INVITRO ANTICANCER ACTIVITY OF MEDICINAL PLANTS

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ABSTRACT:

Plant materials have been used for medical purposes in human life. Cancer diseases severely effect the human populations in worldwide. There is a constant challenge to find out the new therapies and drugs for treating cancer. The new focus is on finding anti cancer drugs with less side effects compared to current treatments such as chemotherapy. Historical use of plant materials might contain anticancer activities. This study evaluated three plant extracts on cancer cells. The essential oils extracts of Hedyotis corymbosa, Inula racemosa and Holostemma adakodien were studied against cancer cells. All the three plant extracts have shown dose dependent cancer cell killing activity.

INTRODUCTION

The word *in vitro* came from Latin term which means “in glass”. *In vitro* tests are performed with cells or biological molecules studied outside their normal biological context. Colloquially called “test tube experiments”, these studies in biology and its sub disciplines are traditionally done in test tubes, flasks, petri dishes etc. However, when compared to the *in vivo* studies these in vitro studies are faster, cost effective and can be done with less ethical and safety concerns.

When the free radical reacts with other molecules, in an attempt to achieve stability, it can do extensive damage to components of the cell, especially the genetic material in the nucleus. Free radicals damage the DNA by inducing mutations, leading to the uncontrolled proliferation of cells that characterizes cancer. Free radicals also can directly activate oncogenes (cancer genes). An additional type of damage done by free radicals is the conversion of some relatively harmless substances (pre-carcinogens) into dangerous cancer-producing chemicals (carcinogens). Furthermore, free radicals can interfere with cellular mechanisms that can potentially limit abnormal cell growth and also can interfere with immune defenses that could help keep the onset, growth and spread of cancer in check (Devasagayam *et al.,* 2004; Halliwell 1994).

Cancer is a potentially fatal genetic disease characterized by the irregular proliferation of cell. It is a major human health problem in both developed and developing countries and it is the second leading cause of death in the modern World. Cancer results from a series of molecular events that alters the normal properties of cells. For the past three decades significant process has been achieved in understanding the molecular basis of cancer (Jesse *et al.,* 2003).

Among all cancers, lung cancer is the leading cause of cancer mortality observed both in men and women Worldwide. More people die of lung cancer than from colon, breast and

prostate cancers. Lung cancer is the uncontrolled growth of abnormal cells in one or both of the lungs. According to a report by World Health Organization lung cancer is of four main types namely Squamous Cell Carcinoma, Small Cell Carcinoma, Adenocarcinoma and Large Cell Carcinoma. On the basis of its biology, prognosis and therapy lung cancer is further divided into two major classes namely Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). Of this NSCLC accounts for 75-80% of lung cancer cases whereas, SCLC accounts only to about 15-25% of lung cancer cases (Yang *et al.,* 2008).

Conventional treatment for these two forms of lung cancer was found to be ineffective. Although, cancer chemotherapy and radiotherapy are the therapeutic methods in the case of many malignancies, it causes undesirable changes and side effects. Hence, any discovery of anti-cancer agents should be effective against specific types of cancer cells but less toxic to normal cells or it should have a unique mechanism of action for specific type of cancer. In this case, medicinal plants have received a considerable interest in the anti-cancer therapy as they do not have these drawbacks (Greenwell and Rahman, 2015).

Natural products especially plants have been used widely in the treatment of various diseases for more than many centuries and recent attention has been turned towards natural active compounds form plant products. So far available chemotherapeutic agents are products derived from medicinal plants. But, the interesting fact is that the plants have been used in the treatment of various cancers for a long period of time and their therapeutic effects have been evaluated both *in-vitro* and *in-vivo*. Therefore, plants have a long history of their use in the treatment of cancer (David *et al.,* 2000).

Use of plants in traditional and folk medicine has been extensively documented by many researchers. From the beginning of 19th century onwards 25% of the prescriptions dispensed from community pharmacies of well developed nations like Unites States contain either plant extracts or their bioactive principles derived from plant sources. Nearly, to about 119 anti-cancer drugs have been derived from 90 plant species of which 74% of these drugs are derived by extracting and isolating the active principles (Chemical compounds) of the plants based on its traditional medicinal uses (Amr *et al.,* 2009). Out of many plant derived anti-cancer drugs that are commonly known to us Vinblastine and Vincristine from *Catharanthus roseus* L are more familiar and are commonly used for cancer therapy. Therefore, the discovery of any anti-cancer drug is indirectly attributed to the medicinal use of the plant with reference to traditional and folk medicine practitioner's knowledge (Tamrat *et al*., 2018).

Most of the cancer chemotherapeutic treatment involves the use of highly cytotoxic drugs that target proliferating cells. But during recent years the therapeutic use of essential oils has attracted the attention of many researchers to test for anti-cancer activity. Variety of dietary monoterpenes has been shown to be very effective in the chemoprevention and chemotherapy of cancer. For example in an earlier report monoterpenes have been documented for its chemopreventive as well as chemotherapeutic activities in mammary tumor models and they represent a new class of therapeutic agents. Another study shows that the essential oil of nutmeg (Myristica fragrans) showed a potent hepatoprotective activity against liver damage and it is also found to be showing an effective anti-cancer activity by

suppressing angiogenic mechanism. Monoterpenes includes compounds like Carvocrol, Thymol, limonene, Citral, Citronellol and Linalool are a proven compound to be an ideal chemopreventive agent due to the reason that they have efficient anti-tumor activity, commercially available, lower cost and treatment can be done by giving oral doses and most of all it has a low toxicity. Hence, these characteristics of monoterpenes made researchers to begin their cancer chemoprevention research by using monoterpenes. Researchers are now progressing towards human clinical trials for chemotherapeutic activity with the help of monoterpenes (*Rita et al*., 2013).

OBJECTIVE

To analyze the *in vitro* anti-cancer potency of the selected three plant's *Holostemma adakodien, Inula racemosa* and *Hedyotis corymbosa* essential oil extracts.

RATIONALE

In an effort to find out the possible mechanisms by which these plants exerted their anti-cancer effects, we intended to study their reported as potent anti-cancer.

MATERIALS AND METHODS

Preparation of Plant Extracts:

Extraction of essential oils from the selected three medicinal plants is done by hydro distillation method using Clevenger type apparatus for 3 hours (Faiza, *et al.*, 2014).

In Vitro Cytotoxicity Assay

Human cancer cells were obtained from National Centre for Cell Science (NCCS) pune. The cells were maintained in 25 to 75 cm^2 tissue culture flasks (Corning, U.S.A) in complete growth medium containing RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum along with 100 U/ml pencillin and streptomycin in incubator maintained at 5% CO_2, 37°C temperature and with 90% humidity. The cells were harvested at near confluence with 0.2% trypsin and counted for cell viability by using tryphan blue. The sub confluent stage healthy and no sign of contamination were used for *in vitro* cyto-toxicity assay.

Cell lines: Cancer cell lines used in this project were

- Human Lung Carcinoma Cell line (COR-L23)
- Human Brest Adenocarcinoma Cell line (MCF7)
- Murine Melanoma Cell line (B16F10)

Preparation of Cell Suspension:

The cultured flasks reaches near confluence stage, the cell lines were washed with phosphate buffer saline (PBS). The cells were trypsinized with 2.5 mL of 0.2% trypsin to detach from the flask surface. To this 10 mL of PBS was added and transferred to the centrifuge tube. The cell suspension was centrifuged and cell pellet was obtained. The cell density was counted by tryphan blue staining method in hemocytometer. The cell density was adjusted to 10,000 cells /100 µL (1 X 10^5 cells/ mL) from cell suspension. 100 µL of cell suspension was added to

each well of flat bottom 96 well plates and incubated at 37°C in 5% CO_2 and 90% relative humidity for 24 hours (La-ongthong and Suwanna2018).

Determination of Cytotoxicty by Sulforhodamine-B (SRB) Assay

SRB assay has been widely used to investigate cytotoxicity in cell based studies. SRB is a colorimetric assay for cytotoxic screening. The screening is based on cell line active growing under mitotic division. The cells are cultured in 96 well plates and the growth which depends upon the rate of multiplication is measured indirectly by the intensity of the colour which directly proportional to the number of cells present. In this assay the rate of proliferation of a cancer cell line in the presence and absence of the test substance is compared at specific time. Sulphorphodamine Blue (SRB) is a water soluble, pink aminoxanthine dye that binds to the basic amino acid residues of cellular proteins in the plasma membrane. The adsorb dye is dissolved in alkaline medium and thus, colorimetric measurement of the bound dye provides an estimate of the total protein mass that is related to the cell number. The greater the number of cells, the greater amount of dye is adsorbed and greater absorbance.

Procedure:

The cell suspension (procedure described in cell suspension preparation) 1 X 10^4 cells/ well were added in 96 well cell culture plate was incubated for 24 hours in CO_2 incubator at 37ºC. Add different concentrations of 100 µL of test item to each well in triplicates and incubated for 48 hours in CO_2 incubator at 37ºC. The cells were fixed by the addition 50 µL/well of chilled 50% TCA allowed for 1 hour at 4ºC to stop the cell growth. The plates were washed with distilled water for 5 times and allowed for drying. To each well 0.4% SRB 100 µL/well was added and incubated for 30 minutes at room temperature. Then the plates were washed with 1% acetic acid for 5 times and allowed for drying. To this plates 100 µL of Tris buffer (10 mM, pH 10.4) to dissolve the dye and read the plates at 540 nm in ELISA plate reader (Esteban *et al.*, 2016; Baraga *et al.*, 2007).

Cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of various extracts of three study plants was calculated as the following equation:

$$\textbf{Percentage of Cell growth} = \left\{ \frac{\text{Mean Test item OD}}{\text{Mean control OD}} \right\} \text{X } 100$$

Percent of Cell growth Inhibition = 100 - Percentage of Cell growth

Determination Cytotoxicty by MTT Assay

Cell viability of different cancer cell lines were evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric Assay. The MTT assay determines the ability of viable cells to convert a soluble tetrazolium salt, MTT into an insoluble formanzan precipitate. Tetrazolium salt accept electron from oxidized electron from oxidized substrate or appropriate enzyme such a2Q1s NADH and NADPH. In particular, MTT is reduced at the ubiquinone and cytochrome B and C sites for the mitochondrial electron transport system and is the result of succinate dehydrogenase activity. This reaction converts the yellow salt to blue coloured formazan crystals that can be dissolved in an organic solvent whose concentration can be spectrophotometrically determined.

Procedure:

The cancer cell lines were seeded at density of 1 X 10^4 cells/ well in 96 well cell culture plate and incubated for 24 hours. Then the medium was replaced with serum free RPMI 1640 medium and treated with various concentrations of plant extracts in triplicates and incubated for 48 hours in CO_2 incubator at 37ºC. Then the media was replaced with 100 µL of fresh media containing 1 mg/ mL MTT and incubated for 3 hours at 37ºC. After incubation the wells were aspirated and added the 100 µL of DMSO to dissolve the formazan crystals. The colour intensity was measured at 540 nm (Durvanei *et al.,* 2014; Mahmoud *et al.,* 2017).

Cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of various extracts of three study plants was calculated as the following equation:

$$\textbf{Percentage of Cell growth} = \left\{ \frac{\text{Mean Test item OD}}{\text{Mean control OD}} \right\} \text{X } 100$$

Percent of Cell growth Inhibition = 100 - Percentage of Cell growth

RESULTS

Screening of *in vitro* cytotoxicity activities of the selected three plant species oil extracts revealed a concentration dependent inhibitory activity resulting from reduction of Cytotoxicity test by SRB and MTT methods on three different types cancer cell lines COR-L23, MCF-7 and B16F-10.

Sulforhodamine-B (SRB) Assay

Hedyotis corymbosa, Inula racemosa and Holostemma adakodien for cytotoxicity was reported in **Table 1** and **Figure 2** respectively. The *Hedyotis corymbosa, Inula racemosa and Holostemma adakodien* was showed LC_{50} values in COR-L23 as 52.09, 43.04 and 31.27 µg/mL respectively. The three medicinal plants essential oil extracts screened for cytotoxicity against MCF-7 was showed LC_{50} as 54.16, 38.05 and 36.66 µg/mL respectively. The three medicinal plants essential oil extracts screened for cytotoxicity against B16-F10 was showed LC_{50} as 50.25, 45.35 and 40.16 µg/mL respectively.

The essential oil of three plants showed potent cytotoxic effect against the three different cancer cell line COR-L23, MCF-7 and B16-F10.

5.1.1.1 MTT Assay

Hedyotis corymbosa, Inula racemosa and Holostemma adakodien for cytotoxicity was reported in **Table 5.8** and **Figure 5.8** respectively. The *Hedyotis corymbosa, Inula racemosa and Holostemma adakodien* was showed LC_{50} values in COR-L23 as 34.77, 28.24 and 19.12 µg/mL respectively. The three medicinal plants essential oil extracts screened for cytotoxicity against MCF-7 was showed LC_{50} as 39.99, 28.98 and 30.16 µg/mL respectively. The three medicinal plants essential oil extracts screened for cytotoxicity against B16-F10 was showed LC_{50} as 30.59, 26.19 and 19.16 µg/mL respectively.

The essential oil of three plants showed potent cytotoxic effect against the three different cancer cell line COR-L23, MCF-7 and B16-F10

Table 1: *In vitro* Cyotoxicity data by SRB Assay

Cell Line	Conc (µg/mL)	% Cell Leathality	Conc (µg/mL)	% Cell Leathality	Conc (µg/mL)	% Cell Leathality
	Hedyotis corymbosa		***Inula racemosa***		***Holostemma adakodien***	
COR-L23	10	11.20	10	16.85	10	14.76
	20	28.74	20	30.31	20	48.30
	40	42.05	40	47.80	40	59.14
	80	60.82	80	66.20	80	71.33
LC_{50}	52.09		43.04		31.27	
MCF-7	10	13.39	10	11.37	10	19.06
	20	26.93	20	40.69	20	35.61
	40	42.77	40	54.49	40	53.96
	80	59.24	80	66.64	80	68.10
LC_{50}	54.16		38.05		36.66	
B16F10	10	8.37	10	6.13	10	14.29
	20	23.99	20	37.11	20	37.73
	40	41.77	40	47.43	40	46.67
	80	64.76	80	63.01	80	68.40
LC_{50}	50.25		45.35		40.16	

Table 2: *In vitro* Cyotoxicity MTT Assay

	Conc (µg/mL)	% Cell Leathality	Conc (µg/mL)	% Cell Leathality	Conc (µg/mL)	% Cell Leathality
	Hedyotis corymbosa		***Inula racemosa***		***Holostemma adakodien***	
COR-L23	6.25	9.10	6.25	17.74	6.25	21.92
	12.5	22.01	12.5	27.99	12.5	36.29
	25	38.16	25	45.70	25	60.67
	50	61.97	50	65.82	50	73.73
LC_{50}	34.77		28.24		19.12	
MCF-7	6.25	6.92	6.25	16.20	6.25	8.76
	12.5	18.92	12.5	36.45	12.5	24.70
	25	35.64	25	46.82	25	46.49
	50	56.51	50	60.46	50	64.00
LC_{50}	39.99		29.98		30.16	
B16F10	6.25	11.93	6.25	13.41	6.25	25.84
	12.5	27.00	12.5	35.88	12.5	37.55
	25	42.91	25	51.66	25	57.14
	50	64.30	50	63.60	50	72.23
LC_{50}	30.59		26.17		19.16	

Values are mean of triplicate

Figure 5.7: *In Vitro* Cytotoxicity by SRB Assay

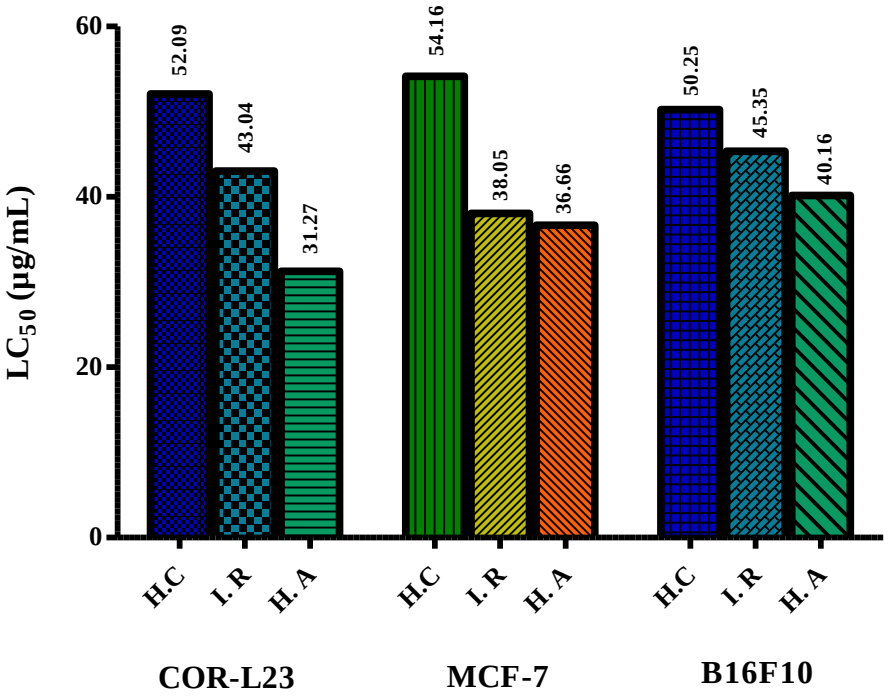

Fig. 5.7: The bars represent 50% inhibitory concentration (IC_{50} in µg/mL) of essential oil extracts; Extracts of *Hedyotis corymbosa, Inula racemosa and Holostemma adakodien*.

Figure 5.8: *In Vitro* Cytotoxicity by MTT Assay

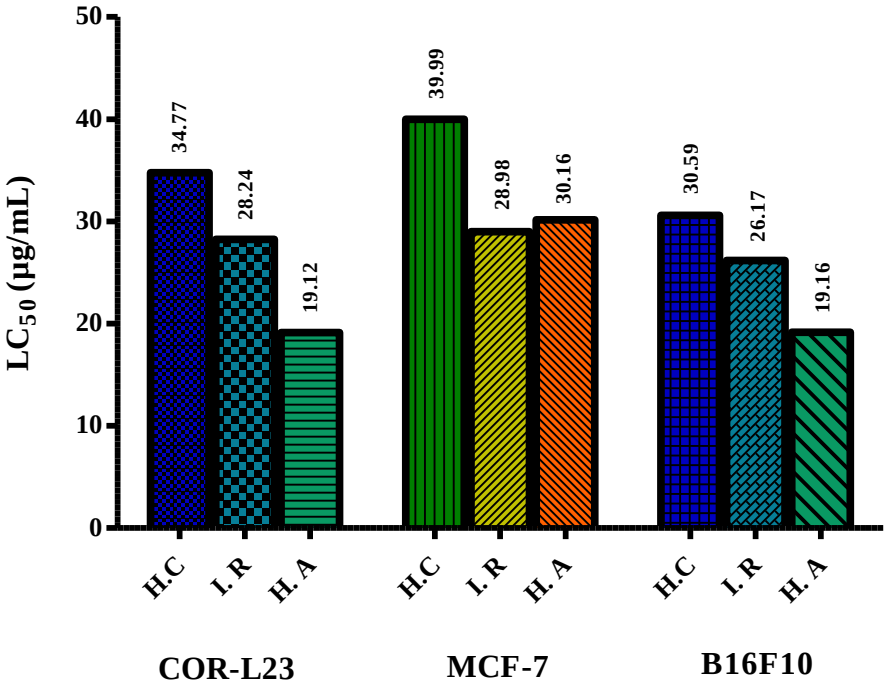

Fig. 5.8: The bars represent α-Amylase enzyme 50% inhibitory concentration (IC_{50} in µg/mL) of essential oil extracts; Extracts of *Hedyotis corymbosa, Inula racemosa and Holostemma adakodien*.

DISCUSSION

Anti-cancer studies that are carried out on the selected medicinal plants have been performed using their extracts, but in the present study essential oil was extracted and evaluated for their *in vitro* cyto-toxicity on cancer cell line models Human lung carcinoma cell line (COR-L23), Human breast adenocarcinoma cell line (MCF7) and melanoma cell line (B16F-10). In the present study anti-proliferative activity (cytotoxicity) of selected medicinal plant oil against cancer cell lines were estimated by SRB and MTT method.

SRB assay has been widely used to investigate cytotoxicity in cell based studies and this method cost effective. This method relies on the property of SRB, which binds stoichiometrically to proteins under mild acidic conditions and then can be extracted using basic conditions. Thus the amount of bound dye can be used as a proxy for cell mass. Which can then be extrapolated to measure cell proliferation. The sulforhodamine B (SRB) protein stain is used for in vitro chemosensitivity testing. The SRB assay appears to be more sensitive than the MTT assay, with a better linearity with cell number and higher reproducibility (Vichai and Kirtikara, 2006). The *Hedyotis corymbosa Inula racemosa and Holostemma adakodien* oil extracts shows high percentage of growth inhibition exhibited (anti-proliferative activity) by inhibiting the COR-L23, MCF-7 and B16F-10 cell proliferation. *Hibiscus cannabinus* L. seed extract and oil against human cancer cell lines and found significant activity (Yu *et al.,* 2014).

The MTT assay is a colorimetric method for assessing cell metabolic activity. NADPH dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. The MTT assay is based on the coversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells. The *Hedyotis corymbosa Inula racemosa and Holostemma adakodien* oil extracts shows high percentage of growth inhibition exhibited (anti-proliferative activity) by inhibiting the COR-L23, MCF-7 and B16F-10 cell proliferation. Different wild plant oil extracts of Saudi Arabia on human breast cancer cell lines were analyzed by MTT assay (Ali *et al.,* 2013).

CONCLUSION:

The *Hedyotis corymbosa Inula racemosa and Holostemma adakodien* oil extracts shows significant exhibited significant percentage of growth inhibition (anti-proliferative activity) by inhibiting the COR-L23, MCF-7 and B16F-10 cell proliferation. *In vivo* anti cancer activity of all three plant extracts will be tested. Finally, all the above mentioned literature evidence and our study results using the essential oils of the three study plants warrants for further study on the significant anti-cancer activity.

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