RESEARCH ARTICLE

MINOR FRUITS OF JAMMU SUBTROPICS POSSESS *IN VITRO* CYTOTOXICITY AGAINST HUMAN CANCER CELLS

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Received-07.07.2018, Revised-26.07.2018

Abstract: The present research work was carried out to examine the *in vitro* cytotoxic effect of four minor fruits of kandi belt of Jammu region *viz.*, *Emblica officinalis* (amla), *Morinda citrifolia* (shehtoot), *Olea europaea* (jaitun), *Ziziphus mauritiana* (ber) against nine human cancer cell lines from eight different origins such as A-549 (lung), A-498 (renal), HCT-116 (colon), MCF-7 (breast), MDA-MB-435 (melanoma), OVCAR-5 (ovarian), PC-3 (prostate), SF-295 (CNS), T-47D (breast). Methanolic extracts of fruits were used as test material and *in vitro* cytotoxicity was determined at 100 µg/ml *via* SRB assay. Results revealed that all the minor fruits (except shehtoot) showed *in vitro* cytotoxic efficacy against one or the other human cancer cell line with growth inhibition range of 70-99%. Maximum growth inhibition against more number of human cancer cell lines was produced by amla followed by ber and jaitun. When evaluated at lower concentrations, ber fruit exhibits significant *in vitro* cytotoxic potential (79% & 70%) at 50 and 30 µg/ml respectively against lung cancer cell line (A-549). Ber also suppressed 70% proliferation of melanoma cancer cell line (MDA-MB-435) at 50 µg/ml. To conclude, ber possesses certain constituents with cytotoxic properties that will be effective against melanoma and lung cancer cells.

Keywords: Amla, Ber, Cancer Cells, *In vitro* cytotoxicity, SRB assay

INTRODUCTION

Now a days, many options for the treatment of cancer exist (surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy), but the sure-shot, perfect cure is yet to be brought into the world of medicine. Fruits consumed in our daily diet could be a solution to this deadly disease by providing chemo protective and chemotherapeutic remedy. Fruit of *E. officinalis* is of immense use in various folk / traditional systems of medicine (Zhang *et al*., 2003; Khan, 2009), is reported to possess hepatoprotective, cardioprotective, stomachic, anti-inflammatory properties and is used for a variety of ailments such as anemia, hyperacidity, diarrhea, jaundice, nerve debility, liver complaints, cough (Pandey, 2002). Aqueous extract of amla inhibited the growth of A-549 (lung), HepG2 (liver), HeLa (cervical), MDA-MB-231 (breast), SK-OV-3 (ovarian), SW-620 (colorectal) cells *in vitro* and also induced apoptosis in HeLa, A-549, MDA-MB-231, SK-OV-3 cells (Ngamkitidechakul *et al*., 2010). Moreover, the amla extract possesses antiproliferative activity in MCF-7 and MDA-MB-231 - breast cancer cell lines (Lambertini *et al*., 2004). The extract was devoid of cytotoxic effect on normal ovarian cell line, suggesting it to be selectively cytotoxic to only neoplastic cells (Sumantran *et al.,* 2007). *M. citrifolia* fruits, rich in phenolic compounds including flavonoids, anthocyanins, carotenoids (Lin and Tang, 2007; Sass-Kiss, 2005) has been reported to have not only antioxidative, antitumor, antidiabetic effects, but also cardiovascular, hepato and neuro-protective properties (Andallu *et al.*, 2001; Huang *et al*., 2008; Kimura *et al.*, 2007; Isabelle *et al.*, 2008). Mulberry fruit extracts result in human glioma cell death *in vitro* through the reactive oxygen species (ROS)-dependent mitochondrial pathway and glioma tumor growth *in vivo via* reduction of tumor cell proliferation and the induction of apoptosis (Jeong *et al.*,2010). *O. europaea* fruit extracts inhibit proliferation and induce apoptosis in HT-29 human colon cancer cells (Juan *et al.*, 2006). Antioxidants are present in olive/olive oil and consumption of antioxidants is believed to reduce the risk of mutagenesis / carcinogenesis (Waterman and Lockwood 2007; Visioli *et al.*, 2004). Olive oil intake has been shown to induce significant levels of apoptosis in various cancer cells including breast, prostate, colon and these anti-cancer properties are thought to be mediated by phenolic compounds present in olive (Han *et al.*, 2009). A polyphenolic fraction extracted from olive oil was investigated on proliferation, the cell cycle distribution profile, apoptosis and differentiation of the promyelocytic

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leukemia cells (HL-60) and it showed antiproliferative effect in a time and concentration-dependent manner (Fabiani *et al.,* 2006; Fabiani *et al.,* 2008). The high content of squalene in olive oil could supply a chemo-protective effect to skin and may also provide defense against many other forms of cancer (Newmark, 1997; Smith, 2000). Different parts of *Z. mauritiana* have been used for the treatment of asthma, allergies, depression, ulcers, diabetes and inflammation (Marwat *et al*., 2009; Bhatia & Mishra, 2010; Cisse *et al*., 2000; Gupta *et al.,* 2012; Memon *et al.,* 2012; Siddharth *et al.,* 2010). A study was conducted to investigate the chemical composition as well as biological (antioxidant, antimicrobial, antitumor, anticancer) attributes of different solvent extracts from the leaves of *Z. mauritiana* and it was established that methanol extract exhibited higher DPPH free radical scavenging potential (IC_{50} = 0.11 mg/mL) and antimicrobial (antibacterial / antifungal) activity among others. Chloroform extract showed strongest antitumor (IC_{50} = 70.74 µg/mL) and anticancer (IC_{50} values of 27.78 and 18.32 µg/mL against human cancer cell lines U937 and HCT-116 respectively) activity and significantly inhibited the viability of these cell lines (Ashraf *et al*., 2015). There is strong, consistent evidence that high intake of fruits protect against various cancers and these protective effects of high fruit consumption are attributed to the active micronutrients (vitamins and minerals) and non-nutritive components (phytochemicals) that exhibit a potential for modulating human metabolism in a manner favorable for the prevention of cancer. In the present study, *in vitro* anticancer potential of *E. officinalis*, *M. citrifolia*, *O. europaea*, *Z. Mauritiana* has been investigated against nine human cancer cell lines (A-498, A-549, HCT-116, MCF-7, MDA-MB-435, OVCAR-5, PC-3, SF-295, T-47D) from eight different tissues (renal, lung, colon, breast, melanoma, ovarian, prostate, CNS).

MATERIALS AND METHODS

Chemicals

RPMI-1640 medium, dimethyl sulfoxide (DMSO), EDTA, fetal bovine serum (FBS), sulphorhodamine blue (SRB) dye, phosphate buffer saline (PBS), trypsin, gentamycin, penicillin and 5-flurouracil were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally with the brand Sigma-Aldrich Chemicals Pvt. Ltd. and S.D. Fine Chemicals Pvt. Ltd. from Ramesh Traders, Panjthirthi-Jammu, J&K.

Fruit material and preparation of extracts

E. officinalis, *M. citrifolia*, *O. europaea* and *Z. Mauritiana* were authenticated at site by Dr. Vijay Bahadhur Singh, *I/c* Regional Research Station for Subtropical Fruits, Raya (District Samba), SKUAST-Jammu and enough quantity of fresh fruits were collected.The freshly collected fruits were chopped, shade-dried and ground into powdered form. The methanolic extracts of all the fruits were prepared by percolating the dried ground plant material (100 g) with 99% methanol and then concentrating it to dryness under reduced pressure. Stock solutions of 20 mg/ml were prepared by dissolving methanolic extract in DMSO (Kandil *et al*., 1994). Stock solutions were prepared at least one day in advance and were not filtered. The microbial contamination was controlled by addition of 1% gentamycin in complete growth medium *i.e.* used for dilution of stock solutions to make working test solutions of 200 µg/ml.

Cell lines /cultures and positive controls

The human cancer cells were obtained from National Centre for Cell Science, Pune, India and National Cancer Institute, Frederick, USA. These human cancer cells were further grown and maintained in RPMI-1640 medium. Doxorubicin, 5-Fluorouracil, Mitomycin-C, Paclitaxel and Tamoxifen were used as positive controls.

In vitro assay for cytotoxic activity

Extracts were subjected to *in vitro* anticancer activity against various human cancer cell lines (Monks *et al*., 1991). In brief, the cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator (Hera Cell, Heraeus; Asheville, NCI, USA). The cells at sub-confluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium. Cells with more than 97% viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 µl of cells (10^5 cells/ml) was transferred to a well of 96-well tissue culture plate. The cells were allowed to grow for 24 h. Extracts (100 µl/well) were then added to the wells and cells were further allowed to grow for another 48 h.

The anti-proliferative SRB assay which estimates cell number indirectly by staining total cellular protein with the dye SRB was performed to assess growth inhibition. The SRB staining method is simpler, faster and provides better linearity with cell number. It is less sensitive to environmental fluctuations and does not require a time sensitive measurement of initial reaction velocity (Skehan *et al*., 1990). The cell growth was stopped by gently layering 50 µl of 50% (ice cold) trichloroacetic acid on the top of growth medium in all the wells. The plates were incubated at 4°C for 1 h to fix the cells attached to the bottom of the wells. Liquid of all the wells was then gently pipetted out and discarded. The plates were washed five-times with distilled water

and air-dried. SRB 100 µl (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min.

The unbound SRB was quickly removed by washing the cells five-times with 1% acetic acid. Plates were air-dried, tris buffer (100 µl, 0.01 M, pH 10.5) was added to all the wells to solubilize the dye and then plates were gently stirred for 5 min on a mechanical stirrer. The optical density (OD) was recorded on ELSIA reader at 540 nm. Suitable blanks (growth medium and DMSO) and positive controls (prepared in DMSO and distilled water) were also included. Each test was done in triplicate and the values reported were mean values of three experiments.

Data analysis

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Percent growth in presence of test material was calculated as under:

- OD Change in presence of control = Mean OD of control – Mean OD of blank
- OD Change in presence of test sample = Mean OD of test sample – Mean OD of blank
- % Growth in presence of control = 100/OD change in presence of control
- % Growth in presence of test sample = % Growth in presence of control × OD change in presence of test sample
- % Inhibition by test sample = 100 – % Growth in presence of test sample

The growth inhibition of 70% or above was considered active while testing extracts, but in testing of active ingredients at different molar concentrations, the growth inhibition of 50% or above was the criteria of activity.

RESULTS AND DISCUSSION

In the present investigation, it was observed that amla fruit suppressed the proliferation of six human cancer cell lines from six different tissues in the range of 72-99% *via* its methanolic extract at 100 µg/ml. Maximum growth inhibition of 99% was observed against ovarian cancer cell line (OVCAR-5) followed by colon (HCT-116) with 96% of growth inhibition. The fruit also showed 84% growth inhibition of lung cancer cell line (A-549), 82% of renal (A-498), 78% of CNS (SF-295) and 72% of melanoma (MDA-MB-435) cancer cell line lines (Fig.1a). However, at lower concentrations the fruit did not exhibit any significant *in vitro* cytotoxic effect against any of the tested human cancer cell lines (Fig.1b).

The fruit part of ber was observed to be cytotoxic to four human cancer cell lines from four different tissues as it showed 81% growth inhibition of HCT-116 & A-549, 76% growth inhibition of MDA-MB-435 and 75% growth inhibition of A-498 cancer cells (Fig.2a). When evaluated at lower concentrations, ber fruit extract exhibited 79% (at 50 µg/ml) and 70% (at 30 µg/ml) growth inhibition against lung (A-549) cancer cells. The fruit extract also exhibited 70% *in vitro* cytotoxic effect against melanoma (MDA-MB-435) cancer cells at 50 µg/ml (Fig.2b). Surprisingly, olive displayed *in vitro* anticancer efficacy against only one human cancer cell line *i.e.*, A-549 at 100 µg/ml as 79% growth inhibition was observed in this case (Fig. 3). The growth inhibition by mulberry fruit was observed in the range of 00-49% which is not considered active (Fig. 4). Therefore, the extracts of olive and mulberry were not further evaluated at lower concentrations against any of the human cancer cell lines.

According to World Health Organization's recent estimate, the cumulative death toll due to cancer will be 12 million by 2030. Most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non tumor cells. Despite the recent advances in surgery, endocrine therapy, radiotherapy, chemotherapy, it is considered that the management of cancer is still not up to the mark and we are in emergent need of drugs for the treatment of cancer having no side effects. Therefore, the research for alternative drugs of natural origin, which are less toxic, endowed with fewer side effects and more potent in their mechanism of action, is an important research line. A number of exciting researches suggest that vegetables and fruits, nuts and seeds contain an abundance of polyphenolic compounds, terpenoids, sulphur compounds, pigments and other natural antioxidants, that have been associated with protection from or treatment of conditions such as cancer. Therefore, we can say that natural products have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer. High intake of fruits protect against various cancers as synergistic effects of phytochemicals in fruits are responsible for their potent anticancer activities. Jammu region has diversity of subtropical fruits, however, only a relatively small number of fruits have been subjected to accepted scientific evaluation for their potential anticancer effects. In view of the above, the present investigation was carried out in which significant results were produced by ber and amla.

The methanolic extract derived from amla fruit showed strong antiproliferative potential against six human cancer cell lines and the striking observation was produced by this extract against ovarian cancer cell line. The methanolic extract from ber fruit also suppressed the proliferation of four human cancer

cell lines and at lower concentrations, the fruit produced significant *in vitro* cytotoxic effect against lung (A-549) and melanoma (MDA-MB-435) cancer cells. What is quite remarkable in these observations is that the cytotoxic effect shown by these extracts was much stronger than that shown by standard drugs for cancer (serving as positive controls).

The data was compared with literature values and it was found that the data was in good agreement with the published data. The triterponic acid and betulinic acid extracted from *Z. jujuba* and *Z. mauritiana* showed selective toxicity against cultured human melanoma cells (Kim *et al*., 1998). Recently, considerable *in vitro* evidence has demonstrated that betulinic acid is effective against small and non small cell lung, ovarian, cervical and head / neck carcinomas (Pisha *et al*., 1995). Published data suggest that betulinic acid induces apoptosis (Liu *et al*., 2004) in sensitive cells in a p53-and CD95-independent fashion (Eiznhamer and Xu, 2004). Chemopreventive potential of amla against skin carcinogenesis and liver cancer has been established (Sancheti *et al*., 2005; Sultana *et al*., 2008). Kaempferol (an amla phytochemical) inhibited the expression of stromelysin 1(MMP-3) in the MDA-MB-231 breast cancer cell line (Phromnoi *et al*., 2009). The polyphenol gallic acid is also reported to possess inhibitory effects on gastric adenocarcinoma cell migration, decreased expression of MMP-2/9 *in vitro* (Ho *et al*., 2010) and metastasis of P815 mastocytoma cells to the liver of DBA/2 mice (Ohno *et al*., 2001). Olive fruits and olive oil are helpful in cancer prevention (Owen *et al*., 2004). Potential anticancer effects of olive oil on colorectal carcinogenesis models *in vitro* (Gill *et al*., 2005) and on human adenocarcinoma caco-2 cells (Mateos *et al*., 2013) has also reported.

The results from the investigation forms a good basis for the selection of these minor fruits from kandi belt of Jammu region for further phytochemical and pharmacological analysis. To conclude, ber has shown remarkable activity against lung cancer cells (A-549) at lower concentrations and could offer new drugs from natural sources which would be less toxic and more potent for the efficient management of cancer.

Fig. 1a *In vitro* cytotoxic effect of amla (100 µg/ml) along with positive controls against human cancer cells

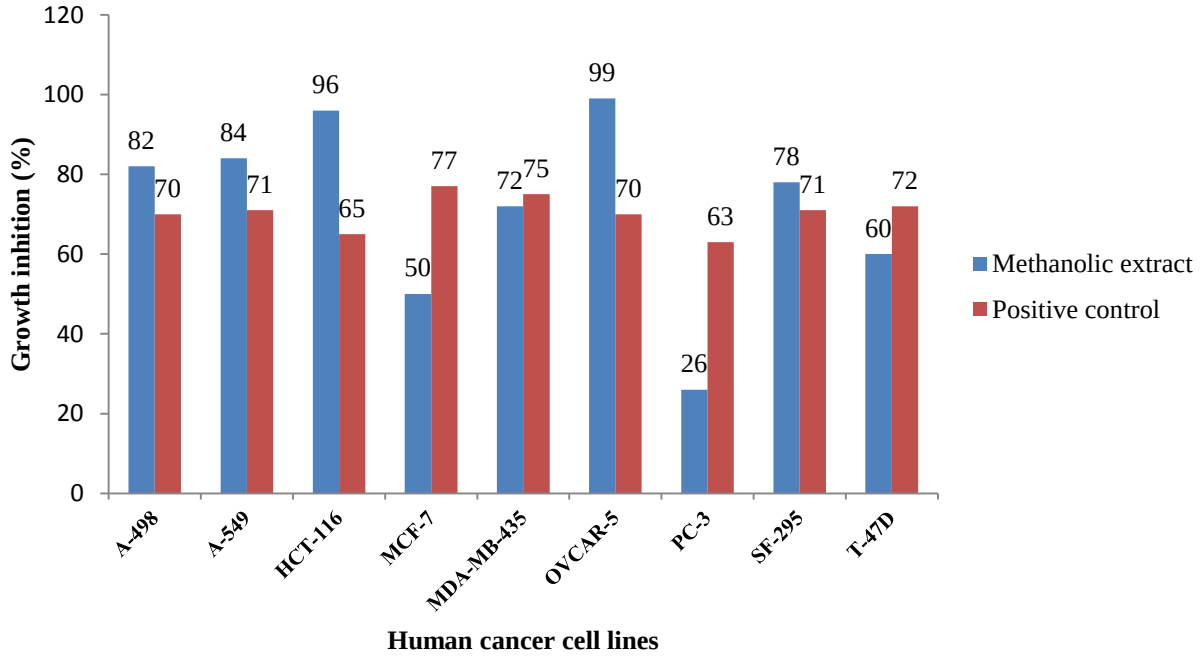


Fig. 1b *In vitro* cytotoxic effect of amla (lower concentrations) along with positive controls against human cancer cells

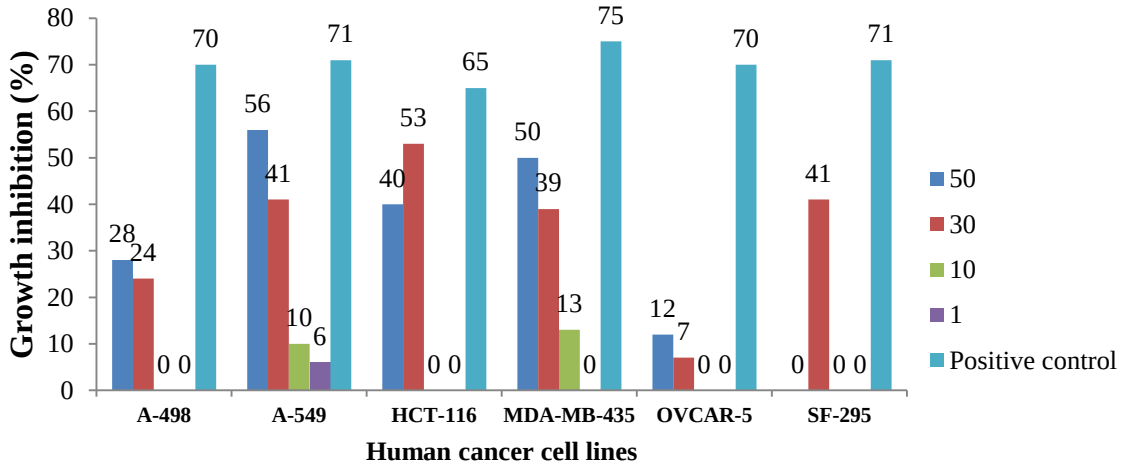


Fig. 2a *In vitro* cytotoxic effect of ber (100 µg/ml) along positive controls against human cancer cells

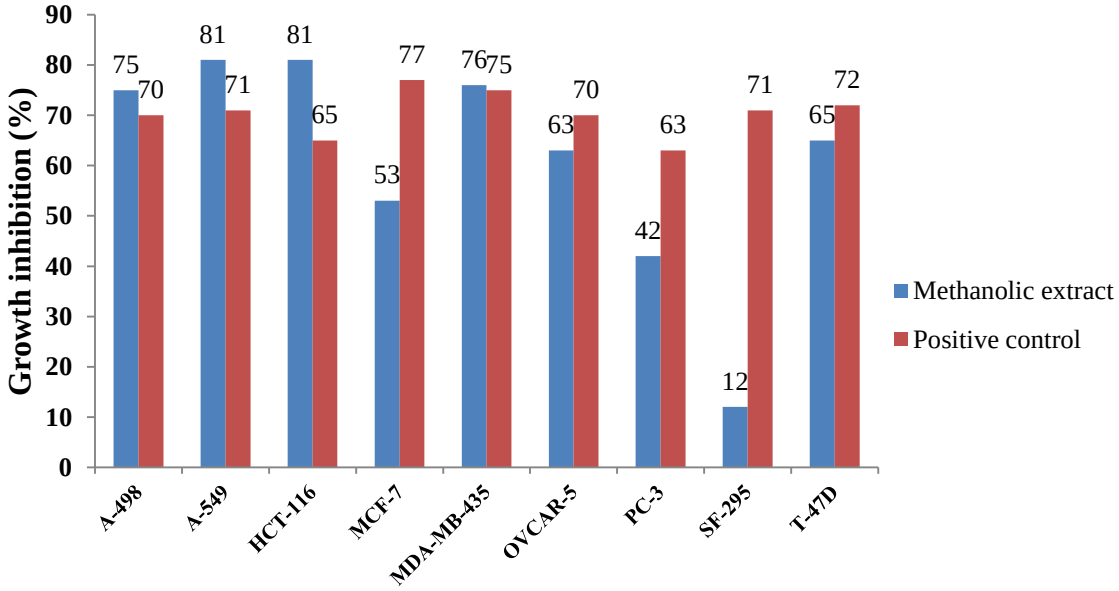


Fig. 2b *In vitro* cytotoxic effect of ber (lower concentrations) along with positive controls against human cancer cells

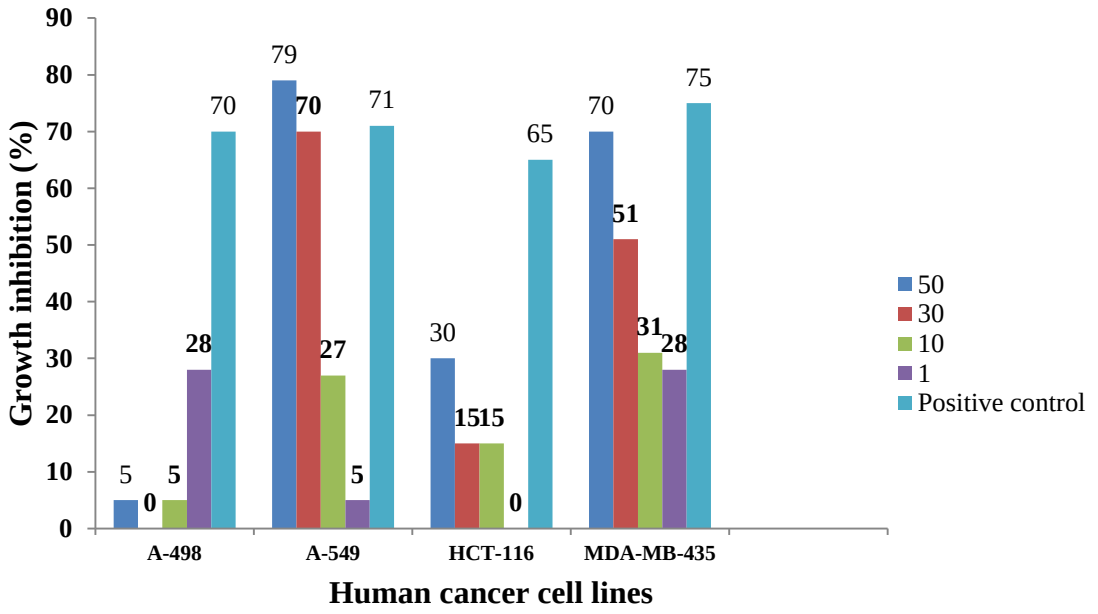


Fig. 3 *In vitro* cytotoxic effect of olive (100 µg/ml) along with positive controls against human cancer cells

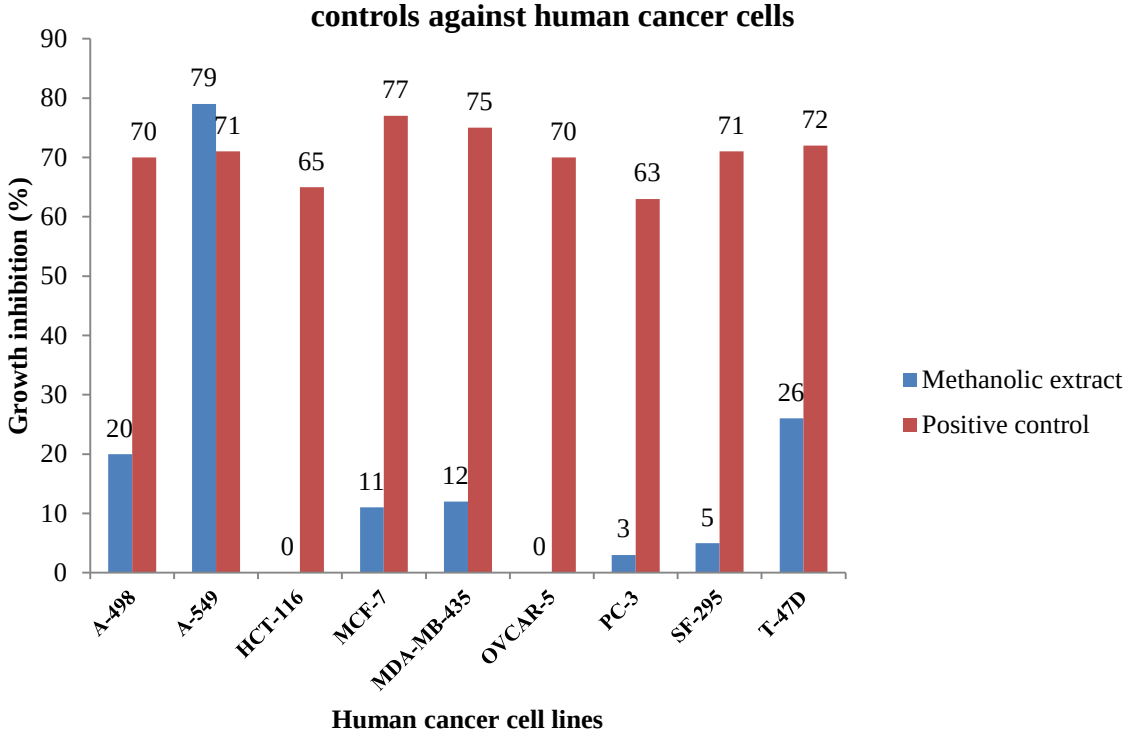
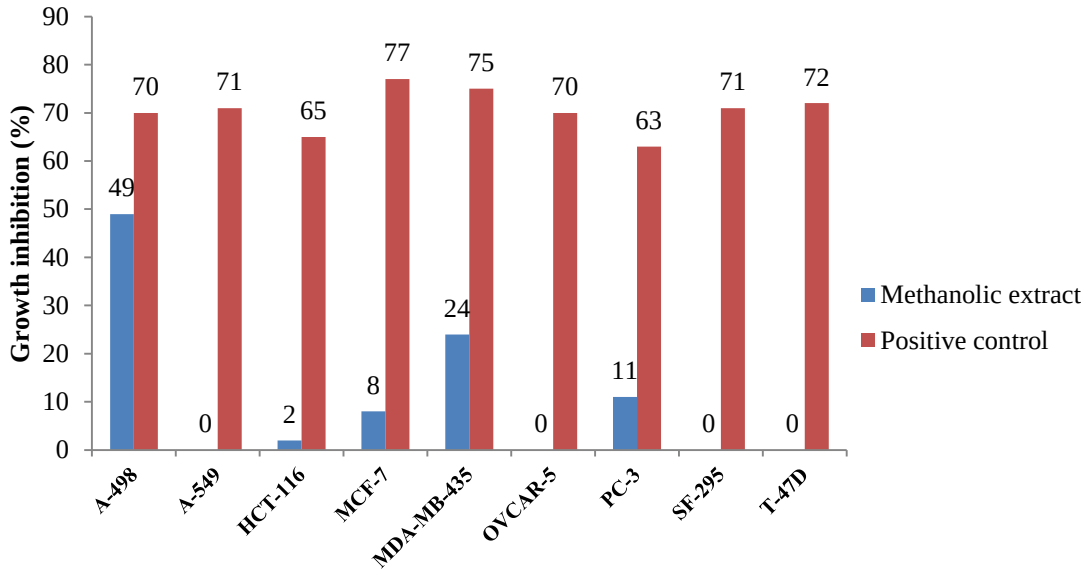


Fig. 4 *In vitro* cytotoxic effect of mulberry (100 µg/ml) along with positive controls against human cancer cells



ACKNOWLEDGEMENT

The authors are thankful to Dr. Neeraj Gupta, Junior Scientist (PHT), Regional Research Station for Subtropical Fruits, Raya (District Samba), SKUAST-Jammu for providing the raw/test material.

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