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Inhibition of human breast and colorectal cancer cells by *Viburnum foetens* L. extracts *in vitro*

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PEER REVIEW

Peer reviewer

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Comments

The overall the manuscript is written well and the results are explained in a good way by providing the suitable references. So this will be helpful for new anticancer drug's exploration in the future.

(Details on Page 35)

ABSTRACT

Objective: To investigate efficacy of *Viburnum foetens* (*V. foetens*) extracts against different cancer lines. **Methods:** The crude extract and fractions of *V. foetens* are evaluated against MDA MB-468 and Caco-2 cancer cell lines by using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assays. These extracts are also tested against breast carcinoma and human colon adenocarcinoma through NRU (neutral red uptake) assay. **Results:** The crude extract inhibited the cancerous cell growth in a dose dependent manner. From the MTT assay it is obvious that the ethylacetate fraction significantly inhibited the growth of Caco-2 (93.44%) cell. Similarly, the methanol and ethylacetate fractions shows 99% and 96% inhibition of MCF-7 and Caco-2 cell lines by NRU assay. Furthermore, the ethylacetate fraction also exhibited momentous inhibition of MDA MB-468 cells in both assays. Other fractions i.e. chloroform, hexane also inhibited cancer cell proliferation at a significant level. Natural products exhibited significant activity against multiple cancerous cells. **Conclusions:** In this framework, we can speculate that the present study will be helpful in the identification and isolation of novel anticancer drug compounds from the crude extract (*i.e.*, methanol and ethyl acetate fractions) of *V. foetens*.

KEYWORDS

Anticancer, MTT assay, NRU assay, *Viburnum foetens*

1. Introduction

Among the various types of cancer, breast cancer is the most commonly found in the women, while colorectal cancer is second most common type which propagate in both women and men[1]. Normally, a chemotherapy technique is employed for the treatment of almost all types of cancers. On the other hand, anti-cancer drugs can only be used effectively for the treatment of a specific type of cancer in the effected patients. For example a drug known as tamoxifen, a synthetic drug, is only effective against estrogen receptor positive breast cancer cells, but ineffective against estrogen receptor negative breast cancer cells[2,3]. Secondly, the resistance developed in tumour cells against chemotherapeutic drugs, and the side effects of chemotheuraptics are the major limitations in cancer treatment[4]. Thus, there is a need for finding broad range and novel natural products.

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The natural products may act as chemoprotective and therapeutic agent against cancers. The active compounds derived from medicinally important plants inhibit either few type of cancer cells and/or found effective against wide array of cancers[5]. Therefore, the screening of plant extracts is worth demanding to explore hidden treasures of nature[6]. The *Viburnum foetens* L. (*V. foetens*), a deciduous shrub is abundantly found in Himalaya Pakistan at an altitude ranges between 1 500-3 000 m. The ethanobotanical studies revealed purgative and sedative properties of *V. foetens* in addition to their use as tooth brush and blood purifier by local people[7,8]. Moreover, this plant also exhibit antidiabetic[9], and antibacterial properties[10].

In our previous findings, we have reported the efficacy of *V. foetens* against MCF-7 cell line[10]. Inspired from the previous finding, we are further interested to investigate its efficacy against other cancer cell lines, *e.g.*, MDA MB-468 and Caco-2 by performing MTT and NRU assays.

2. Materials and methods

2.1. Plant material preparation

The fresh *V. foetens* plant material was collected from Ayubia KPK Pakistan. The plant was identified by Dr Mir Ajab Khan, Department of Plant Sciences Quaid-i-Azam University Islamabad, Pakistan, after examining preserved specimens in the herbarium. The plant material was washed thoroughly under running tap water and subsequently, shade dried.

2.2. Extraction and fractionation

The extraction and related fractionations were prepared by following the procedure developed by Bibi *et al*[11]. Briefly, the dried plant material (1.5 kg) was first powdered and dipped in methanol (2 L) for a period of 7 d at room temperature. Then the extract was filtered, the residue again immersed in methanol for additional seven days and, thereafter, filtered. The extracts were combined and evaporated under reduced pressure at 40 °C. A dark green semi-solid residue (475 g) was obtained. The crude extract (470 g) was dispersed in 500 ml H_2O, partitioned into four organic fractions in increasing order of the polarity starting with hexane followed by chloroform and the ethylacetate fractions. Aqueous part was dried and methanol was added to obtain methanol soluble part as methanol fraction and methanol insoluble as aqueous fraction. This procedure resulted in the hexane fraction (43 g), chloroform fraction (172 g), ethyl acetate fraction (96 g), methanol fraction (98 g) and aqueous fraction (50 g).

2.3. Cell culture

Breast cancer cell lines MCF-7 (human breast estrogen-dependent adenocarcinoma) and MDA-MB-468 (human breast estrogen-independent adenocarcinoma) cells were purchased from LGC Standards, Teddington, UK. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) provided with 15% heat inactivated foetal bovine serum (FBS), 40 μg/mL gentamycin, 100 units/mL penicillin and 1.04 mg/mL streptomycin.

Human colon cancer cell line Caco-2 (human colon adenocarcinoma) was obtained from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK) and cultured in complete growth medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS and 2 mmol/L l-glutamine (Sigma Aldrich, UK). All cell lines were maintained in a humidified incubator at 37°C in an atmosphere containing 5% CO_2 and in the logarithmic phase of growth at the time at which cytotoxic assays were performed.

The cells were seeded into 96-well tissue culture plates at a density of 1×10^4 cells per well in 200 μL aliquots of medium after successful harvesting. The cells were allowed to attach on the plates surface for 24 h at 37 °C and 5% CO_2 in a humidified atmosphere. The plant extracts dissolved in DMSO to prepare different concentrations of crude extract and fractions were added in seeded plates and left for an exposure period of 24 h. The control groups received the same amount of DMSO. The positive control used was Actinomycin-D (4 μmol), in 200 μL media as a final concentration in well.

2.4. Neutral red uptake assay

The inhibition of cell growth was determined by neutral red uptake (NRU) assay; the method developed by Borenfreund and Puerner[12]. The living cells can only take up red dye and incorporate in lysosomes, while dead or damaged cells did not show any activity.

After incubating the cells with extracts, the medium was removed and neutral red solution (40 μg/mL) was added to each well including control samples. Then the plates were incubated for further 2.5 h. The neutral red solution was carefully removed and rinsed with pre-warmed D-PBS. The ethanol/acetic acid (1% glacial acetic acid in 5% ethanol) at 200 μL per well was added. The plates were covered with aluminium foil, and placed on a plate shaker for 30 min in order to extract neutral red from the cells and form a homogeneous solution. The absorbance was measured at 540 nm in a microplate reader (Labtech LT-4000MS, Labtech International Ltd., Acorn House, East Sussex, UK).

2.5. MTT assay

The assay for 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide (MTT) involves the quantification of growth of cancer cells by the ability of living cell's mitochondrial succinic dehydrogenase enzyme to reduce the yellow dye MTT to a blue insoluble product formazan[13].

After 24 h exposure period of extracts, cells were washed twice with PBS and 10 μL MTT reagents (5 mg/mL in PBS) were added in each well. The plates were incubated for

additional 4 h. The cells were again washed twice with PBS and DMSO (100 μL/well) was added. DMSO dissolves the insoluble crystalline formazan products. The drug efficacy was determined as the percentage of control absorbance of reduced dye at 550 nm.

The experiments for each extract were carried out in triplicate including untreated cell control and a blank cell-free control. Each concentration was tested in triplicate. The inhibitory rate of cell proliferation was calculated by the following formula;

$$\text{Percentage inhibition} = \frac{\text{OD control} - \text{OD treated} \times 100}{\text{OD control}}$$

2.6. Statistical analysis

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

3. Results

The cells died during the different developmental and stress processes. However, in the cancer cells such apoptotic processes remain inactivate. This is the main reason to find effective strategies against tumour propagation. The studies showed that the plants and their extracts have a great potential to find new strategies in anticancer research[14]. The present study was focused to determine the ability of *V. foetens* extract against proliferation of human breast cancer cell lines and human colon endocarcinoma. In our case the crude extract of *V. foetens* exhibited good activity in dose dependent manner against MCF-7, MDA-MB-468 and Caco-2 cell lines, while the different fractions also confirmed the significant inhibition.

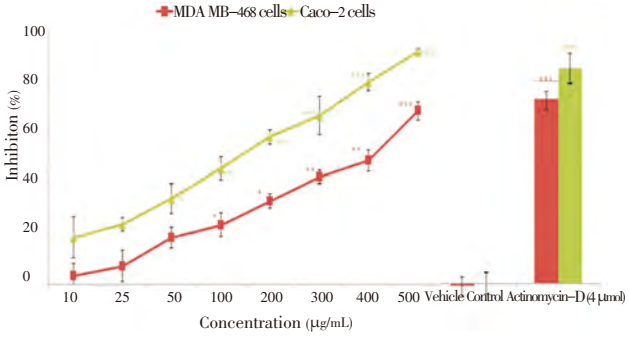

Figure 1. Cytotoxic activity of crude extract of *V. foetens* using MTT assay.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

A concentration dependent inhibition of malignant cells was evaluated showing apparent IC_{50} value of 100 μg/mL in NRU assay. The maximum inhibition with the value of 98.8% and 96.5% exhibited by the MCF-7 and Caco-2 cells, respectively. This was measured by using NRU assay at the concentration 500 μg/mL. While 81.4% inhibition of MDA-MB cells was recorded by *V. foetens* crude extract (Figure 1). The dose dependent inhibition has also been reported by several other researchers working in the same field[15-18].

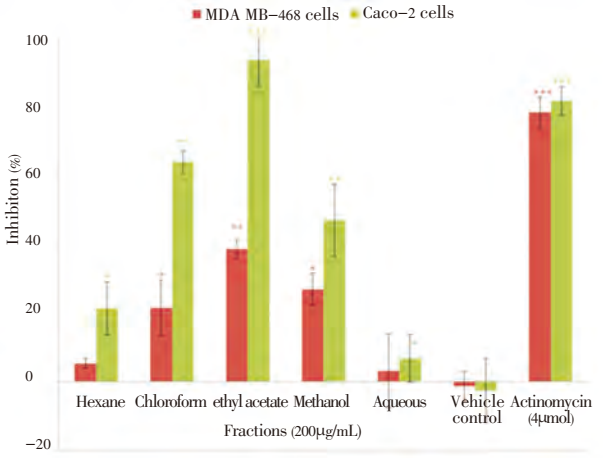

Figure 2. Cytotoxic activity of fractions from *Viburnum foetens* crude extract using MTT assay.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

The methanol fraction of *V. foetens* crude extract exhibited maximum inhibition 99% against MCF-7 cell line. While significant results were also presented by the ethylacetate fraction with 75% inhibition against MCF-7 cell line. The ethylacetate fraction also showed 96% inhibition against Caco-2 cells (Figure 2). The chloroform fraction also showed noteworthy results against MCF-7 and Caco-2 cell line. A substantial inhibition of cancer cell proliferation was also in the case of chloroform, methanol and ethylacetate fractions against MDA-MB cells. A number of plant extracts have also shown to regulate epidermal growth factor receptor that overexpressed in breast cancer[19]. The inhibition of cell proliferation by these fractions indicates the presence of anticancer compounds.

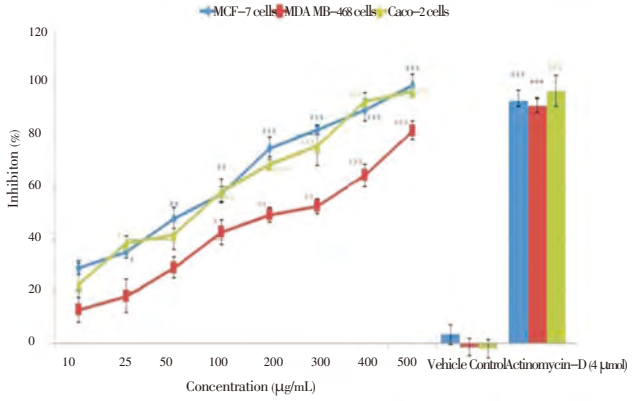

Figure 3. Cytotoxic activity of crude extract of *Viburnum foetens* using neutral red uptake assay.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

In cell proliferation and cytotoxicity assays, cell viability can also be determined using MTT assay[20]. We have already reported the MTT assay results for *V.*

foetens against MCF-7 cells, where a dose dependent inhibition effect was observed[10]. Crude extract also inhibited the growth of MDA-MB-468 (68.1% inhibition) and Caco-2 cells (91.4% inhibition) at 500 μg/mL (Figure 3). The apparent value of IC_{50} was found to be 200 μg/mL in MTT assay against all the three cell lines tested. It was observed that MCF-7 cells sharply inhibited by crude extract up to 50 μg/mL while against Caco-2 and MDA-MB-468 cells, it was a linear inhibition up to 500 μg/mL. The low activity of extract against MDA-MB-468 cell might be due to resistance towards apoptosis. The disruptions in the signaling pathways or changes in the expression of enzymes related to apoptosis are associated with tumor resistance[21]. It has also been reported that plants extracts might be selectively toxic to tumor cell not to normal cell[22]. The failure of normal apoptotic mechanism in cancerous cells supports the course of carcinogenesis. Cancerous cells undergo molecular and biochemical changes that resist their susceptibility to apoptosis. Thus for the chemotherapeutic agent to be accepted and developed as a potential anti-cancer drug, it should possess direct cytotoxic activity on the cancer cells[23].

MCF-7 cell line with maximum inhibition (83%) was shown by methanol fraction of *V. foetens*[10]. The ethylacetate fraction was found active against MDA-MB-468 and Caco-2 cell lines. Against MDA-MB-468, 38.47% inhibition was observed which was highest among the other fractions (Figure 4). However, 93.4% inhibition was exhibited by this fraction against Caco-2 cell line that was significantly high. The differential efficacy of the same fraction against different cancer cell lines might be due to their differential properties[24]. The chloroform fraction was also found to be significantly active against Caco-2, representing an inhibition of 63.8%. The aqueous fraction, however, proved inactive in MTT assay against all the three cell lines. The lack of activity in aqueous extracts might be due to insolubility or the absence of insufficient quantities of active components[11]. Many therapeutic agents are known to suppress human cancerous cells growth. However they might be cytotoxic to normal cells or might not be effective against multiple cancer cell lines. Our results clearly demonstrate that the active constituents of *V. foetens* can be used effectively against different cancerous cells.

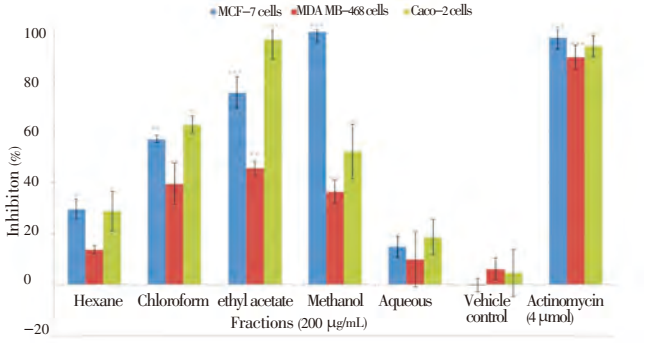

Figure 4. Cytotoxic activity of fractions from *Viburnum foetens* crude extract using neutral red uptake assay.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

4. Discussion

In this study we have showed for the first time that *V. foetens* exhibits significant antiproliferative activity against MCF-7, Caco-2 and MBA-MB-468 cell lines. Recently, Bae et al. reported that *V. awabuki* contain 9′-O-methylvibsanol that inhibit MCF-7 and A-549 cells[25]. *V. foetens* might also contain this compound along with others. However, *Viburnum* species have potent antiproliferative compounds that can be used against a number of cell lines. These results also provide a basis for the potential therapeutic application of 9′-O-methylvibsanol and/or its related compounds to cancer therapy.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The manuscript describes screening of plant extract against different cancer cell lines using two different assays, MTT and NRU. Human cancer is increasing day by day and there is dire need to locate effective therapies. This manuscript will be handful addition in its field of research.

Research frontiers

The author reported the efficacy of *V. foetens* extracts for the inhibition of human breast cancer and colorectal cancer cells. The extract fractions are studied against MDA MB-468 and Caco-2 cancer cell lines by using MTT and NRU assays. For MTT assay it author have used ethylacetate fraction which exhibit significant inhibition for the growth of Caco-2 cell. Also methanol and ethylacetate fractions also shows good inhibition of MCF-7 and Caco-2 cell lines by NRU assay. The manuscript could be published as a full length article by considering the following minor corrections in the manuscript.

Related reports

MTT and NRU assays provide basic screening of natural extracts or compounds. The methodology in the manuscript is well defined and results are presented in a good way.

Innovations & breakthroughs

The authors for the first time reported efficacy of *V. foetens* extract and its fractions against multiple cancer cell lines. This study can provide basis to isolate active constituents that could be used against different cancer therapies.

Applications

Applicable, however further studies are required.

Peer review

The overall the manuscript is written well and the results are explained in a good way by providing the suitable references. So this will be helpful for new anticancer drug's exploration in the future.

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