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IN-VITRO CYTOTOXIC STUDIES OF *Viburnum punctatum* Buch. - Ham. ex D. Don ETHANOLIC LEAF EXTRACT AGAINST MCF-7 BREAST CANCER CELL LINES

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Abstract

Viburnum punctatum Buch.-Ham. ex D. Don belongs to Caprifoliaceae family is a medicinally important plant also known as "Konakaram" commonly in Tamil. It is a small evergreen tree, commonly found in moist forests and in sholas, above 1200 m in South East Asia. Many species of *Viburnum* are recognized for their medicinal properties from very early times of this century. The *Viburnum punctatum* leaves were traditionally used for the treatment of fever, stomach disorder and mentioned to possess the anti - periodic effect. The ethanol extract of *V. punctatum* leaves were evaluated for *invitro* cytotoxicity studies against breast cancer MCF – 7 cell lines using MTT cellular viability assay. The result showed a remarkable anticancerous activity at all concentrations in a dose dependent manner. By increasing the concentration of the ethanolic extract, the average absorbance is decreased. When determining 6.45 % inhibition at 10 µg/ ml, the highest absorbance of 1.102 was recorded while 0.429 absorbance was recorded with 63.58 % inhibition at the concentration of 100 µg/ m of ethanol extract. The IC_{50} value is 56.73 µg/ ml.

Keywords: *Viburnum punctatum* Buch.-Ham. ex D. Don, Ethanolic leaf extract, MCF – 7 cell lines, MTT assay

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INTRODUCTION

Most of the reasons seem worldwide that the cause of death next to cardiovascular disease as well as the second leading root of death is Cancer. Mortality (the death rate) is still unaccceptably high despite many therapeutic advances [1]. Drugs used today in the clinics mostly were first discovered from microorganisms and plants [2]. Natural products are of increasing interest and is important to cancer patients. It has been reported that these natural production that is the plant products have gained increasing interests and importance to cancer patients. Apoptosis is an event that plays important role in organism development and homeostasis. Apoptosis is characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation that is consistent with DNA cleavage in ladders [3, 4]. In the anticancer properties of many drugs, the induction of apoptotic cell death is an important mechanism. Breast cancer is the most commonly found cancer in women when observed among the various types of cancer [5]. For the treatment of almost all types of cancer, normally a chemotherapy technique is employed. Anti - cancer drugs can only be used effectively on the other hand for the treatment of a specific type of cancer in the affected patients. A synthetic drug for example, tamoxifen that is only effective against receptor positive breast cancer cells. They are found to be ineffective against oestrogen receptor negative breast cancer cells [6, 7]. Secondly, in chemotherapy in tumour cells resistance developed against chemotherapeutics are the major limitations in cancer treatment [8]. Hence a necessity has arisen for finding novel natural products and broad range products. Against cancer, these natural products may act as chemoprotective and therapeutic agents. From medicinally important plants, active compounds are derived. These active compounds inhibit either a few types of cancer cells and/ or found effective against a wide array of cancers [1]. Thus to explore the hidden treasures of nature, the screening of plant extracts is worth demanding [9, 10, 11]. *Viburnum punctatum* Buch.-Ham.ex D.Don (*Viburnum acuminatum* Wall) belonging to Caprifoliaceae family, is a medicinal plant under the order Dipsacales [12]. It belongs to the monotypic genus Viburnum, native to India, Nepal, Bhutan, Thailand, Cambodia, Vietnam, Indonesia and China. It is a shrub or medium sized tree, growing at an altitude not less than 1500 m; profusely with other plants in Nilgiri, Himalaya and Coimbatore. The leaves were traditionally used for the treatment of fever, stomach disorder and mentioned to possess antiperiodic effect [13]. The aim of the study was to evaluate the invitro cytotoxicity studies of leaf extracts of *Viburnum punctatum* Buch.-Ham.ex D.Don growing in Nilgiri Hills, Tamilnadu, India.

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MATERIALS AND METHODS

Collection of plant material

The fresh healthy plant leaves were collected from the Nilgiri Hills, Tamil Nadu, India during the month of June - July. The collected plant material was identified and authenticated by Dr. M. Palanisamy, Scientist, Botanical Survey of India, Southern Regional Centre, Coimbatore, India (Voucher No. BSI/ SRC/ 5/ 23/ 2014-15/ Tech/ 513) and a voucher specimen has been reserved in the Department of Biotechnology, Sri Ramakrishna College of Arts and Science (Autonomous), Coimbatore, Tamil Nadu, India.

Processing of plant material

Freshly collected leaves of *Viburnum punctatum* were washed thoroughly with tap water, shade dried and then homogenized to a fine powder using a mechanical grinder and stored in airtight bottles. The powdered leaves were seived through a No. 40 sieve for powder analysis.

Extraction of plant material

50 grams of pulverized leaf material were soaked separately in 250 ml ethanol and kept on a rotary shaker for 24 hours. The extracted material from solvents was filtered through a Whatmann No. 1 Filter paper in separate flasks and the process was repeated until all the soluble compounds had been extracted. The extract was then concentrated under reduced pressure in a rotary evaporator, weighed and stored at 4°C until further analysis.

Cell lines and culture

The human breast cancer cell line (MCF - 7) was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Eagle's minimum essential medium containing 10% Fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO2, 95% air and 100% relative humidity. Maintenance of cultures was passaged weekly and the culture medium was changed twice a week.

Cytotoxicity assessment (MTT assay)

The cytotoxic effect of compounds against human tumor cell lines was determined by a rapid colorimetric assay, using 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay and compared with untreated controls [14].

For screening experiment, the cells were seeded in 96 - well plates in 100µL of medium containing 5% FBS, at plating density 10,000 cells/ well and incubated at 37 °C, 5% CO2, 95% air and relative humidity (100%) for 48 h prior to addition of compounds.

After 48h, compounds at various concentrations was added and incubated at 37 °C, 5% CO2, 95% air and relative humidity (100%) for 48 h. Triplicate was maintained and the medium containing without the sample were served as control. After 48 h, 50 µL of MTT (5 mg/ml) in

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triple distilled water was added to each well and incubated at 37 °C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µL of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage cell inhibition was determined using the following formula

Cell inhibition (%) = 100 - Absorbance (Sample)/Absorbance (control) x 100

The results were expressed as the mean percentage of cell growth inhibition. The IC50 value was expressed as the concentration of compounds that inhibited the growth of cells by 50%.

Statistical Analysis

All the experiments were performed in triplicate. Each replicate was considered as individual unit. The results were statistically analyzed using Dunnett's multiple comparison test. The results were analyzed at $p < 0.05$ (significant); $p < 0.01$ (highly significant) and $p < 0.001$ (very highly significant).

RESULTS

To determine the cytotoxicity of the leaf extracts from *Viburnum punctatum* on breast cancer cell line MCF - 7 cells, the tetrazolium cellular viability assay was used in this study [14]. MTT cellular viability assay assisted in obtaining the determination of the effect of cancer cell proliferation. A tetrazolium salt dye used in a colorimetric assay is the 3-[4, 5-dimethylthiazol-2-yl] -2, 5-diphenyl -2H- tetrazolium bromide. It measures the modification of the yellow substrate to an insoluble dark blue/ purple formazan product.

A linear increase in the production of formazan dye is due an increase in the mitochondrial enzyme activity. The number of metabolically active cells and the measured quantity of formed formazan dye is directly correlated, yielding an accurate measurement of cell viability and thus toxicity. This formazan is solubilized by the addition of DMSO or isopropanol as the formazan dye is insoluble in the reaction medium, and the intensity of colour is measured spectrophotmetrically.

Plants serve as a base for modern medicines and have been used as traditional medicinal agents. A remarkable anticancerous activity at all concentrations in a dose dependent manner is shown by the ethanolic extract of V. punctatum (Table 1.1, Fig. 1). By increasing the concentration of the ethanolic extract, the average absorbance is decreased. When determining 6.45 % inhibition at 10 µg/ ml, the highest absorbance of 1.102 was recorded while 0.429 absorbance was recorded with 63.58 % inhibition at the concentration of 100 µg/ m of ethanol extract. The IC50 value is 56.73 µg/ ml.

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Table 1: *In-vitro* Cytotoxicity (MTT Assay) of VPL extract on MCF - 7 Cell Line

Concentration (µg/ml)	OD at 575 nm	% of viable cells	% of inhibition
10	1.102	93.54 ± 0.178	6.45 ± 0.085
20	1.028	87.26 ± 0.415	12.73 ± 0.150
30	0.985	83.61 ± 0.663	16.38 ± 0.145
40	0.965	81.91 ± 0.138	18.08 ± 0.167
50	0.758	64.34 ± 0.115	35.65 ± 0.149
60	0.555	47.11 ± 0.479	52.88 ± 0.250
70	0.534	45.33 ± 0.381	54.66 ± 0.165
80	0.479	40.66 ± 0.219	59.33 ± 0.180
90	0.447	37.94 ± 0.282	62.05 ± 0.434
100	0.429	36.41 ± 0.305	63.58 ± 0.172

The values are mean ± Standard Error Mean (n = 3).

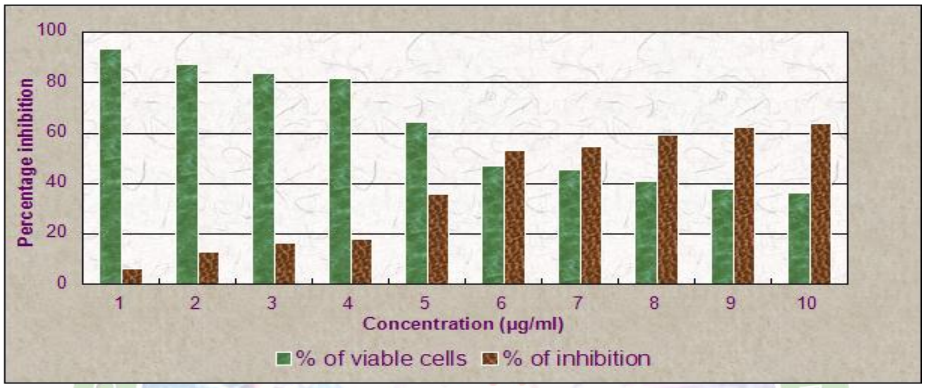


Fig. 1: *Invitro* Cytotoxicity (MTT Assay) of VPL extract on MCF - 7 Cell line

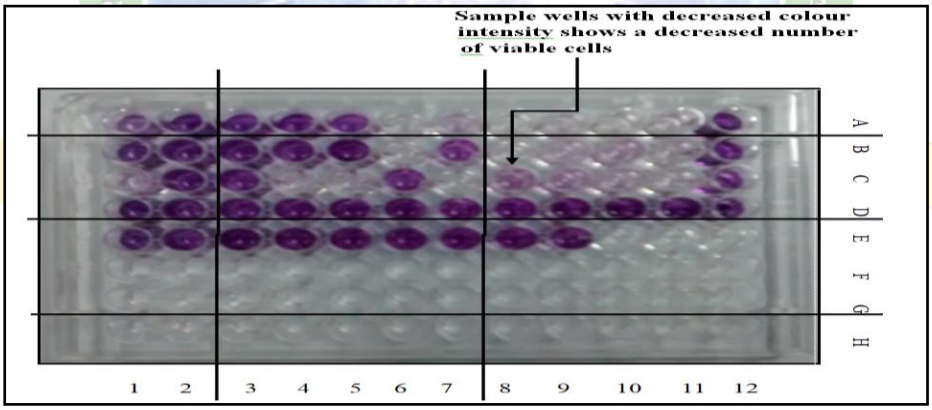
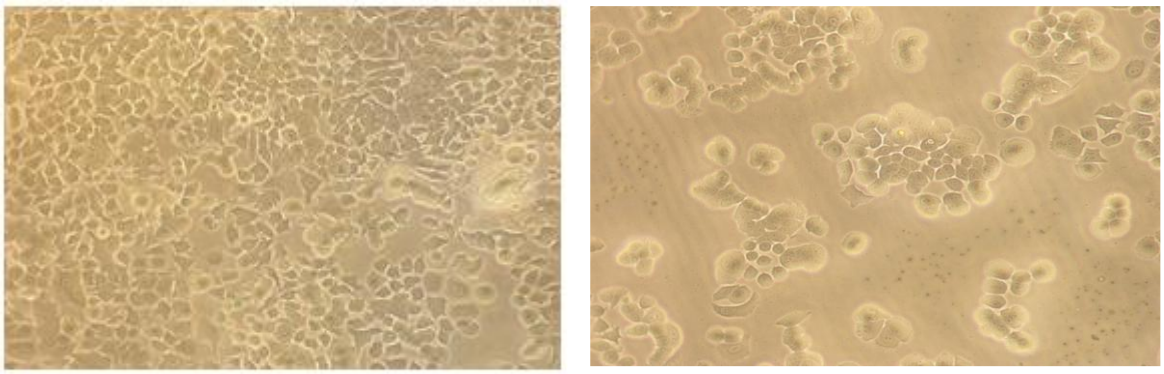


Fig. 2: Microtitre plate showing the colour intensity of viable cells



A **B**

Fig. 3: (A) Untreated MCF – 7 cell line (B) Treated MCF – 7 cell line with VPL extract

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DISCUSSION

A cellular malignancy that results in the destruction of normal cell - cycle control is cancer. Loss of normal cell - cycle includes unregulated growth and the lack of differentiation. This can develop at any time in any time of any organ. The oesophagus, lungs, liver, stomach, pancreas, ovaries and breasts are mostly affected by cancer [15]. There has been a continuous struggle by the researchers to explore in order to find plants with a wide range of activities from antibiotic to anticancer. The use of natural products in controlling cancer among other diseases has gained popularity [16]. This is because drugs used in the treatment of cancer adversely affect the normal body cells [17]. To evaluate the anticancer potential of extracts by MTT assay breast cancer cell line, MCF - 7 was used. MCF - 7 is known as an estrogen receptor positive cell line. It serves as a model for studying response of therapeutic agents against breast cancer cells and it has the ability to show inhibition effects which are the quick responses [18, 19]. A guide for screening potent plants against cancer is the cytotoxic assays [20]. To evaluate the reduction of viability of MCF - 7 cell line culture either in the presence or absence of extracts is the standard MTT assay (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide) method [21]. The ability of mitochondrial dehydrogenase only in viable cells is to reduce MTT to a blue formazan product [22]. An extract sample has the capability to impure reduction potential of mitochondrial dehydrogenase in cancer cells. This has been proved to be effective to be used in cancer therapy. This would require the inhibitory concentration at which 50% of the cells are inhibited [23]. Each tumour has its own individual drug response. This was conducted by the researchers. They are highly individualistic [24]. The most sensitivity to indigenous Viburnum species was exhibited by the MCF - 7 cells. Cancer cell specificity against the MCF - 7 cell line was displayed by extracts from Viburnum punctatum leaves. Here the percentage cell growth inhibition at 10 µg/ ml was found to be 93.54 ± 6.45 % and 36.41 ± 63.58 % for 100 µg/ ml, respectively. Extracts that presented activity against the MCF - 7 cell lines was promising. An inhibitory effect was exhibited and the IC50 value for this species that was determined was 56.73 µg/ ml. From these extracts, the isolation of anti - inflammatory agents may lead to promising anti - cancer compounds. For screening of bioactive components, Cordell (1995) and Kusumoto et al. (1995) also suggested that solvent extracts testing is beneficial [25, 26]. Ethanol extract of V. punctatum exhibits anticancerous activity against breast cancer cell line MCF - 7. On the basis of this significance, the leaf extract was fractionated. At a concentration of 100 µg/ ml, the fractions were again tested against MCF - 7 cell line. Inhibition at the highest percentage (63.58 %) was measured by ethanol fraction. Regarding bio - activities, the role played by phytochemicals is

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well established [27, 13]. In antioxidant studies, the ethanolic extract of V. punctatum leaves exhibited better free radical scavenging property [28]. This suggests that the antioxidant property of V. punctatum leaves might contribute to their anticancer effect on MCF – 7 cell lines. The leaves and twigs of Viburnum awabuki showed cytotoxicity against selected cancer cells was measured [29]. It is also reported that the methanol fraction of V. foetens Dene is a good candidate for isolation of anticancerous compounds [30]. The inhibition against the MCF - 7 cell line with 99% and 96% from the methanol and ethyl acetate fraction of V. foetens crude extract was also stated in previous studies [11]. A number of plant extracts have also shown to regulate the epidermal growth factor receptor that overexpressed in breast cancer [31]. The inhibition of cell proliferation by these fractions indicates the presence of anticancer compounds. The plant species V. foetens also exhibits anticancerous property and efficacy against MCF - 7 cell line [32]. The stems and roots of V. grandiflorum was reported to contain anti nociceptive activity, cancer and cytotoxic effect [33]. The plant agents against cancer, microbes and tumours are considered as the flavonoids [34, 35]. A strong inhibition of tumours is also reported by tannins. Another class of cytotoxic compounds showing activity in plants is the coumarins and their derivatives [36]. Maximum inhibition of ethanol fraction in view of strong cytotoxic potential of flavonoids, tannins and coumarins is implied strongly to be owed to phytochemicals either synergistically as suggested in previous studies [37]. A conclusion was arrived from the activity of a fraction of plant extract that says this activity might be due to synergistic effect of phytochemical constituents present in the extract sample or individually has led to the isolation of cytotoxic compounds from cytotoxic fractions due to bioactivity [38].

CONCLUSION

This study aimed to evaluate the *invitro* cytotoxicity study of *V. punctatum* ethanolic leaf extract against MCF - 7 breast cancer cell lines. This present investigation provide an important information that the ethanolic extract of *V. punctatum* leaves have potent cytotoxic activity against MCF – 7 cells. The outcome of the present study encourage to carry out further studies in other cell lines and *invivo* cytotoxic investigation required to identify anticancer activity.

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