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ORIGINAL ARTICLE

In Vitro Investigation of Anticancer Activity Of Bark Extracts Of Plant *Ehretia laevis* on selective cancer cell lines by using SRB Assay

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ABSTRACT
Cancer is a major public health problem in all over the world. Medicinal herbs have been always on the forefront whenever we talk about anticancer properties. Many herbal plants have been evaluated and are currently being investigated phytochemically to understand their anticancer actions against various types of cancers. The aim of the present study is to evaluate the effect of in-vitro anticancer activity of plant extracts by SRB Assay. The petroleum ether, chloroform, methanolic, aqueous and hydroalcoholic extract of bark of plant Ehretia laevis against five human cancer cell lines such as Cervical cancer cell lines (Hela), Colon cancer cell lines (Colo-205), Lung cancer cell lines (A-549), Prostate cancer cell lines (PC-3) and Breast cancer cell lines (MCF-7) and it was compared with Adriamycin as Positive control compound by using Sulforhodamine B (SRB) assay. Anticancer activity of petroleum ether, chloroform, methanolic, aqueous and hydroalcoholic extract of plant Ehretia laevis showed cytotoxic activity on selective cancer cell lines Hela, Colo-205, A-549, PC-3 and MCF-7. The anticancer activity results were expressed in Percentage Control Growth, LC50, TGI (Total Growth Inhibition) and GI 50. The present study suggests Ehretia laevis as a potential natural source of cancer chemotherapeutics and present work may be beneficial for the development of anticancer agents of plant origin.
Keywords: *Adriamycin, Ehretia laevis, In-vitro anticancer activity, Human cancer cell lines, Sulforhodamine B (SRB) assay.*

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INTRODUCTION

Medicinal plants have been used since ancient times as medicines for the treatment of a range of diseases [1, 2]. Herbal plants have played a key role in world health. An increasing number of research papers and reviews clearly indicate that medicinal plants exhibit a variety of therapeutic properties and provide health security to rural people in primary health care [3, 4]. Cancer is group of disease characterized by uncontrolled growth and spread of abnormal forms of body's own cell [5]. If the spread is not controlled it can leads to death. It is second most common cause of death in developed countries and one in the three population will be diagnosed with cancer during their whole lifetime [6]. Cancer is majorly caused by both external factor like as Aflotoxins, cytotoxics, tobacco, chemicals, radiation and infectious organism and internal factors like as inherited mutations, hormones, immune conditions, schistosomiasis infection and mutation that occur due to metabolism. These factors may acts together or in sequence to initiate or promote carcinogenesis [7]. The development of cancer in human body requires multi steps that occur over many years. Certain types of cancer can be prevented by eliminating exposure to tobacco, chemicals and other factors that accelerates this process [8]. Any other potential malignancies can be detected before cells become cancerous, when the disease is most treatable. Cancer is treated by surgery, radiation, chemotherapy, hormones and immunotherapy. Cancer is one of the most life threatening diseases and possess many health hazard in developed as well as developing countries [9]. It can confirm that struggle to combat cancer is one of the greatest challenges of mankind [10]. From the traditional times, herbal plants have been prized for their pain-relieving and healing abilities and today upto we rely largely on the

curative properties of plants. According to World Health Organization (WHO), 85 % of the population living in rural areas depends on medicinal herbs as primary healthcare system. The available synthetic anticancer remedies are beyond the reach of common population because of cost factor. Herbal medicines have played a vital role in the prevention and treatment of cancer and medicinal herbs are commonly available and comparatively economical [11]. Screening models for In-vitro anticancer activity may provide the important preliminary data to help in selection of plant extracts with potential anticancer properties for future work. Screening and isolation of active chemical compounds from plants which possess potential anticancer activity appears to be a promising way of discovering novel therapeutic compound [12, 13]. Phytoconstituents which are obtained from flowering plants play a significant role in cancer chemotherapy. Anticancer drugs like as vincristine sulphate and vinblastine sulphate from Vinca plant, palitaxel (Taxol) and taxotere from species of yew (Taxus), etoposide derived from lignans of Podophyllum plant and camptothecin analogues, such as topotecan, from *Camptotheca acuminate* all of these are fundamentally shows cytotoxic and act principally by inhibiting cell proliferation, but by different mechanisms. Some natural substances from plants have been found to act by novel mechanisms and so have enabled to get novel targets to developed for screening, it can be explained by the discovery that paclitaxel inhibited mitosis by stabilising microtubules and so preventing their depolymerisation back to tubulin, in contrast to many other anticancer agents which inhibit the formation of microtubules in the first place [14].

Ehretia laevis plant is fast-growing and small tree which is belonging to family Ehretiaceae. The plant is cultivated mainly in India, Pakistan, Myanmar, Vietnam, China, Bhutan. The plant *Ehretia laevis* is located mainly at hilly forests, in ravine and on hill slopes. The plant is known as Dant-Rang, Vadhvarni, Chamror [15]. The inner bark of *E. laevis* is used as food. Leaves are applied to ulcers, skin diseases and in headache. Fruit are used in urinary passage, lung and spleen diseases, astringent, anthelmintic, diuretic, demulcent, expectorant [16, 17, 18]. Powdered kernel mixed with oil is a remedy in ringworm. Seeds are anthelmintic. Barks are used in throat infection. Root for veneral diseases. The plant contains chemical constituents like as fatty acids, phenolic acids, flavonoids, cyanogenetic glycosides and benzoquinones [19, 20, 21].

MATERIAL AND METHODS

Plant Collection

The fresh barks of plant *Ehretia laevis* were collected from region of Taluka Yawal, District Jalgaon, India. The selected plant was authenticated by Dr. D. A. Dhale, Asst. Professor, PG & Research Dept. of Botany SSVPS's, L. K. Dr. P. R. Ghogrey Science College, Dhule, Maharashtra. Barks were dried at room temperature to avoid loss of chemical constituents and milled with the aid of grinding machine.

Preparation of Plant extract

The bark of plant were thoroughly washed with tap water, dried at room temperature and transformed to coarse powder. The bark powder was extracted with solvents like as Petroleum ether (60-80^{o}C), Chloroform, Methanol, Water-methanol and water separately by Soxhlet extraction method. Finally, the extracts were evaporated and dried under vacuum and tray dryer to obtain thick sticky extract [22, 23].

Cell lines

Various human cancer cell lines used for in vitro Sulforhodamine B (SRB) Assay are Cervical cancer cell lines (Hela), Colon cancer cell lines (Colo-205), Lung cancer cell lines (A-549), Prostate cancer cell lines (PC-3) and Breast cancer cell lines (MCF-7). The stock cultures were grown in T-75 flasks containing 50 mL of RPMI-1640 (Roswell Park Memorial Institute) medium with 2 mM L-glutamine, bicarbonate and 10 % fetal calf serum. Medium was changed at 48 hours intervals. Cell were dissociated with 0.25 % trypsin and 3 mM 1,2-cyclohexanediaminetetraacetic acid in NKT buffer (137 mM Nacl, 5.4 mM Kcl and 10 mM Tris; pH 7.4). For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs [24].

Sulforhodamine B (SRB) Assay procedure [25, 26]

The anticancer activities of extracts were studied at Advanced Center for Treatment, Research and Education in Cancer (ACTREC), Mumbai where all cell lines were maintained in ideal laboratory conditions. All cell lines were selected grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs (Extracts and Positive

control). Experimental drugs (Extracts and Positive control) were initially solubilized in dimethyl sulfoxide (DMSO) at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquote of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml.

Positive control

Adriamycin (Doxorubicin) a known anticancer was used as a positive control for each of the experiments.

Endpoint measurement

After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA (Trichloroacetic acid). Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was then discarded and the plates were washed 5 to 6 times with water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After making the staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm wavelength kept as reference. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as: [Ti/C] x 100 % for concentrations for which Ti>/=Tz (Ti-Tz) positive or zero [(Ti-Tz)/Tz] x 100 for concentrations for which Ti<Tz. (Ti-Tz) negative. The dose response parameters were calculated for each test article. The experiment data were estimated using linear regression method of plots of the cell viability against the molar drug concentration of tested compounds and results were given in terms of LC50, TGI and GI50 values. The summary of the parameters is as follows

GI50- Concentration of drugs causing 50 % inhibition of cell growth, calculated from [(Ti-Tz)/(C-Tz)] X 100= 50

LC50- Concentration of drugs causing 50 % cell kill, calculated from [(Ti-Tz)/Tz] X 100= 50

TGI- Concentration of drugs causing total inhibition of cell growth, calculated from Ti=Tz GI50 value of ≤ 20 µg/ml is considered to demonstrate activity.

Statistical analysis

Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

RESULTS AND DISCUSSION

The Plant extracts inhibited percent control growth of all the human cancer cell lines in dose dependent manner. The anticancer effect of plant extracts is more in case of Human Colon Cancer Cell Line and Human Breast Cancer Cell Line, moderate in case of Human Lung Cancer Cell Line and Human Prostate Cancer Cell Line and least in case of Human Cervical Cancer Cell Line. Petroleum ether and Chloroform extract of plant *Ehretia laevis* were showed prominent activity against Human Colon Cancer Cell Line (Colo-205) with % control growth were found to be -26 and -25.6 at 80 ug/ml respectively (Table No. 3). Chlorofom, Methanolic, Aqueous and Hydroalcoholic extract of plant *Ehretia laevis* were showed prominent activity against Human Breast Cancer Cell Line (MCF-7) with % control growth were found to be 34.6, -12.8, -32.6 and -34.8 at 80 ug/ml respectively (Table No. 4). Chloroform extract showed moderate anticancer activity against Human Lung Cancer Cell Line (A-549) and Human Prostate Cancer Cell Line (PC-3) with % control growth were found to be 9.9 and 28 at 80 ug/ml respectively (Table No. 3 and 4). All GI_{50} ≤ 10 ug/ml is considered to demonstrate activity in case of Standard Adriamycin and GI_{50} ≤ 20 ug/ml is considered to demonstrate activity in case of Plant extracts. All GI_{50} values are averages of three experiments. GI_{50} value of Petroleum ether and Chloroform extract of plant *Ehretia laevis* were found to be ≤ 10 ug/ml each against Human Colon Cancer Cell Line (Colo-205) as shown in Table No. 1. GI_{50} value of Chlorofom extract were found to be 4.75 ug/ml, while Methanolic, Aqueous and Hydroalcoholic extract were found to be ≤ 10 each against Human Breast Cancer Cell Line (MCF-7) as shown in Table No. 2. GI_{50} value Chloroform extract were found to be 29.2 ug/ml each against Human Lung Cancer Cell Line (A-549) and Human Prostate Cancer Cell Line (PC-3) as shown in Table No. 1 and 2.

The results showing GI_{50} values for anticancer activity of various extracts of *Ehretia laevis* against human cancer cell lines such as Human Cervical cancer cell lines (Hela), Human Colon cancer cell lines (Colo-205), Human Lung cancer cell lines (A-549), Human Prostate cancer cell lines (PC-3) and Human Breast cancer cell lines (MCF-7) are presented in Table No. 1 and 2. The Growth curve of different Human Cancer Cell Lines were plotted % Control growth vs Drug Concentration (ug/ml) as given in Figure No. 1, 2, 3, 4 and 5.

Modifications in Morphology of Human Cervical cancer cell lines (Hela), Human Colon cancer cell lines (Colo-205), Human Lung cancer cell lines (A-549), Human Prostate cancer cell lines (PC-3) and Human Breast cancer cell lines (MCF-7) after treatment with Plant extracts and Standard Adriamycin were observed after 72 hours. However, when all Five Human Cancer Cell Lines were exposed to cytotoxic components like plant extracts and Standard Adriamycin, two distinct mode of cell death were recognized, mainly, Apoptosis and Necrosis. The majority of Human Cancer Cell Lines treated with cytotoxic components like plant extracts and Standard Adriamycin showed features of Apoptosis like as shrinkage of cells, blebbing of membrane and formation of apoptotic body all changes were viewed under an inverted light microscope. The Micrograph images of the Five Human cancer cell lines used for studying anticancer activity after treatment with Plant extracts and Standard Adriamycin are shown in Figure No. 6-10. Anticancer activity is the capability of the natural or synthetic agents to suppress carcinogenic progression. Several cytotoxic therapies mentioned in Ayurveda consist of herbal remedies as they are the potent medicines in the traditional system and majority of the cancer cells are treated by herbal medicines. One of the approaches for finding the anticancer agent is the selection of medicinal plant based on their ethno medicinal value [27]. Efforts are made to make the treatment of cancer more suitable and satisfying, herbal medicines have been in use because they reported with minimum side effects in compared to synthetic drugs [28]. Several earlier anticancer studies showed that plant extract contains rich number of phytochemicals which are possess anticancer properties and may be responsible for anticancer effect of these plants extract [29].

Table No. 1: *In Vitro* Anticancer Activity of Extracts of *Ehretia laevis* Using SRB Assay on Cervical Cancer Cell Lines (Hela), Colon Cancer Cell Lines (Colo-205) and Lung Cancer Cell Lines (A-549)

Extracts	Cell Lines								
	Cervical cancer cell lines (Hela)			Colon cancer cell lines (Colo-205)			Lung cancer cell lines (A-549)		
	LC50	TGI	GI50	LC50	TGI	GI50	LC50	TGI	GI50
PET-2	NE	NE	63.6	NE	55.7	<10	NE	NE	NE
CHL-2	NE	>80	35.94	NE	34.1	<10	NE	>80	29.2
MET-2	NE	NE	>80	NE	NE	54	NE	NE	>80
AQ-2	NE	NE	70.4	NE	74	40.9	>80	76.2	39.6
HAL-2	NE	>80	54	76.6	49.4	22.2	>80	69.5	35.3
ADR	NE	<10	<10	NE	<10	<10	NE	<10	<10

Table No. 2: *In Vitro* Anticancer Activity of Extracts of *Ehretia laevis* Using SRB Assay Prostate Cancer Cell Lines (Pc-3) and Breast Cancer Cell Lines (Mcf-7)

Extracts	Cell Lines					
	Prostate cancer cell lines (PC-3)			Breast cancer cell lines (MCF-7)		
	LC50	TGI	GI50	LC50	TGI	GI50
PET-2	NE	NE	NE	NE	NE	>80
CHL-2	NE	>80	29.2	NE	NE	4.75
MET-2	NE	NE	>80	NE	45.1	<10
AQ-2	NE	NE	NE	>80	7.4	<10
HAL-2	>80	>80	59.1	NE	NE	<10
ADR	<10	<10	<10	NE	NE	<10

Where,

LC50: Concentration of Drug causing 50% cell kill

GI50: Concentration of Drug causing 50% inhibition of cell growth

TGI: Concentration of Drug causing total inhibition of cell growth

NE: Non evaluable data

Table No. 3: % Control Growth of Extracts of Plant *Ehretia laevis* against Cervical Cancer Cell Lines (Hela), Colon Cancer Cell Lines (Colo-205) and Lung Cancer Cell Lines (A-549)

Test Component	% Control growth											
	Drug Concentrations (ug/ml)											
	Cervical cancer cell lines (Hela)				Colon cancer cell lines (Colo-205)				Lung cancer cell lines (A-549)			
	10	20	40	80	10	20	40	80	10	20	40	80
PET-2	95.3	101.1	85.9	27.3	49.9	34.3	18.5	-26.0	81.0	83.1	86.0	78.4
CHL-2	81.4	63.6	45.6	2.7	12.4	10.3	-4.7	-25.6	67.8	58.8	36.1	9.9
MET-2	118.0	127.9	104.4	51.7	93.5	82.9	67.5	22.6	80.5	92.2	83.0	48.7
AQ-2	115.5	116.3	94.8	34.3	83.9	81.6	73.6	-18.7	74.9	71.5	84.6	-19.5
HAL-2	111.8	110.8	67.3	10.8	73.5	54.8	14.6	-55.1	74.6	71.2	66.1	-25.1
ADR	-39.9	-40.7	-44.7	-22.8	-57.9	-51.6	-65.2	-66.5	-7.6	-7.6	-11.3	-10.9

Table No. 4: % Control Growth of Extracts of Plant *Ehretia laevis* against on Prostate Cancer Cell Lines (Pc-3) and Breast Cancer Cell Lines (Mcf-7)

Test Component	% Control growth							
	Drug Concentrations (ug/ml)							
	Prostate cancer cell lines (PC-3)				Breast cancer cell lines (MCF-7)			
	10	20	40	80	10	20	40	80
PET-2	121.3	136.3	144.3	116.9	82.4	91.1	67.4	52.4
CHL-2	109.5	111.4	80.7	28.0	62.7	47.5	6.6	34.6
MET-2	97.1	105.6	101.6	52.2	32.5	15.7	-16.9	-12.8
AQ-2	101.9	111.4	110.8	66.7	7.7	-5.7	-35.8	-32.6
HAL-2	105.1	111.1	83.1	17.4	-18.1	-27.4	-35.7	-34.8
ADR	-54.6	-61.2	-65.1	-54.0	-19.3	-24.7	-18.4	27.3

Where, PET-2- Petroleum ether extract, CHL-2- Chloroform extract, MET-2 Methanolic extract, AQ-2 Aqueous extract, HAL-2 Hydroalcoholic extract and ADR Adriamycin

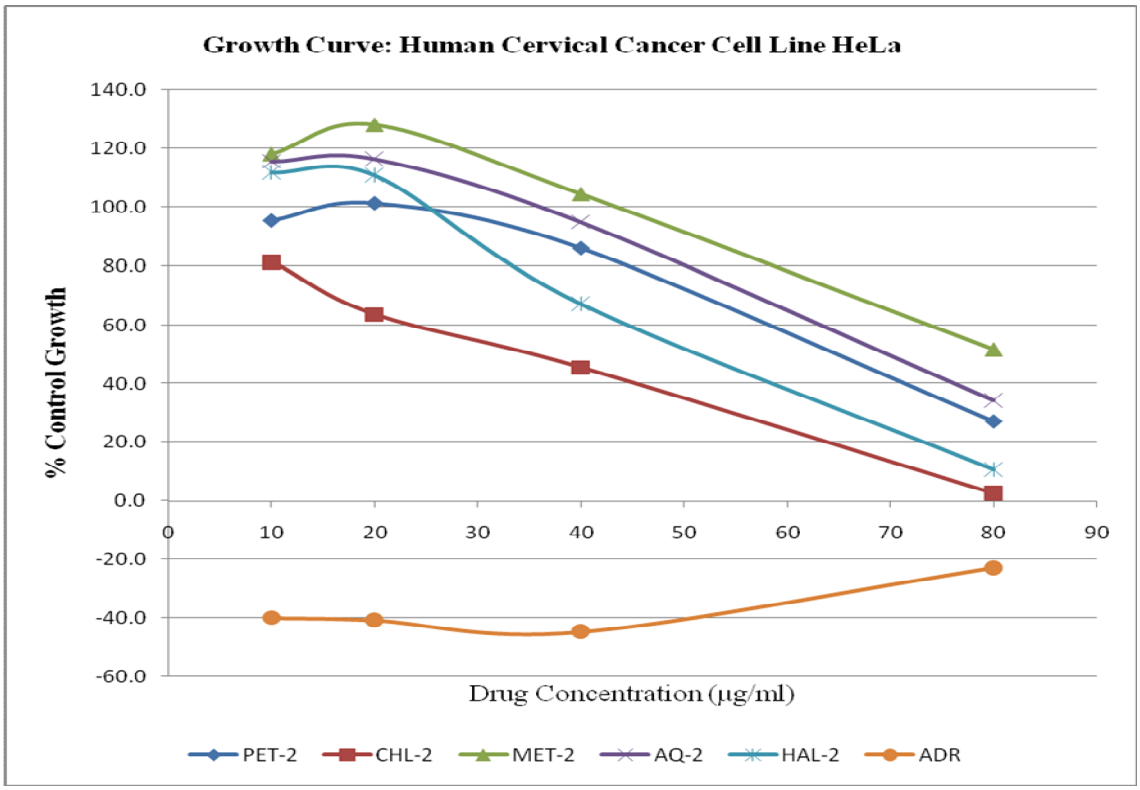

Fig. 1: Percentage control growth of Human Cervical Cancer Cell Line HeLa

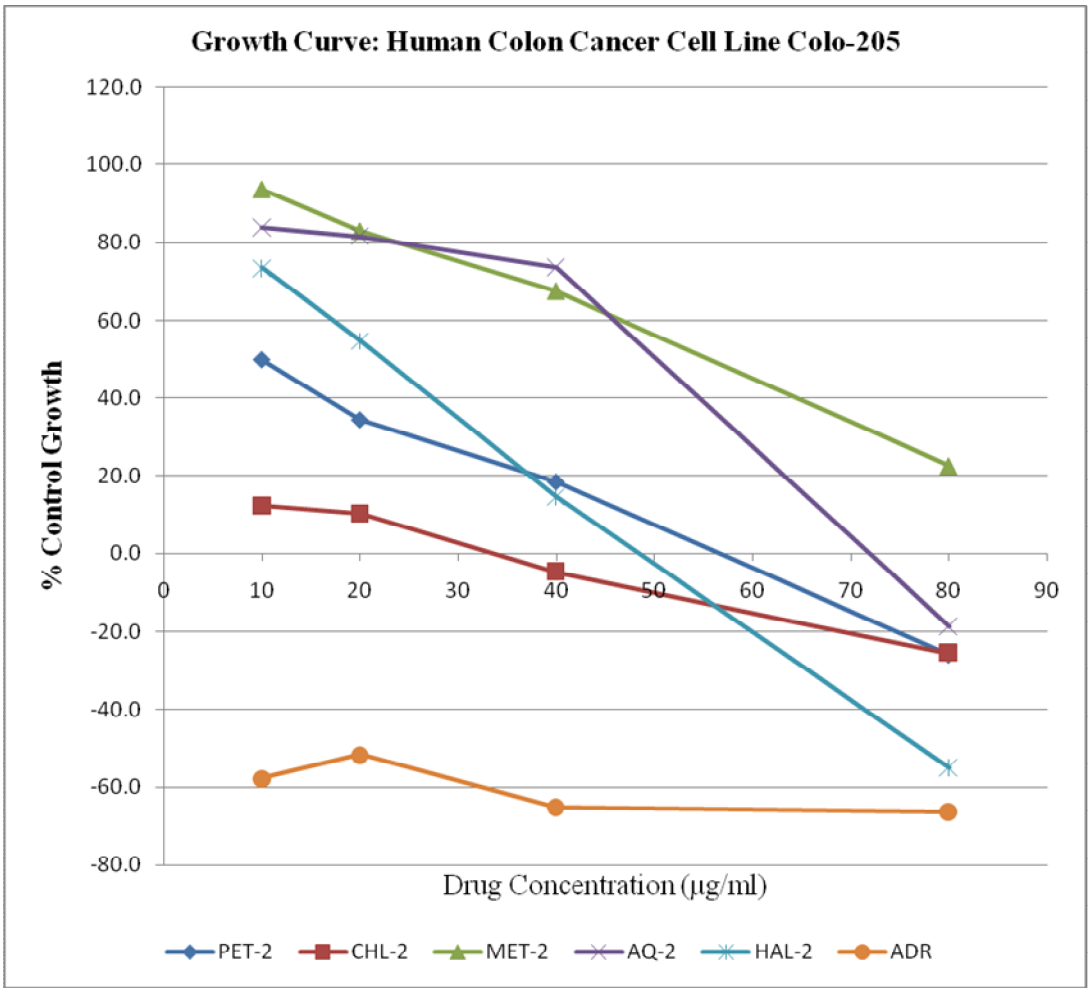

Fig. 2: Percentage control growth of Human Colon Cancer Cell Line Colo-205

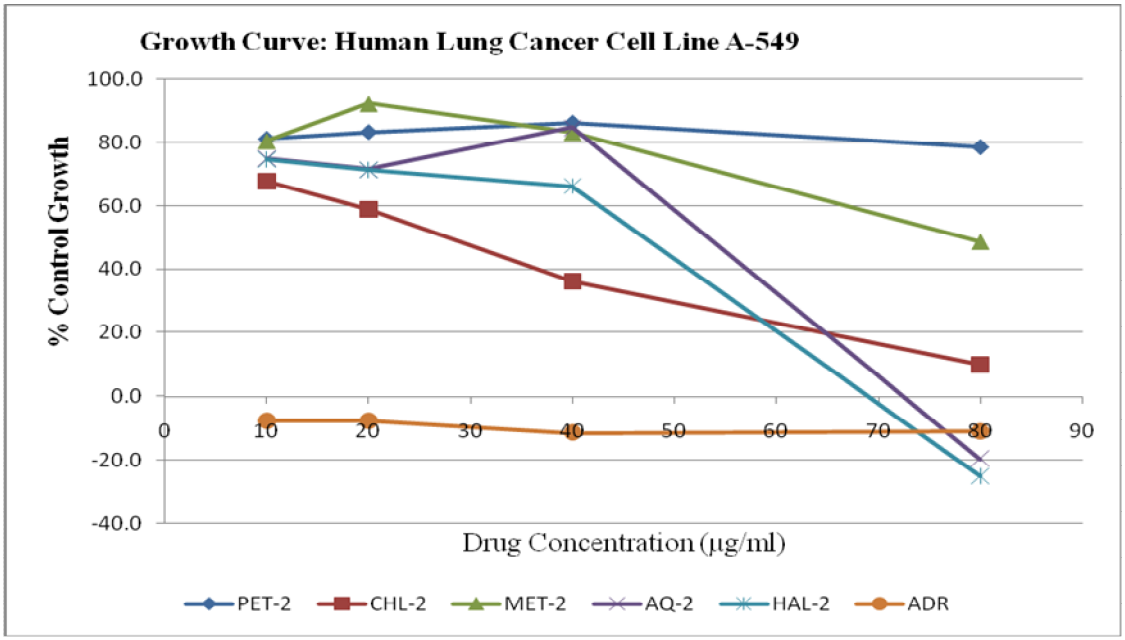

Fig. 3: Percentage control growth of Human Lung Cancer Cell Line A-549

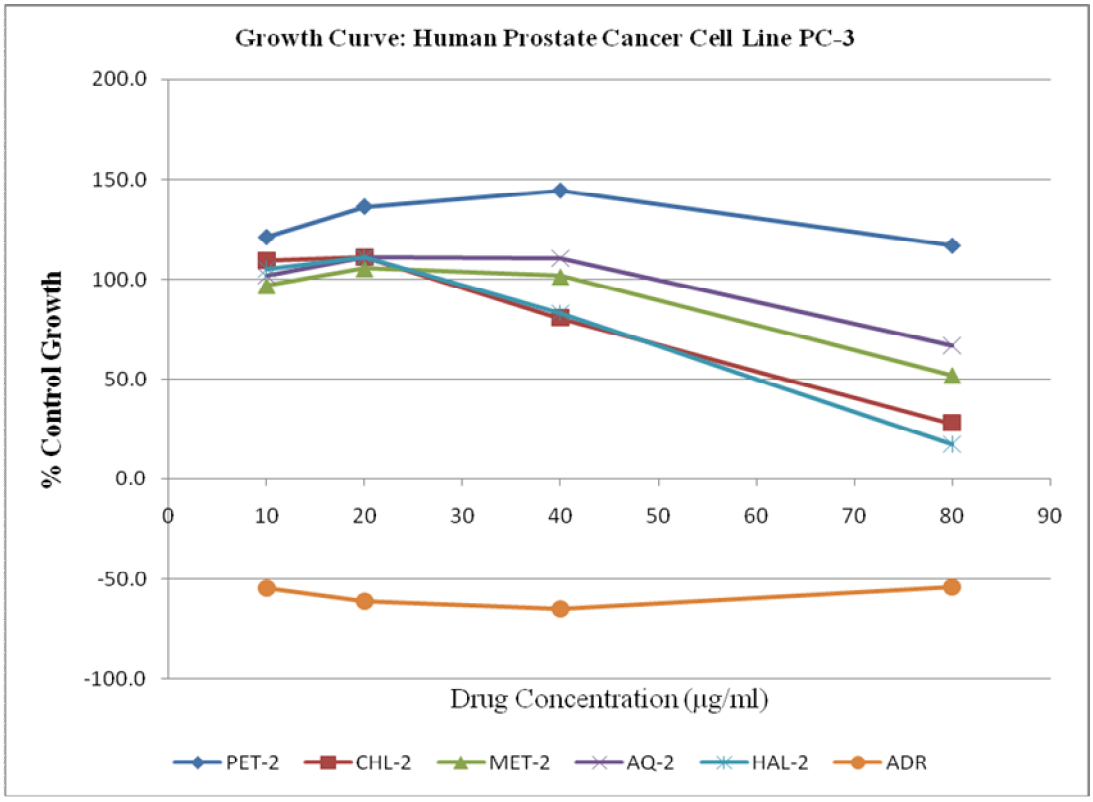

Fig. 4: Percentage control growth of Human Prostate Cancer Cell Line PC-3

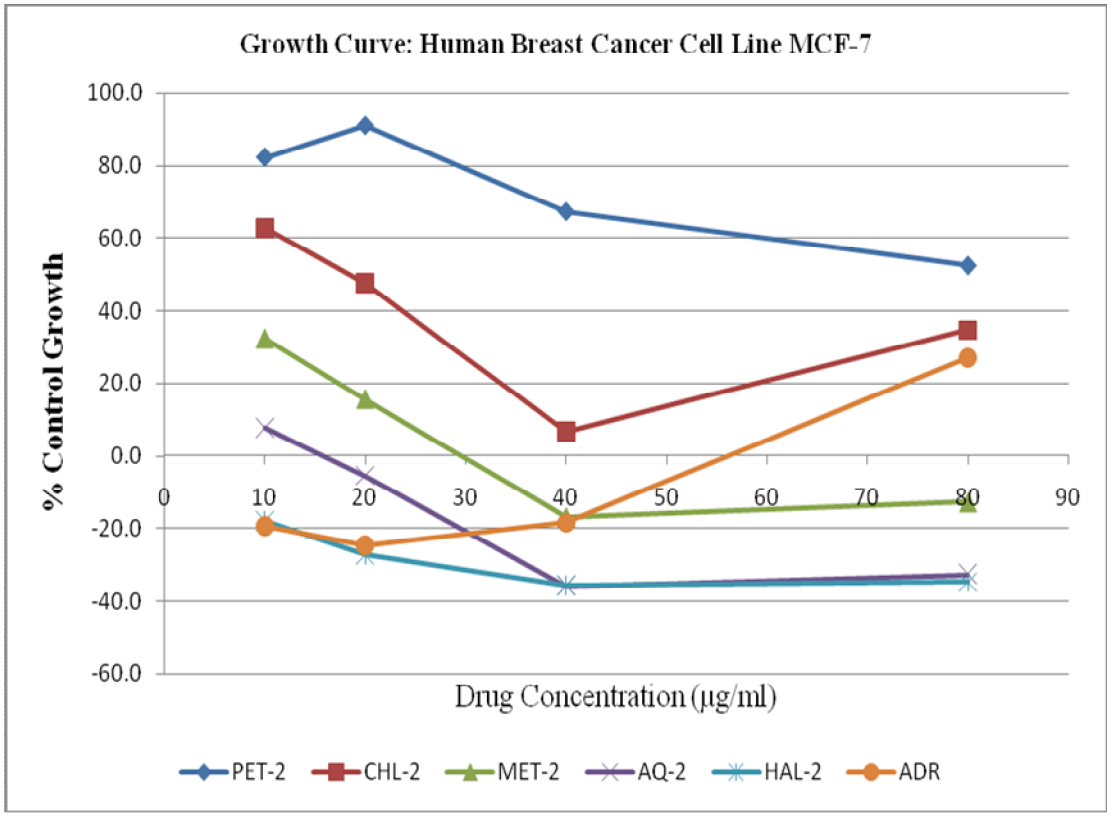

Fig. 5: Percentage control growth of Human Breast Cancer Cell Line MCF-7

Where, PET-2- Petroleum ether extract, CHL-2- Chloroform extract, MET-2 Methanolic extract, AQ-2 Aqueous extract, HAL-2 Hydroalcoholic extract and ADR Adriamycin

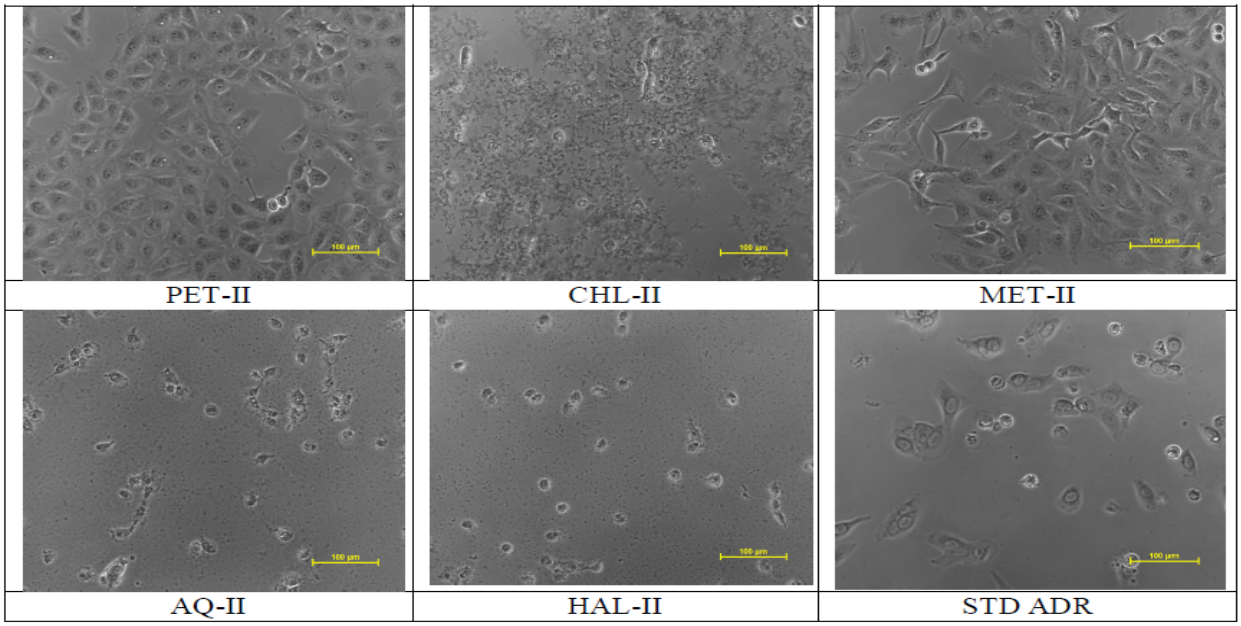

Fig. 6: Micrographs of Lung Cancer cell line (A-549) treated with different extracts of Plant *Ehretia laevis* and Standard

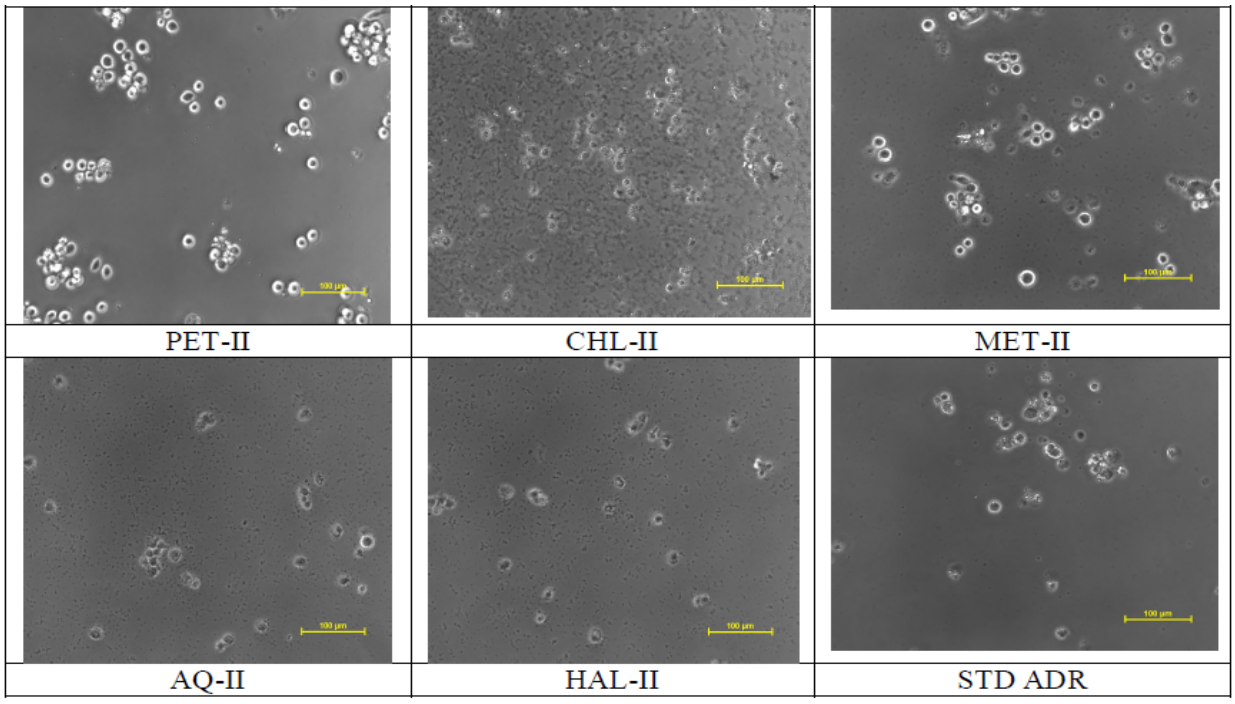

Fig. 7: Micrographs of Colon cancer cell lines (Colo-205) treated with different extracts of Plant *Ehretia laevis* and Standard

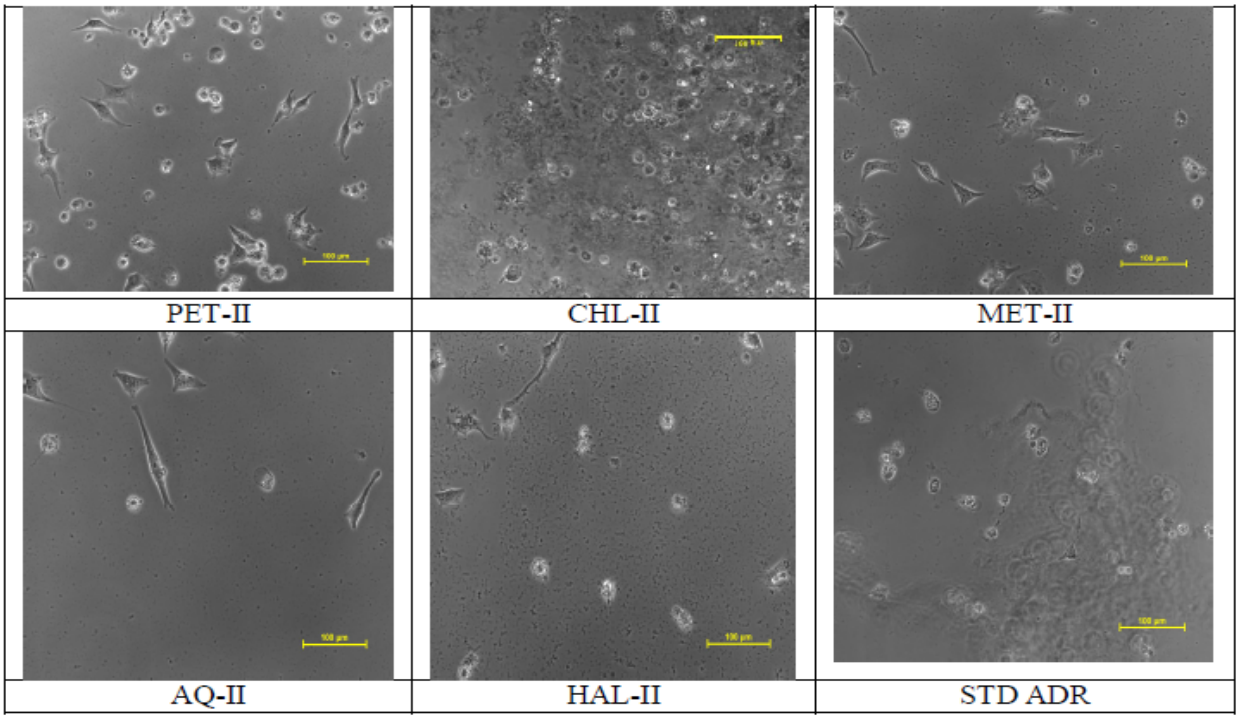

Fig. 8: Micrographs of Cervical cancer cell lines (Hela) treated with different extracts of Plant *Ehretia laevis* and Standard

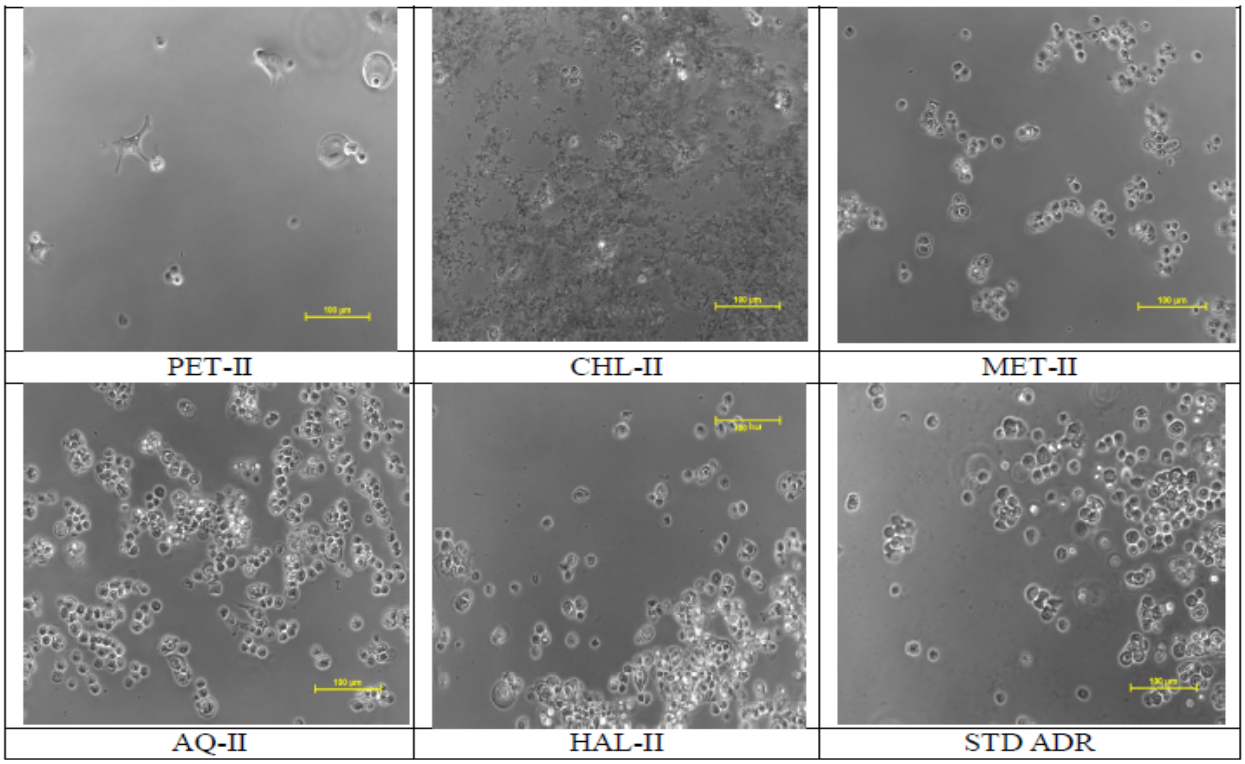

Fig. 9: Micrographs of Breast cancer cell lines (MCF-7) treated with different extracts of Plant *Ehretia laevis* and Standard

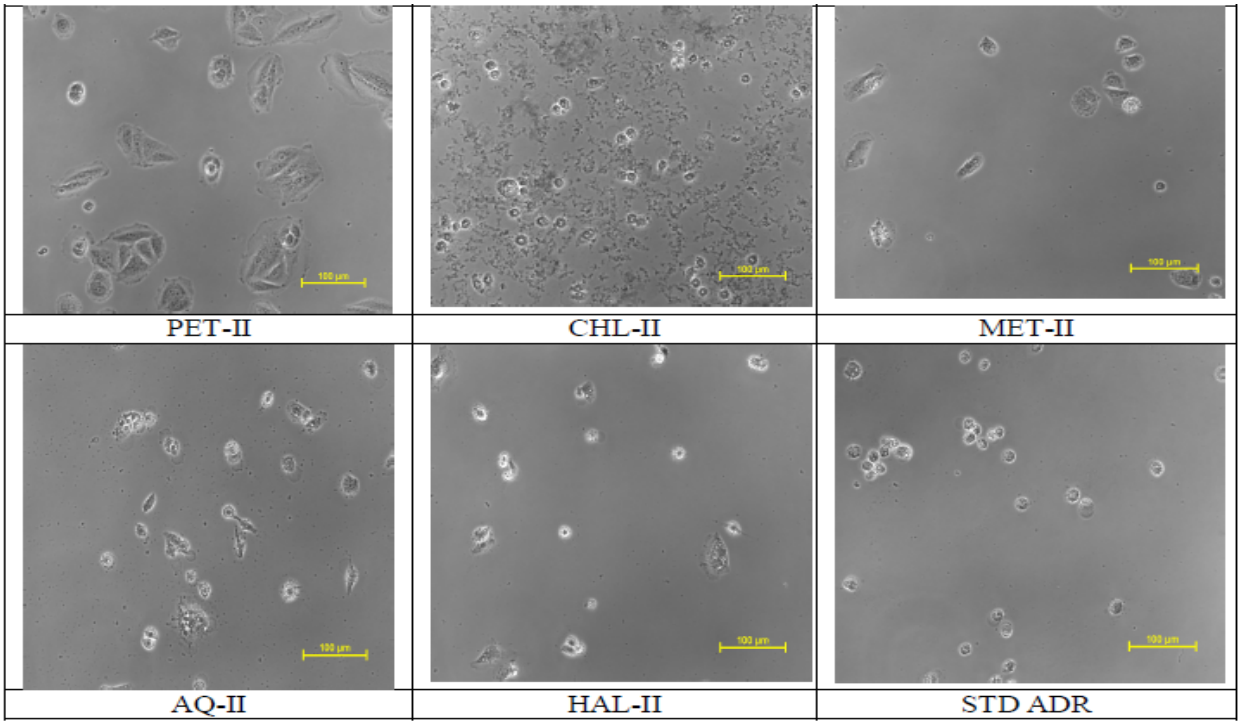

Fig. 10: Micrographs of Prostate cancer cell lines (PC-3) treated with different extracts of Plant *Ehretia laevis* and Standard

CONCLUSION

Cancer is one of the most leading causes of mortality worldwide. There are two in vitro screening techniques like SRB and MTT assays are the reliable techniques used to carry out evaluation of anticancer activity on the human cancer cell lines. The SRB assay provides a better linearity with cell number and a higher sensitivity and its staining is not cell dependent. It is known that, in contrast to the MTT assay the SRB assay stains recently lysed cells. In the present study, we concluded that the various plant extracts of *Ehretia laevis* showed prominent *in vitro* anticancer activities against some selected human cancer cell lines. The activity may be depended upon the morphology of cell lines and mechanism of action of the plant extract. Further, all these plants extracts of plant *Ehretia laevis* need to be screened against different cell lines apart from the selected cell lines to confirm the activity. So finally it can be concluded that plant part can be promising candidates in future anticancer therapy.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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