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Full Length Research Paper

Effect of camptothecin a potent anti cancer drug from *Mappia foetida* on breast cancer cell line MDA-MB-231

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The anti cancer activity of different parts of *Mappia foetida* (leaf, bark and root) on breast cell line (MDA-MB-231) was investigated. It was found that the extracts kill the cancer cell MDA-MB-231 in dose and duration dependent manner. The IC_{50} of the leaf, bark and root extract against MDA-MB-231 cells are 450 and 500 µg/ml respectively. Morphological changes and abnormal nuclear morphology was clearly visible on acridine orange (AO), ethidium bromide (EB) and Hoechst 33258 staining loss of mitochondrial membrane potential, extensive DNA damage and there was a slight change in the expression of the protein levels.

Key words: 3-(4,5-Dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide, microlitre, millilitre, fetal bovine serum, inhibition concentration, 5,5', 6,6' – tetracholo-1, 1', 3, 3' – tetraethyl benzimidazole carbocyanine.

INTRODUCTION

Cancer is a scourge-afflicting mankind from the time immemorial. It is a major killer disease not only in developed countries, but also in developing and under developed countries. Every year about 7 million new cases are diagnosed, half of the developing countries and about 5 million people are dying out of cancer. Breast cancer and ovarian cancer are very common among females. Breast cancer is commonly noted in women frequently traced in the age group beyond 40. World wide breast cancer is the second most common type of cancer after lung cancer (World Cancer Report, 2008).

In 2005, breast cancer caused 502,000 deaths world wide (7% of cancer deaths, almost 1% of all deaths, WHO, 2007). Estrogen receptor (ER) is a very important biomarker of breast cancer. Estrogen receptors are over expressed around 70% of breast cancer cases referred to as ER positive. Unfortunately women in the early stages of breast cancer may not have any symptoms. However as the cancer grows in size a women may notice a number of symptoms.

1. A lump or thickening in her breast or underarm area.
2. A change in size or shape of her breast.
3. Nipple discharge or nipple turning inward.
4. Redness or scaling of the skin on or around the nipple.
5. Ridges or pitting of the breast skin.

Treatment for breast cancer includes lumperctomy, mastectomy, chemotherapy, hormone therapy and radiation therapy. Surgical resection of primary breast tumors aims to control local disease and prevent recurrence in regional draining lymph nodes.

A large number of higher plants have been used as a source of drugs by mankind for several thousand years. It is estimated that 35,000 to 70,000 plant species have at one time or another been used in some culture for medicinal purposes. Camptothecin a monoterpene indulge alkaloid is a promising plant based metabolite known for its anti tumor activity. It was first isolated from a Chinese deciduous tree *Camptotheca acuminatea.* Other sources of camptothecin includes: *Nothapodytes nimmoniana* (*Mappia foetida*), *Ophirrohiza mungos* and *M*. *foetida.* In India it is distributed in the Western Ghats in Satara, Pune, Kolhapur, Raigad, Ratangiri and Jargon. It is a small tree or large shrub with strong fetid smell

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particularly the flowers.

Camptothecin contains a pentacyclic ring system that includes a pyrroll (3,4-b) quinoline moiety (ring A, B and C). Camptothecin and its derivatives are unique in their ability to inhibit DNA topoisomerase I by stabilizing a covalent reaction termed the cleavable complex which ultimately causes tumor cell death. Clinically it also exhibit remarkable anti-tumor and anti-leukemia activity. Hence the study has been undertaken to find out the effect of camptothecin on breast cancer cell line MDA-MB-231. To check the cytotoxicity of monobolic extract of leaf, bark and root in MDA-MB-231 by MTT assay, to find the morphological changes in the cells after treatment by acridine orange (AO), ethidiumbromide (EO) staining. The cytotoxicity of monobolic extract of leaf, bark and root in MDA-MB-231 was checked using MTT assay while the morphological changes in the cells were assessed after treatment with acridine orange (AO) and ethidiumbromide (EO) staining. Hoechst 33258 staining was used to find the mitochondrial transmembrane potential; by JC-1 staining, to find the extent extract of DNA damage by single cell gel electrophoresis (Comet assay) and one dimensional SDSPAGE was used to find the expression of protein levels.

MATERIALS AND METHODS

Studies on anti cancer effect of methanolic extracts of the leaf, bark and root of *Mappia foetida* against a breast cancer cell line MDA-MB-231 (ER negative) and a cervical cancer cell line SiHa was carried out during 2007 to 2008 at the Department of Biotechnology, Mother Teresa Women's University, Kodaikanal, Cell culture works were carried out in the Department of Animal Sciences, Bharathidasan University, Tiruchirapalli. A brief account of experimental methodology is presented as follows:

Plant material

The root and bark of *M*. *foetida* was collected from Gunduru, Hills of Kodaikanal, Tamil Nadu, India and leaves of *M*. *foetida* were collected from Herbal Garden, Department of Biotechnology, Mother Terasa Women's University, Kodaikanal. The leaves, bark and root were washed, shade-dried and powdered in mixer.

Extraction

The powdered leaf, bark and root was individually (10 g) extracted with methanol. Each extract was concentrated to a paste in vacuum at 40°C using a rotary evaporator (BUCHI, Germany). The paste was transferred to a vial and kept at 4°C until use.

Maintenance of MDA-MB-231 and SiHa cells

MDA-MB-231 and SiHa cells were grown as monolayer in RPM1 1640 medium with 10% FBS and 2% antibiotics. Stock cultures were sub-cultured every 7th day after harvesting the cells with trypsin EDTA and then seeding them in tissue culture flask to maintain in exponential phase.

Tumor cell line

The breast cancer cell line (ER negative) and cervical cancer cellline namely: SiHa, used in the present study, was procured from National Centre for Cell Science, Pune.

Culture media

RPMI 1640 medium with glutamine without sodiumbicarbonate was used for growing the cells and was obtained from Medox Agencies, India and serum of Fetal bovine serum was used for the growth of H460 and was obtained from Sigma Aldrich Chemicals, USA and antibiotics of penicillin and streptomycin were obtained from Sigma Aldrich Chemicals, USA.

Chemicals

0.25% trypsin EDTA was obtained from Himedia Lab Limited, Mumbai. All other chemicals used in the present investigation were of analytical grade and obtained from SD Fine Chemical Limited and SISCO Lab Limited, India.

Plastic-wares

Tissue culture flasks, tissue culture plates, centrifuge tubes, etc. were obtained from Medox Agencies, Chennai.

Cell counting

0.04% trypan blue in PBS, haemocytometer and inverted microscope was used and taken for the study. The cell suspension was mixed gently and an aliquot was added to the trypan blue solution-1 (100 L cell suspension; 100 uL dye) and was then counted in haemocytometer. MTT, 3- (4,5-Dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) is cleaved by mitochondrial dehydrogenase of viable cells, yielding a measurable purple Formosan product. This Formosan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity (Mosmann, 1983). MDA-MB-231 and SiHa cells were grown in 96 well microtiter plate (5 × 103 cells/well) for 24 h after seeding.

The plates were incubated with leaf, bark and root extracts at different concentrations ranging from 50 to 500 µg and without extracts for 24 and 48 h respectively (triplicates for each concentration for leaf, bark extracts and duplicates for root extract). The medium was refreshed and 20 µl of MTT (5 µg/ml) was added. The plates were incubated for 3 h in dark. The Formosan crystals developed were solubilized with 100 µl of DMSO and the plate was kept in the dark for another 5 to 10 min. The colour developed was measured in an ELISA reader (Bio Rad, USA) at wavelength 570 nm and with reference wavelength at 630 nm (Table 1).

Assessment of cell morphology

Morphological changes of treated cells were assessed by AO/EB staining and Hoechst staining under fluorescence microscope. Acridine orange stains only live cells whereas "ethidium bromide"

Table 1. Determination of IC_{50} concentrations of extracts on MDA-MB-231 and SiHa cancer cell line through assay.

MDA-MB-231 cancer cell line			SiHa cancer cell line		
Extract	**IC_{50} concentration (µg/ml)**		**Extract**	**24 h**	**48 h**
Leaf extract	450	300	Leaf extract	500	300
Bark extract	400	250	Bark extract	300	150
Root extract	500	400	Root extract	500	350

Table 2. Assessment of cell morphology on MDA-MB-231 and SiHa cancer cell lines treated with extracts of *Mappia foetida* by Acridine orange/Ethidium bromide staining.

Treated cell line (%)	24 h treatment with IC_{50} concentration of extract				48 h treatment with IC_{50} concentration of extract			
	Control	**Leaf**	**Bark**	**Root**	**Control**	**Leaf**	**Bark**	**Root**
MDA-MB-231								
Live cell	99	56	45	61	99	32	29	46
Necrotic cell	0.76	32	14	19	0.57	53	17	29
Apoptotic cell	0.24	12	41	20	0.43	15	54	26
SiHa								
Live cell	98	52	42	57	98	31	34	47
Necrotic cell	0.9	38	18	23	1.2	59	11	26
Apoptotic cell	1.1	10	40	20	0.8	10	55	27

stains dead cells and cells were appeared in green and orange colour respectively. Type of cell death (apoptosis and necrosis) was also assessed by nuclear morphology. Hoechst 33258 stains nuclei of both live and dead cells blue and apoptotic and necrotic cells and clearly distinguished by nuclear abnormalities in stained cells. MDA-MB-231 and SiHa cells were grown in 6 well plates (5 × 10^3 cells/ well) for 24 h. The cells were than incubated with the IC_{50} dose of the extracts for 24 and 48 h. The medium was discarded and the cells were washed with PBS. The cells were then trypsinized and placed on a glass slide and stained with acridine orange, ethidium bromide (Spector et al., 1998) and Hoechst stain (Kasibhatla et al., 2006). The cells were then viewed in an epifluorescent microscope (Olympus, Japan) (Tables 2 and 3).

Measurement of mitochondrial transmembrane potential (Δψm)

Mitochondrial swelling is often associated with the loss of mitochondrial membrane potential, a phenomenon readily measured using the mitochondrial dye JC1 (5.5' 6.6' – tetrachloro –1, 1' 3, 3' – tetraethyl benzimidazol carbocyanine iodide). In normal cells JCI accumulates in the inner mitochondrial membrane in which it oligomerizes and fluoresces red. If there is a reduction in mitochondrial transmembrane potential in cells results in diffusion of the dye from the mitochondria and fluoresces green.

The cells were grown in glass cover slips (22 × 22 cm) placed into 6 well plates and treated with the IC_{50} concentration of the extracts. The cells were stained with JC-1 dye after 6 and 12 h exposure. The mitochondrial depolarization patterns of the cells were observed in a fluorescent microscope (Carl Zeds, Jena, Germany) fitted with a 377 to 355 nm filter, at ×400 magnification.

Comet assay

The comet assay (single cell gel electrophoresis) is the sensitive method for evaluating the DNA damage (Mohan et al., 2002). The alkaline comet assay is applied to detect both single and double strand breaks and formation of

Table 3. Assessment of cell morphology on MDA-MB-231 and SiHa cancer cell lines treated with extracts of *Mappia foetida* by Hoechst staining.

Treated cell line (%)	24 h treatment with IC_{50} concentration of extract				48 h treatment with IC_{50} concentration of extract			
	Control	Leaf	Bark	Root	Control	Leaf	Bark	Root
MDA-MB-231								
Normal nuclei	99.38	54	51	68	99.67	38	30	43
Abnormal nuclei	0.62	46	49	32	0.33	62	70	57
SiHa								
Normal nuclei	98.9	62	53	66	98	44	48	58
Abnormal nuclei	1.1	38	47	34	2	56	52	42

comets depends upon the extension of DNA damage after treatment.

Protein profile analysis

1. The treated MDA-MB-231 and SiHa cells were pelleted out and homogenized with PBS (pH 7.2). The amount of protein was estimated by Bradford's method and 60 µg of protein was electrophoresed in 10% gel using SDS-PAGE for 4 h under 100 V.
2. The gel was stained with Coomassie Brilliant Blue R-250 overnight.
3. It was then destained using destained solution.
4. The bands were visualized and documented in an automated gel documentation system.

RESULTS AND DISCUSSION

In the present study we focused on the anti tumor potential of leaf, bark and root extracts of *M*. *foetida*. Camptothecin, an anti-cancer agent is present in *M*. *foetida*. The tree is reported to be a major source of Camptothecin in India. The presence of Camptothecin in this plant was first reported by Govindachari and Viswanathan (1972). This study indicates that the extracts kill the cancer cells MDA-MB231 and SiHa, in dose and duration dependent manner. MTT assay is one of the most established and a standard assay for assessing viability of cells is a true indication of death of cells on treatment with the extracts. The results revealed that the leaf extract was more efficient in killing the cell at low concentration than the other two extracts. At 24 h, the IC_{50} of the leaf extract for MDA-MB-231 was found to be 450 µg/mL and the IC 500 µg/mL respectively. The inhibitory effect on MDA-MB-231 cells was observed with Topotecan, an analogue of Camptothecin. The IC_{50} concentration of Topotecan was found to be 160 ng/ml in MDA-MB 231 cell line. Treatment with Quercetin enhanced cytotoxicity of Topotectan as 1,3 fold inMDA-MB-231 cell line (Akbas et al., 2005). Analysis of the mode of cell death is one of the important aspects in cancer therapy. There are several approaches to detect the mode of cell death. Here, staining the cells with acridine orange (AO) and ethidium bromide (EF) and Hoechst 33258 was practiced. In this study it was found that cells stained with the fluorescent dyes revealed abnormal nuclear morphology revealing both apoptosis and necrosis. The treatment with bark extract induced higher level of apoptotic death.

Treatment with the root extract induced both the type of cell death at an equal ratio. The development of resistance to apoptosis is an important step in carcinogenesis. Despite this resistance, cancer therapeutic drugs are effective because of the ability to induce cancer cell selective death. Cancer biologists have now begun to consider whether effective cancer therapeutics might induce cell death by activating necrosis. Approaches reported to be able to induce necrotic death in cancer cells include photodynamic treatment and alkylating DNA damaging agents. Several other chemicals or reagents such as β-lapachone, apoptolidin and honokil also appear to induce cancer cell death through necrosis (Li et al., 1999; Salomon et al., 2000; Tagliarino et al., 2001; Bai et al., 2003). Apoptosis and necrosis are often initiated in response to the same type of insults with different doses of intensities. The dosage dependence of the switch from apoptosis to necrosis in response to the same insults may also alert the organism to the extent of damage and define whether a repair response is required to maintain organismal integrity. Onset of the mitochondrial permeability transition (MTP) is a

key event in both necrotic and apoptotic cell death (Lemasters et al., 1998). Loss of mitochondrial transmembrane potential was observed in both MDA-MB-23 and SiHa cells. All the three extracts led to the loss of mitochondrial potential. Mitochondria are well known as targets led to the loss of mitochondrial potential. Mitochondria are well known as targets for chemotherapeutic agents because they have a central role in the induction and regulation of apoptotic cell death (Brenner et al., 2003; Debatin et al., 2002). However, it has recently become evident that mitochondria can also play a role in primary necrosis (Crompton, 1999; Nieminen, 2003) although few current chemotherapeutic agents induce this form of cell death.

It was also observed in comet assay that the treatment of the cells with all the three extracts induced DNA damage which was clearly seen by the appearance of comets with tails. These observations clearly indicate that the extracts have anti-cancer activity in both cell lines via apoptotic and necrotic cell death. The results of the study in respect of proteins indicate that there is a slight alteration in the expression of proteins. These proteins should be those that are expressed by pro-apoptotic genes. These extrapolations are only circumstantial. The specific methodologies would have been analysis of proteins adopting Western blotting; analysis of mRNA through Northern blotting and expression analysis of the genes through PCR amplified hybridization techniques. This being a highly preliminary study, conducted over a short period, these approaches could not be applied. The anti-proliferative and morphological effects of the extracts show the target-specific killing of cells. These extracts show target based cancer therapy since the mechanism of cell death, appears to be both apoptosis and necrosis. Necrosis also is one of the desired endpoints in cancer therapy. Currently, there is no effective therapy for estrogen independent breast cancers (Thangapazham et al., 2007). Estrogen receptor negative (ER) breast cancer is optically more aggressive with poorer progenies (McLean et al., 2007). Breast tumors that express ER are generally more amenable to endocrine therapy compared with ER negative tumors that exhibit “de novo” resistance. Because all endocrine therapies are designed to block ER function in someway, the identification of new therapies or strategies for sensitization of ER-negative breast cancer cells has become very important (Sharma et al., 2006). The leaf, bark and root extracts of *M*. *foetida* were effective against estrogen receptor negative breast cancer cell line MDA-MB-231. In the same way treatment of cervical cancer caused due to HPV infection can be treated with surgery, chemo- and radiotherapy which have a few harmful side effects. The recurrence rate is also higher (Souhami and Tobias, 2005).

The treatment of cervical cancer cell line, SiHa, (HPV-16 viral genome integrated) with the leaf, bark and root extracts of *M*. *foetida* showed inhibitory effect on the cells and also cell death.

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