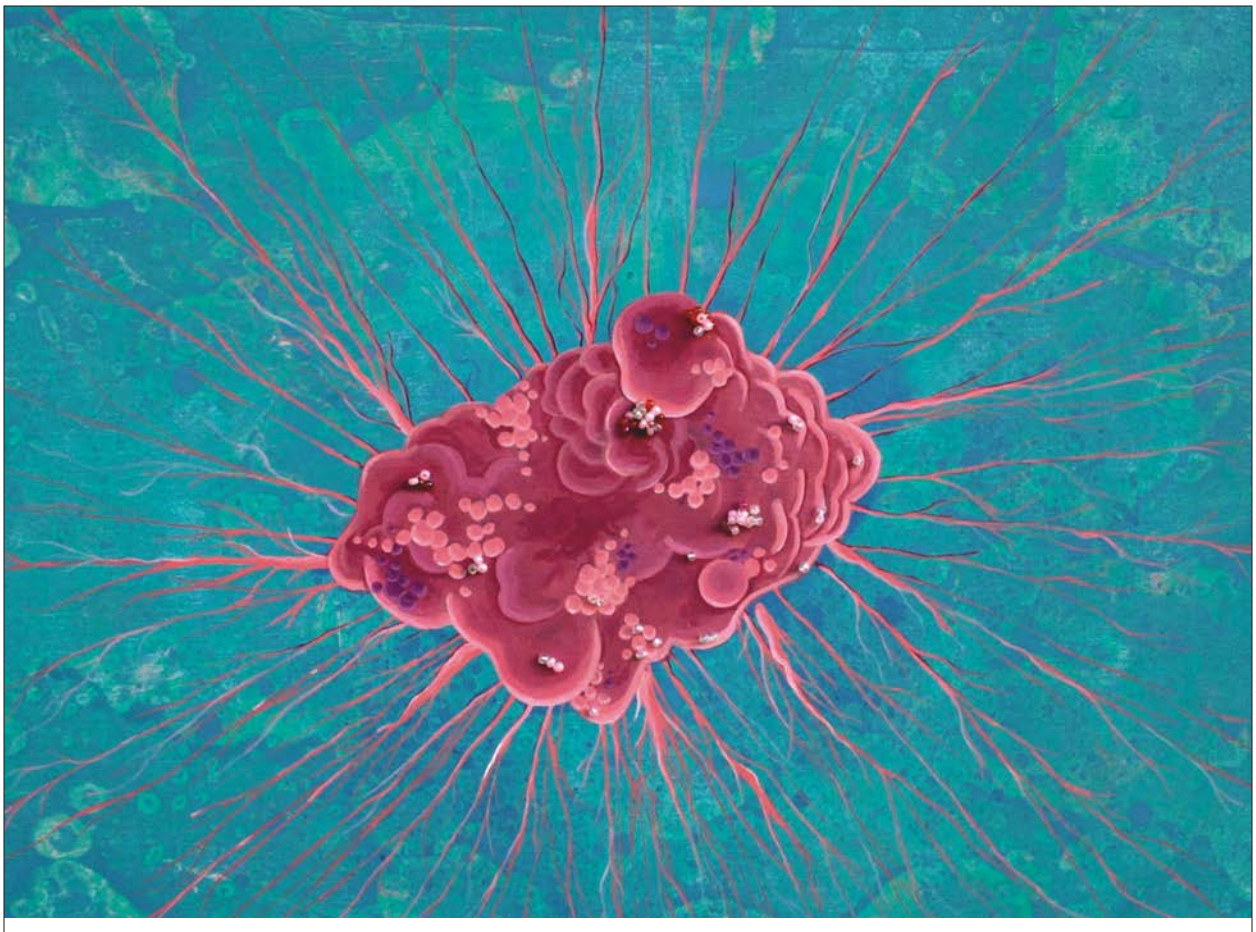

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In vitro Cytotoxic Activity of *Dendrophthoe falcata* on Human Breast Adenocarcinoma Cells-MCF-7

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ABSTRACT

In vitro studies were carried out to evaluate the cytotoxic potential of the ethanolic and aqueous extracts from stem of *Dendrophthoe falcata* against human breast cancer cells (MCF-7). Screening of these extracts was done using the brine shrimp lethality bioassay, MTT assay and SRB assay. The results showed the significant decrease of the viability of the cells in a concentration-dependent manner. The ethanolic extract of *D. falcata* showed IC_{50} 107 μg mL^{-1} in MTT assay and 112 μg mL^{-1} in SRB assay for MCF-7 cells and thus showed significant cytotoxic activity. The aqueous extract of *D. falcata* demonstrated higher activity with IC_{50} 90 μg mL^{-1} in MTT and 98 μg mL^{-1} in SRB assay for MCF-7 cells, respectively after 48 h of exposure. In brine shrimp lethality bioassay also aqueous extract showed more potent cytotoxicity with LC_{50} 90 μg mL^{-1} as compared to ethanolic extract with LC_{50} 120 μg mL^{-1}. From results obtained it was observed that both extracts of *D. falcata* may be a promising alternative to synthetic substances as natural compound with high antiproliferative activities and to be useful in cancer treatment and prevention.

Key words: Carcinogenesity, methanolic and aqueous extract, foetal bovine serum, *Artemia salina*

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years, herbal medicines which formed the basis of health care throughout the world since the earliest days of mankind are still widely used. Recognition of their clinical, pharmaceutical and economic value is still growing, although this varies widely between countries (Lewis and Elvin-Lewis, 2003; World Health Organization, 1998). An impressive number of modern drugs have been isolated from natural sources, many of them based on their uses in traditional medicine. At least 119 chemical substances derived from 90 plant species are important drugs currently in use, of these 119 drugs, 74% were discovered as a result of research directed at the isolation of active compounds from plants used in traditional medicine. Many researchers have discussed the importance of medicinal plants as sources of new therapeutic agents and others have effectively focused on the potential of specific chemical classes (e.g., alkaloids) in drug discovery, recently research continues to validate an ethnobotanically targeted approach to the initial discovery of pharmaceuticals (Lewis and Elvin-Lewis, 2003).

Cancer is one of the most dreaded diseases of the 20th century and spreading further continuously and increasing incidence in 21st century. Cancer is a group of more than 100 different

diseases, characterized by uncontrolled cellular growth, local tissue invasion and distant metastases (Kaufman and Chabner, 1996). In the pursuit of determining the cure for cancer, especially breast cancer which results in 40,000 deaths yearly, making it the second leading cause of death from cancer in women (Nor Aini *et al*., 2008). Breast cancer is the commonest form of malignancies in females in the U.K and metastasis of breast cancer is common. About 7% of patients with breast cancer present with widespread metastases at the initial presentation. The sites most frequently visited by metastasis in breast cancer are bone, lungs, liver, chest wall and central nervous system. Less common sites are the adrenals, ovaries, pericardium, thyroid and bone marrow (Zia *et al*., 2007). It was found that genetic alterations play an important role in the development of invasive carcinoma. Preinvasive lesions indicate the presence of intermediate stages in the development of invasive carcinoma in some cases but we still have little understanding of what genetic/epigenetic events are likely to be associated with the earliest phases of the disease. Cyclin D1 is claimed to be one of the genes that are known to be involved in preinvasive breast lesions (Abd El-Maqsoud and Aly, 2010).

Plants have a long history of use in the treatment of cancer (Kinzler and Vogelstein, 2002). The search for anti-cancer agents from plant sources started in the late 1950's, with the discovery and development of the Vinca alkaloids, (vinblastine and vincristine) and isolation of cytotoxic podophyllotoxins from *Podophyllum hexandrum* (Cragg *et al*., 2005; Newman *et al*., 2003). As a result, the United States National Cancer Institute (NCI) initiated an extensive plant collection program in 1960. This led to the discovery of many other compounds such as taxanes, camptothecins (Cassady and Dourus, 1980) and combrestatins (Pinney *et al*., 2005) which are being used in cancer treatment with varied degrees of success.

Association between occurrence of cancer and diet is strong. Cancer chemopreventive potential of naturally occurring phytochemicals is gaining lot of importance and interest. Although it may be impossible to avoid exposure to xenobiotics and their toxic effects, it may be possible to modify their effects by consuming diet rich in protective phytonutrients that will enhance the cellular protective mechanism and reverse the effects of xenobiotics. Antigenotoxicants have antimutagenic property and inhibit or deactivate the mutagens/carcinogens. It has been suggested that chemoprevention should be considered as an inexpensive, easily applicable approach to cancer control. Different classes of antigenotoxicants have various mechanisms of action. Antigenotoxic activities of various phytochemicals have great potential in the fight against human cancer (Nirmala *et al*., 2007).

Over 60% of currently used anti-cancer agents are derived in one-way or another from natural sources, including plants, marine organisms and microorganisms (Cragg *et al*., 2005; Newman *et al*., 2003). It is estimated that more than 50% of all the patients diagnosed with cancer explore complementary and alternative medicine, especially herbal medicine (Nelson *et al*., 2004). Plant foods with single dietary components are not exclusively responsible for the health benefits of food rather; dietary plant compounds have a synergistic effect in disease prevention. Food synergy attempts to identify the food components with relevant health effects suggested that additive and synergistic effects of phytochemicals in whole foods improve health benefits when compared to an isolated purified phytochemical extract or a dietary supplement which may be responsible for their anti-carcinogenic properties (Gourineni *et al*., 2010).

Dendrophthoe falcata Ettingsh is a perennial climbing woody parasitic plant. It is indigenous to tropical regions especially in India, Srilanka, Thailand, China, Australia, Bangladesh, Malayasia and Myanmar. In India it is widely distributed throughout upto 900 m (Pattanayak *et al*., 2008).

Preliminary phytochemical screening conducted in our department revealed the presence of carbohydrates, phytosterols, flavonoids, glycosides and phenolic compounds.

Warrier *et al.* (1993) reported that the entire plant is used extensively in traditional system of medicine as an aphrodisiac, astringent, narcotic, diuretic and for the treatment of pulmonary tuberculosis, asthma, menstrual disorders, swellings, wounds, ulcers, strangury, renal and vesical calculi and vitiated conditions of *kapha* and *pitta*. In view of its wide use and its chemical composition, the ethanolic and aqueous extracts of *D. falcata* stem were determined for their *in vitro* cytotoxic and anti oxidative activities.

The present study aimed to investigate the *in vitro* cytotoxic potential of the ethanolic and aqueous extracts from stem of *Dendrophthoe falcata* against human breast cancer cells (MCF-7), by brine shrimp lethality bioassay, MTT assay and SRB assay.

MATERIALS AND METHODS

All chemicals and solvents were of analytical grade and were obtained from Nice Chemicals, Mumbai. Human breast adenocarcinoma cells (MCF-7), were obtained from NCCS Pune, India. DMEM (Dulbacco's minimum essential medium) media, 10% Foetal Bovine Serum (FBS) and MTT reagent from sigma Aldrich, USA. Tissue culture flasks, 96 well microculture plates from Tarson and Nunc, USA, antimycotic solution and TPVG solution from Himedia.

Plant material: The stem of *D. falcata* was collected in August 2009 from Manipal. The plant was identified by Dr. Gopalakrishna Bhat, Taxonomist, Poorna Prajna College, Udupi, Karnataka. A voucher specimen (PP 564) has been deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences (Manipal, India).

Plant extract: Ethanolic extract- The stems were shade dried, powdered and about 100 g of powder was extracted with ethanol by hot extraction process (soxhlet) for 72 h. After completion of the extraction the solvent was recovered by distillation and concentrated *in vacuo*.

Aqueous extract (Chloroform: water-1:99). The stems were shade dried, powdered and macerated with chloroform water for seven days.

Cell culture and treatment: MCF-7 cells was procured from NCCS, Pune ,were grown in 75 cm^2 tissue culture flasks containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% Fetal Bovine Serum, Trypsin Phosphate Versene Glucose solution at 37°C in CO_2 incubator in an atmosphere of humidified 5% CO_2 and 95% air. The cells were maintained by routine sub culturing in 75 cm^2 tissue culture flasks. The culture medium was changed every 48 h and the cells were usually split 1:3 when they reached confluence. Plates were changed to FBS-free medium before the beginning of the assay. For the cytotoxicity assay a range of concentrations of extract (62.5-500 μg mL^{-1}) was used for 48 h treatment for the determination of IC_{50}.

Test of cytotoxicity: Cytotoxic activity was determined by brine shrimp lethality bioassay, MTT dye-reduction assay and SRB assay.

Brine shrimp lethality bioassay: The brine shrimp (*Artemia salina*) eggs were brown in colour and very small in size. One hundred milligram of eggs roughly represent 2.5 to 3.0 thousand of eggs. Hatching chamber was fabricated as per the design used by Mayer *et al.* (1982). The

chamber was made of glass, with aluminum lid on top. The chamber was divided into two equal parts with the help of a laminated plywood divider having a number of holes of 2 mm size. One of the compartments was illuminated with a lamp (60 watts) while the other was darkened. Both the chambers were aerated.

Samples of the extracts were prepared by dissolving 5 mg of ethanolic and aqueous extracts in 5 mL of DMSO to get stock solution. From this stock 50, 250, 500 and 750 μL were taken and volume was made up to 5 mL (in seven ml vial capacity) with solution which contain specific volume of brine and yeast suspension to get the final drug conc.10, 50, 100 and 150 ppm. Three replicates were prepared for each dose level. Control vials were prepared by adding equal volumes of distilled water. Nauplli were drawn in a pipette along with water and ten of such shrimps were transferred to each sample vial after they were counted in the stem of pipette against lighted background. The artificial sea water, prepared according to the composition given by Dr.Vasudevappa, Research Officer, FRS, Hesaragatta, Bangalore, was added to each vial to make upto 5 mL, a drop of dry yeast suspension (3 mg in 5 mL sea water) was added to each vial as food for shrimps. The vials were maintained under illumination. After 24 h survivors were counted, by using 3X magnifying glass and the percent deaths and LC_{50} values were calculated by using Finney Computer program (Meyer *et al*., 1982).

MTT assay: The tetrazolium 3- (4, 5 dimethylthiazoly-2)-2, 5-diphenyltetrazolium bromide) (MTT) is reduced to a colored product by the activity of NAD (P) H-dependent deshydrogenases and this indicates the level of energy metabolism in cells. Briefly cells were seeded in 96-well microplates with 1×10^4 cells in 0.1 mL of DMEM medium supplemented with 10% FBS and routinely cultured in a humidified incubator (37°C in 5% CO_2) for 24 h. Herbal extracts were added in serial concentrations (62.5-500 μg mL^{-1}) and incubated for 24 h. Then the medium was discarded and 100 μL of tetrazolium dye (MTT) solution (1 mg mL^{-1} in PBS) was added to every well and re-incubated for an additional 4 h. One hundred microlitter of DMSO was added to dissolve the formazan crystals formed. The plate was then read on a microplate reader at 540 nm. MTT solution with DMSO (without cells and medium) acted as a blank control in microplate reading while the PBS-treated cells served as a control of 100% survival (Mosmann, 1983; Dua and Gude, 2006).

SRB assay: The term cytotoxicity covers both cytostatic and cytocidal effects. The SRB assay can be used to determine which of these takes place for a particular test substance. One hundred microlitter of cell suspension of optimum density was introduced into each well of 96-well plates. A range of concentrations (62.5-500 μg mL^{-1}) of extracts to be tested was made in the culture medium. Added 100 μL of each concentration of test sample in culture medium to the wells containing the cells and 100 μL medium only to the control wells. The cells were incubated with the samples for 48 h and fixed with ice-cold TCA for 1 h at 4°C. The plates were washed five times in distilled water and allowed to dry in the air. Then 50 μL sulphorhodamine (SRB) solution was added to each well of the dry 96-well plates and allow staining at room temperature for 30 min. The sulphorhodamine (SRB) solution was removed by washing the plates quickly with 1% v/v acetic acid five times to remove unbound dye. The bound SRB was solubilised by adding 100 μL of 10 mM unbuffered Tris Base (pH 10.5) to each well and shaking for 5 min on a shaker platform. Plates were read in a 96-well plate reader with the working wavelength 492 nm (Houghton *et al*., 2007).

Statistical analysis: Data represent the mean±standard error (SEM) of the indicated number of experiments. Graphs were prepared by Prism software. Statistical analysis of the data was carried

out by one way ANOVA (Graph Pad Prism 5.02 Software) followed by Dunnet post hoc test. A value of $p< .05$, $p<0.01$, $p<0.0001$ were considered to be significant, very significant and highly significant, respectively. Linear regression analysis was used to calculate IC_{50}.

RESULTS

Brine shrimp lethality bioassay: Both ethanolic and aqueous extracts screened for BSL bioassay were found effective. The LD_{50} of ethanol and aqueous extracts was found to be 120 and 90 μg mL^{-1}, respectively. Since the BSL assay is very preliminary method to assess cytotoxic activity, the extracts were continued to explore in different *in vitro* cytotoxic models (Fig.1).

***In vitro* cytotoxicity in MCF-7 by MTT assay:** Percentage cell death was determined after 48 h. Both the extracts showed significant cytotoxicity in MCF-7 cells in the range of 62.5 to 500 μg mL^{-1}. After 48 hours, ethanolic and aqueous extracts showed potent activity with IC_{50} in the range of 62.5-125 μg mL^{-1} (Fig. 2).

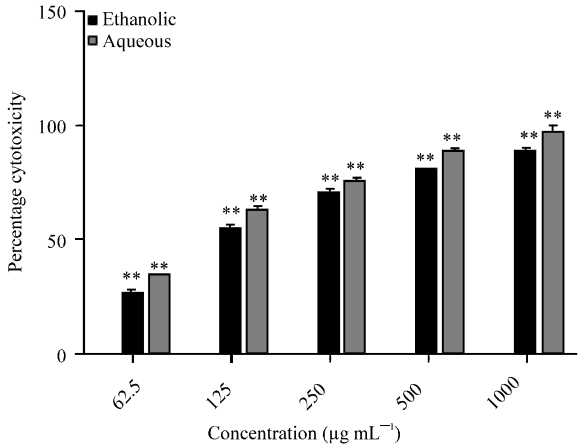

Fig. 1: Effect of various extracts on brine shrimp lethality. All the values are Mean±SEM of three samples, **p<0.01 compared to control

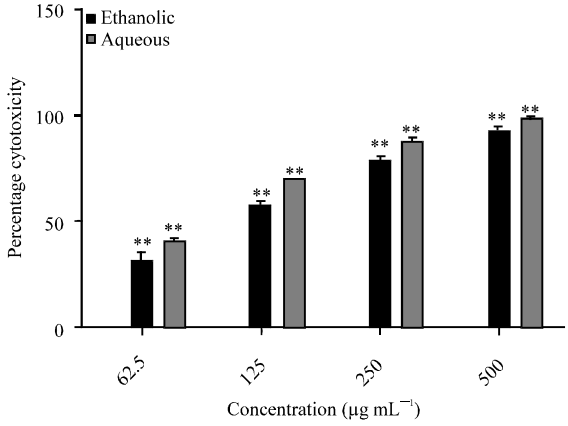

Fig. 2: *In vitro* cytotoxic activity of various extracts in MCF-7 cells (Human breast adenocarcinoma cells) by MTT assay at 48 h of exposure. All the values are Mean±SEM of three samples, **p<0.01 compared to control

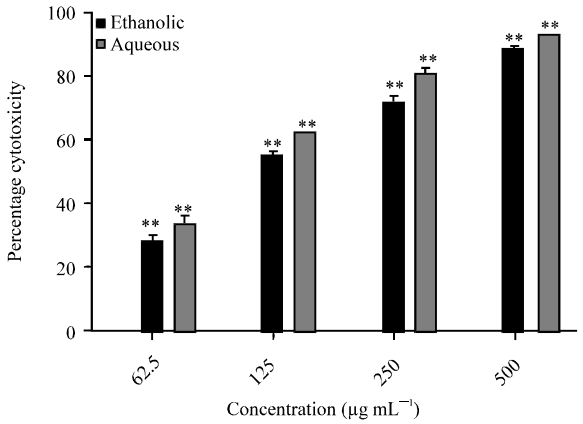

Fig. 3: *In vitro* cytotoxic activity of various extracts in MCF-7 cells (Human breast adenocarcinoma cells) by SRB assay at 48 h of exposure. All the values are Mean±SEM of three samples, **p<0.01 compared to control

***In vitro* cytotoxicity in MCF-7 by SRB assay:** In SRB assay also both the extracts showed significant cytotoxicity in MCF-7 cells. After 48 hours, both extracts showed potent activity with IC_{50} value in range of 62.5-125 µg mL^{-1} but the cytotoxicity of extracts was less in SRB assay as compared to MTT assay (Fig. 3).

DISCUSSION

As cancer is one of the most prevalent diseases second only to cardiovascular disease leading to the mortality. Inspite of tremendous scientific investigations are making best efforts to combat this disease, the sure-shot, perfect cure is yet to be brought into world medicine. Hence, the search for a molecule with the selective antitumor activity devoid of many of the side effects of conventional chemotherapy is ongoing process till the goal is reached. In this context we made an attempt to assess the possible cytotoxic activity of *Dendrophthoe falcata* in various *in vitro* models.

The results obtained in the present study indicate that *D. falcata* stem extracts exhibit potent cytotoxic activity in various *in vitro* models. The activities might be attributed to its polyphenolic content and other phytochemical constituents.

It was reported that the medicinal properties of *D. falcata* is greatly influenced by the host plant. For example, when grown on *Calotropis gigantea*, this parasitic plant is considered useful for improving cognitive function. However, when it is grown on *Tamarindus indicus*, it is used to treat impotence and the plant grown on Shorea robusta (Sal tree) is used to treat paralysis by the tribes of the Bihar state of India In addition to its medicinal value, the fruits taste sweet and are consumed as a food (Pattanayak *et al.*, 2008). The entire plant is used extensively in traditional medicine as an aphrodisiac, astringent, narcotic, diuretic and for the treatment of pulmonary tuberculosis, asthma, menstrual disorders, swellings, wounds, ulcers, strangury, renal and vesical calculi and vitiated conditions of kapha and pitta (Warrier *et al.*, 1993).

The plant-derived extracts containing antioxidant principle showed cytotoxicity toward tumor cells (Gupta *et al.*, 2004). Various phytoconstituents isolated from the plant are flavonoids, viz. quercetin, kaemferol, rutin, quercetrin etc., from *D. falcata* growing on six different host plants (Nair and Krishnakumary, 1989), cardiac glycoside strospeside, neritaloside and odoroside from leaves of *D. falcata* growing on *Nerium oleander* (Rastogi and Mehrotra, 1990), (+) - catechin, leucocyanidin, gallic acid, ellagic acid and chebulinic acid from the leaves and bark of *D. falcata* growing on *Terminalia tomentosa* (Indrani *et al.*, 1980).

Several studies on flavonoids, specifically some kaempferol glycosides, caused cytotoxic activity against human leukemic cell lines *in vitro* and other cancer cells have been demonstrated (Dimas *et al.*, 2000; Nakamura *et al.*, 2005; Schmidt *et al.*, 2006). Two flavonoids, Kaempferol and quercetin present in the extract have been demonstrated to induce apoptosis by releasing of cytochrome c, procaspase-9 processing and through a caspace-3-dependent mechanism. Through one or more of the above biochemical mechanisms, quercetin has been reported to protect against both chemically induced and spontaneous formation of tumors in animals (Lipkin *et al.*, 1999; Duthie *et al.*, 2000) and arrest cell proliferation in a variety of transformed cell lines in culture (Singhal *et al.*, 1995; Richter *et al.*, 1999). The potential antiproliferative effects found in this research might be attributed to quercetin and other compounds present in the extracts of *D. falcata*.

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