ supply. Control wells were then replenished with fresh medium while treatment wells were added with 25, 50, 100 and 200 µg mL⁻¹ of extracts and was subsequently followed by treatment incubation of 24 h. Post-incubation, each well was replenished with fresh medium (200 µL) plus 50 µL of XTT solution (0.6 mg mL⁻¹ containing 25 µM PMS). Further, plates were re-incubated for 4 h maintaining same conditions after which the absorbance was measured at 450 nm (with a 630 nm reference filter) in a Dynex Opsys MRTM Microplate Reader (Dynex Technologies, VA, USA). Percentage cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{Ac - At}{Ac} \times 100$$

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

Apoptosis detection by cellular DNA fragmentation Elisa: The experiment is a photometric detection of 5-bromo-2'-deoxyuridine (BrdU)-labeled apoptotic DNA fragments through enzyme-linked immunosorbent assay (Elisa). Assay was carried out to test the apoptotic potentials of cold-ethyl acetate extract of *O. indicum* (ECO) (12.5, 25, 50, 100 and 200 µg mL⁻¹) on MDA-MB-231 and MCF-7 cells, as per the protocol mentioned previously (Naveen Kumar *et al.*, 2012b).

Cell migration inhibition assay: The extracts were tested to inhibit cell migration according to the method reported by Dimmeler *et al.* (2000) with quite some modifications. MDA-MB-231 cells (6×10⁵ per well) were seeded in 6-well plates and incubated at 37°C for 24 h. After incubation, *in vitro* 'scratch' wounds were created by scrapping the monolayer using sterile cell scrapper. Wells were subsequently washed with growth medium to remove dislodged cells. The plates were added with fresh medium in control wells and medium containing ECO in treatment wells and incubated at 37°C. Plates were then monitored every 4 h intervals (0, 4, 8, 12 and 16 h) to perceive migration of cells which is recorded and quantified by

measuring the decrease in distance between wound edges in a computer-attached inverted phase contrast microscope (Hund wetzlar, Germany) at five distinct positions (every 5 mm). Percentage cell migration was calculated by the following formula:

$$\text{Cell migration (\%)} = \frac{D_0 - D_t}{D_0} \times 100$$

where, D_0 is the mean distance of the scratch wound at time 0 h and D_t is the mean distance of scratch wound after time (0, 4, 8, 12 and 16 h) with extract treatment.

Statistical Analysis: All the experiments were carried out in triplicates and the data expressed were mean values \pm SD. Statistical analyses were performed by one-way (ANOVA) to perceive significant differences between groups at ($p < 0.05$). To evaluate relationships between experimental parameters, results were analyzed for correlation and tested 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 AND DISCUSSION

Carcinogenesis, being a major health problem worldwide, still has a limited means for control. Few important characteristics of cancer cells are uncontrolled growth, lack of apoptosis and its ability to metastasize (Hanahan and Weinberg, 2000). Plants are considered as the best chemopreventive agents to combat cancer cells since antiquities and over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (Cragg and Newman, 2005). Plant resources have received considerable attention as sources of biologically active agents with antioxidant, anti-mutagenic and anti-carcinogenic potential (Shoeb, 2006). Anticancer agents of ethno-pharmacological origin are gaining attention in the treatment of cancer, to combat cancer cells through apoptosis and other mechanisms (Radhakrishna *et al.*, 2004).

Previously, our group has demonstrated the cytotoxic and apoptotic potential of the polar and non-polar extracts from the same plant (Kumar *et al.*, 2010; Rajkumar *et al.*, 2011; Naveen Kumar *et al.*, 2012a). This work on aprotic polar extracts of *O. indicum* to test for chemopreventive potentials, hitherto has not been reported in the literature and is the first of its kind to demonstrate an enhanced,

differential, apoptotic effect in the two breast cancer cell lines (MDA-MB-231 and MCF-7), relative to its cytotoxic potential. The specificity of its relatively higher toxicity has also been demonstrated in comparison with a normal hepatoma cell line. Also, in addition, this is the first report of its kind involving the investigation and evaluation of the anti-metastatic property specifically of the two extracts of *O. indicum*.

Extract yield: Fifty grams of powdered stem bark yielded 0.37 g (percentage extract yield: 0.74% of dry weight) of crude hot ethyl acetate extract (EHO) and 0.19 g (percentage extract yield: 0.38% of dry weight) of crude cold ethyl acetate extract (ECO).

Cytotoxicity: Cytotoxic chemotherapy has common targets such as DNA and microtubules in nucleated human cells, yet it selectively eradicates tumor cells (Chonghaile *et al.*, 2011). As a measure, the ethyl acetate extracts of *O. indicum* has been analysed to exhibit cancer-cell-specific cytotoxicity. The extracts were tested through XTT-formazan assay that results for XTT reduction by live cells, due to active mitochondrial succinate dehydrogenase (Weislow *et al.*, 1989). Both the extracts were tested in a cancer cell line (MDA-MB-231) and a normal cell line (WRL-68) for the stated purpose.

EHO has exhibited low cytotoxic levels against MDA-MB-231 cells ($IC_{50} = 243.00 \pm 1.94 \mu\text{g mL}^{-1}$) with no significant cytotoxicity on WRL-68 cells in the tested concentrations (Fig. 1a), while ECO has exhibited dose-dependent cytotoxicity on both MDA-MB-231 ($IC_{50} = 95.15 \pm 2.41 \mu\text{g mL}^{-1}$) and WRL-68 cells ($IC_{50} = 203.54 \pm 2.96 \mu\text{g mL}^{-1}$) (Fig. 1b). In comparison, ECO had significantly ($p < 0.05$) high cytotoxicity in MDA-MB-231 cells than in WRL-68 cells, thus divulging its cancer-cell-specific cytotoxicity. A similar observation in regard to cancer-cell-specific cytotoxicity has also been noted in our preceding study (Naveen Kumar *et al.*, 2012b). Previously, Cos *et al.* (2006) has specified that relevant and selective activity relates to IC_{50} values below $100 \mu\text{g mL}^{-1}$ for extracts and below $25 \mu\text{M}$ for pure compounds. Taking this into consideration, ECO alone has been taken for further analysis to substantiate its activity against breast cancer.

Apoptosis inductivity: Apoptosis induction is another important criterion for an anticancer agent and is specific as cancer cells lack this ability (Hanahan and Weinberg, 2000). The high cytotoxicity exhibited by ECO was substantiated by testing its apoptotic induction ability to prove the mechanistic base. The assay employed a photometric measurement of BrdU-labeled apoptotic DNA fragments in the cytosolic extract of treated cells.

Consecutively, ECO has been tested on two breast cancer cell lines (MDA-MB-231 and MCF-7 cells) to induce apoptosis and it exhibited a dose dependent increase in apoptosis on both the cell lines as recorded by increase in absorbance, which directly corresponds to apoptotic DNA fragments Fig. 2. This result corroborate with the previous reports given by Zhong *et al.* (2009) and Naveen Kumar *et al.* (2012b).

Anti-metastatic potentials: Metastasis can be regarded as an active, multistep process of migration of neoplastic cells. In the study, ECO has also been tested for its anti-metastatic potential through cell migration inhibition assay and was found to evidently inhibit migration of metastatic breast cancer cells (MDA-MB-231) at a concentration of 95.15 $\mu\text{g mL}^{-1}$ (Fig. 3a). The control wells demonstrated a time dependent (4, 8, 12 and 16 h) gradual

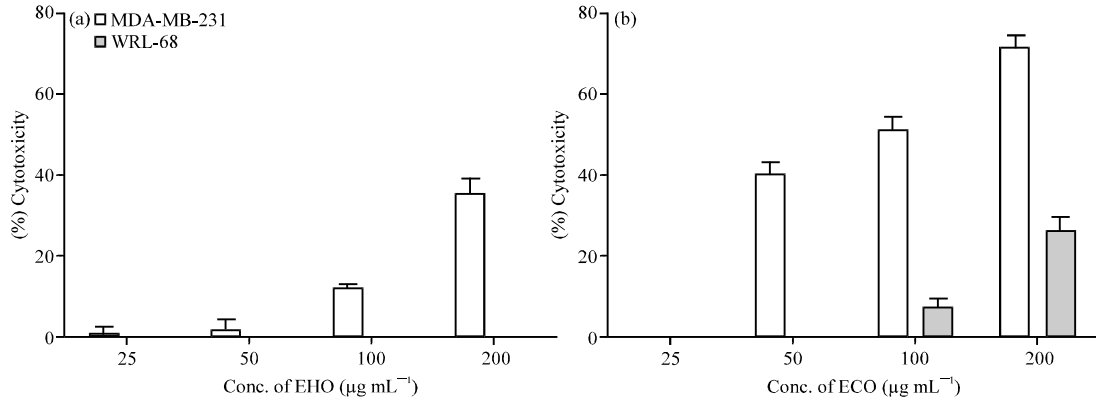


Fig. 1(a-b): Cytotoxicity (%) of (a) EHO and (b) ECO in MDA-MB-231 and WRL-68 cells. Data presented as Mean \pm SD (n = 3, p<0.05). No significant cytotoxicity was exhibited by EHO on WRL-68 cells

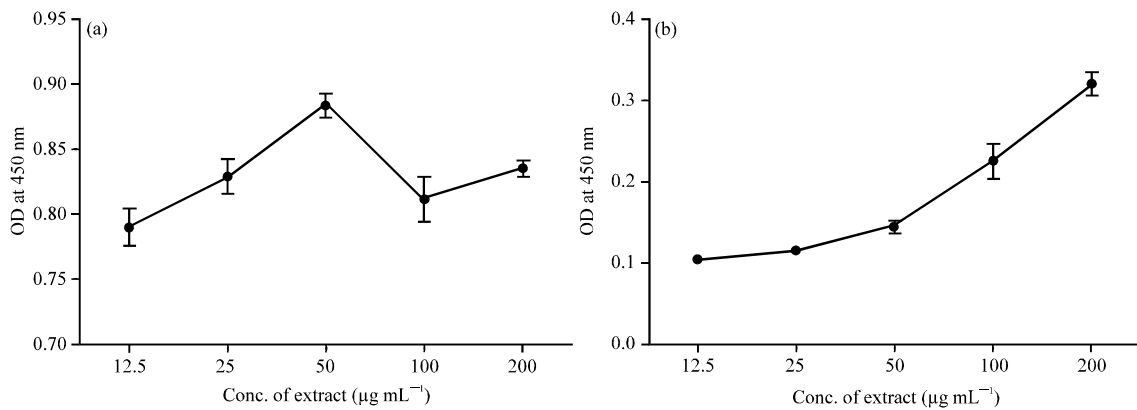


Fig. 2(a-b): Portraying dose-dependent increase of apoptotic DNA fragments in (a) MDA-MB-231 and (b) MCF-7 cells as effect of ECO treatment. Data expressed as mean OD \pm SD (n = 3, p<0.05)

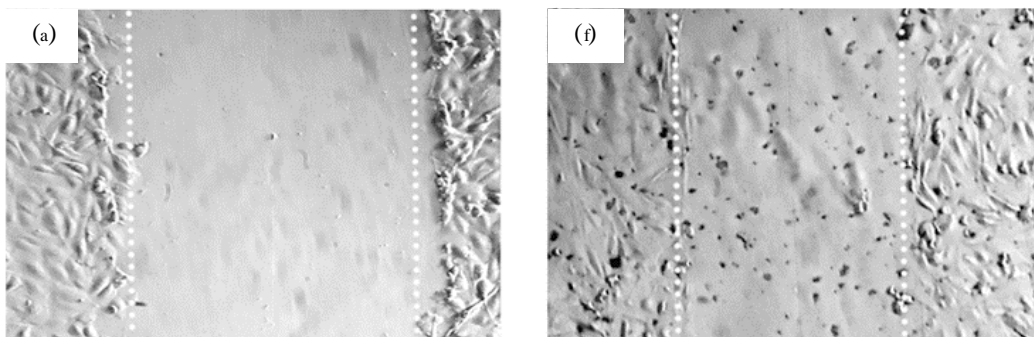


Fig. 3: Continued

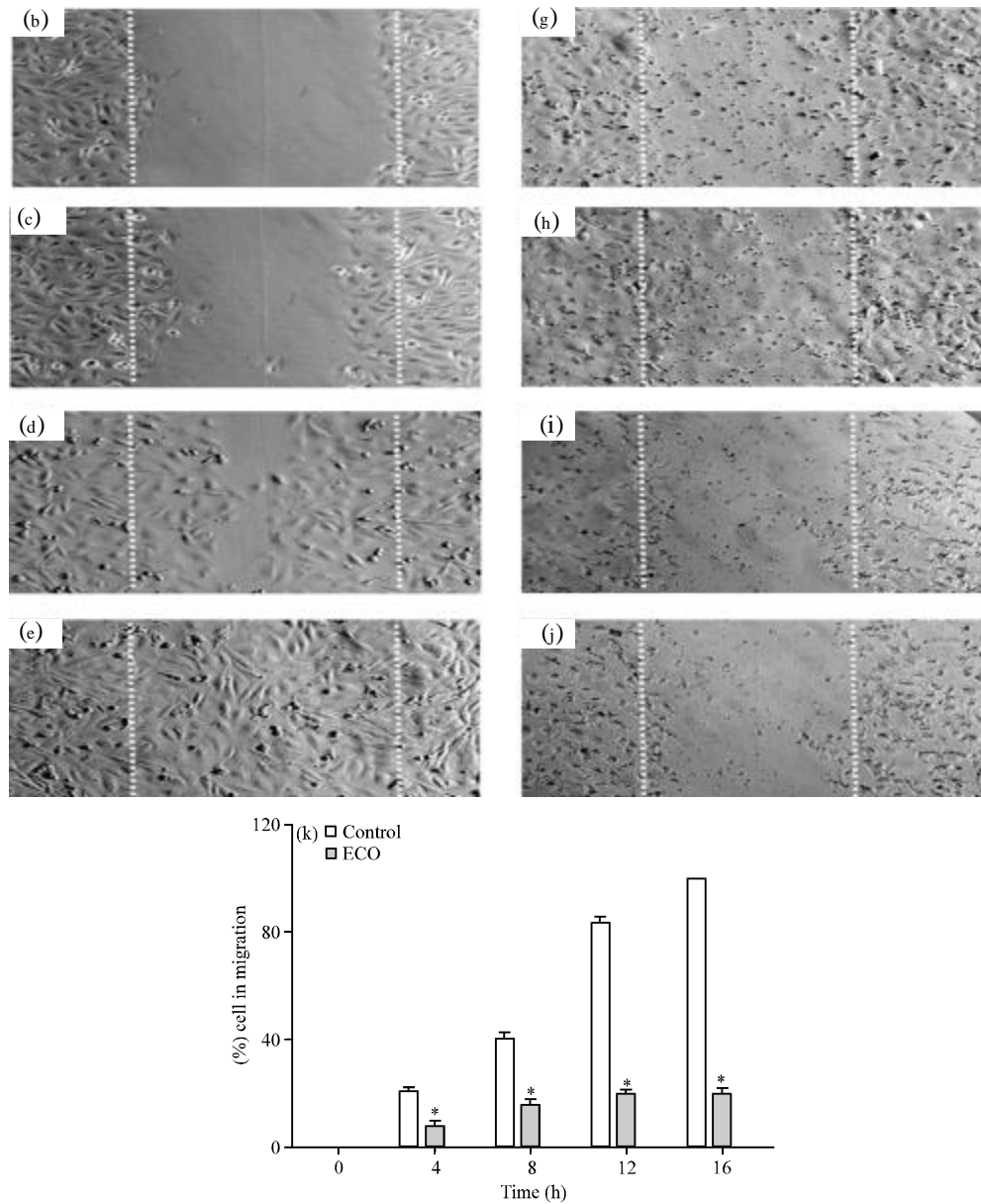


Fig. 3(a-k): (a) Demonstrating the migration of MDA-MB-231 cells from edges of the scratch wound after 0, 4, 8, 12 and 16 h (a, b, c, d, e) control well and (f, g, h, i, j) ECO treated well, respectively (n = 3). (k) Calculated (%) Cell migration of MDA-MB-231 cells, showing significantly ($[p < 0.05]$ marked by Asterisk [*]) less migration in ECO treated cells compared to control cells

migration of cells to protrude into open space of scratch wound and have attained a complete confluency at 16th h. Whereas, the migration of cells was minimized by ECO at 4 and 8th h with few morphological changes and was completely arrested at 12th h after which no further migration was observed in treatment wells (Fig. 3b). Similar results of cell migration inhibition were previously been reported by Hsu *et al.* (2012).

CONCLUSION

From this study, we conclude that the ethyl acetate extracts of *O. indicum* was proven to possess significant ($p < 0.05$) cancer-cell-specific cytotoxicity. The cold ethyl acetate extract have also demonstrated an affirmative apoptosis induction in both breast cancer cell types along with its evident anti-metastatic potential which is reported

for the first time. Consequently, this extract could further be explored to elute active bio-components, which could possibly serve as prototypes in designing specific-chemotherapeutic drugs against metastatic breast cancer.

ACKNOWLEDGMENTS

We are thankful to the management of VIT University, Vellore, Tamil Nadu, India, for funding this research work and for providing the necessary infrastructure for the successful completion of the same.

REFERENCES

- CDC, 2012. Cancer among women. Centers for Disease Control and Prevention.
- Chathoth, S., F. Thayyullathil and S. Galadari, 2008. Curcumin cell signaling: A possible target for chemotherapy. *Curr. Trends Biotechnol. Pharm.*, 2: 226-238.
- Chonghaile, T.N., K.A. Sarosiek, T. Trang, J.A. Ryan and A. Tammareddi *et al.*, 2011. Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science*, 334: 1129-1133.
- Cos, P., A.J. Vlietinck, D.V. Berghe and L. Maes, 2006. Anti-infective potential of natural products: How to develop a stronger *in vitro* proof-of-concept. *J. Ethnopharmacol.*, 19: 290-302.
- Cragg, G.M. and D.J. Newman, 2005. Plants as a source of anti-cancer agents. *J. Ethnopharmacol.*, 100: 72-79.
- Dahiru, D., J.M. Sini and L. John-Africa, 2006. Antidiarrhoeal activity of *Ziziphus mauritiana* root extract in rodents. *Afr. J. Biotechnol.*, 5: 941-945.
- Dimmeler, S., E. Dernbach and A.M. Zeiher, 2000. Phosphorylation of the endothelial nitric oxide synthase at Ser-1177 is required for VEGF-induced endothelial cell migration. *FEBS Lett.*, 477: 258-262.
- Fidler, I.J., 2003. The pathogenesis of cancer metastasis: The seed and soil hypothesis revisited. *Nat. Rev. Cancer*, 3: 453-458.
- Hanahan, D. and R.A. Weinberg, 2000. The hallmarks of cancer review. *Cell*, 100: 57-70.
- Hsu, H.F., Z.H. Chen, S.F. Chang, C.P. Wang and S.J. Chiou *et al.*, 2012. Evaluating the anti-metastatic potential of *Anisomeles indica* extract by using human oral squamous carcinoma FaDu cells. *Afr. J. Pharm. Pharmacol.*, 6: 1782-1791.
- Karikas, G.A., 2011. Chemoprevention molecular and biochemical mechanisms involved in cancer control and management. *Health Sci. J.*, 5: 149-156.
- Kaufmann, S.H. and W.C. Earnshaw, 2000. Induction of apoptosis by cancer chemotherapy. *Exp. Cell Res.*, 256: 42-49.
- Kumar, R.A., V. Rajkumar, G. Guha and L. Mathew, 2010. Therapeutic potentials of *Oroxylum indicum* bark extracts. *Chinese J. Nat. Med.*, 8: 121-126.
- Kwiecinski M.R., K.B. Felipe, T. Schoenfelder, L.P. de Lemos Wiese and M.H. Rossi *et al.*, 2008. Study of the antitumor potential of *Bidens pilosa* (Asteraceae) used in Brazilian folk medicine. *J. Ethnopharmacol.*, 117: 69-75.
- Lambertini, E., R. Piva, M.T. Khan, I. Lampronti, N. Bianchi, M. Borgatti and R. Gambari, 2003. Effects of extracts from Bangladeshi medicinal plants on *In vitro* proliferation of human breast cancer cell lines and expression of estrogen receptor alpha gene. *Int. J. Oncol.*, 24: 419-432.
- Lirdprapamongkol, K., C. Mahidol, S. Thongnest, H. Prawat and S. Ruchirawat *et al.*, 2003. Anti-metastatic effects of aqueous extract of *Helixanthera parasitica*. *J. Ethnopharmacol.*, 86: 253-256.
- Mao, A.A., 2002. *Oroxylum indicum* a potential anticancer medicinal plant. *Ind. J. Trad. Knowl.*, 1: 17-21.
- Naveen Kumar, D.R., S. Shikha, V.C. George, P.K. Suresh and R.A. Kumar, 2012a. Anticancer and anti-metastatic activities of *Rheum emodi* rhizome chloroform extracts. *Asian J. Pharm. Clin. Res.*, 5: 189-194.
- Naveen Kumar, D.R., V.C. George, P.K. Suresh and R.A. Kumar, 2012b. Cytotoxicity, apoptosis induction and anti-metastatic potential of *Oroxylum indicum* in human breast cancer cells. *Asian Pac. J. Can. Prev.*, 13: 2729-2734.
- Radhakrishna, P.G., A.S. Srivastava, T.I. Hassanein, D.P. Chauhan and E. Carrier, 2004. Induction of apoptosis in human lung cancer cells by curcumin. *Cancer Lett.*, 208: 163-170.
- Rajkumar, V., G. Guha and R.A. Kumar, 2011. Induction of apoptosis in MDA-MB-435S, Hep3B and PC-3 cell lines by extracts of *Oroxylum indicum*. *J. Pharm. Res.*, 4: 2054-2056.
- Shoeb, M., 2006. Anticancer agents from medicinal plants. *Bang. J. Pharmacol.*, 1: 35-41.
- Sporn, M.B. and N. Suh, 2000. Chemoprevention of cancer. *Carcinogenesis*, 21: 525-530.
- Weislow, O.S., R. Kiser, D.L. Fine, J. Bader, R.H. Shoemaker and M.R. Boyd, 1989. New soluble-formazan assay for HIV-1 cytopathic effects: Application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. *J. Nat. Cancer Inst.*, 81: 577-586.
- Zhong, Y., G. Xu, G. Ye, D. Lee, J. Modica-Amore and C. Peng, 2009. Nodal and activin receptor-like kinase 7 induce apoptosis in human breast cancer cell lines: Role of caspase 3. *Int. J. Physiol. Pathophysiol. Pharmacol.*, 1: 83-96.