



## ANTICANCER ACTIVITY OF CRUDE EXTRACT OF *EUCALYPTUS GLOBULUS*, *TINOSPORA CORDIFOLIA* ON MCF-7 CELL LINE

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**Abstract:** Methanolic crude extracts of *Eucalyptus globulus* and *Tinospora Cordifolia* grown in natural and industrial polluted conditions were investigated for their anticancer activity against MCF-7 breast cancer cell lines to study the pollution effect on Cytotoxicity. It was carried out by the XTT assay using serial dilutions. The Cytotoxicity of individual plants and also the combined extracts of plants grown in polluted and natural area were carried out separately to check out the differences. The MCF – 7 cell lines were procured from National Centre for Cell Science, Pune. At final concentration of 200ug/ml the results indicated the cytotoxicity of individual plant extracts grown in polluted area was more than that of plants grown in natural conditions. The combined action of both plants at 200ug/ml concentration showed better results than the individual extracts. The cell inhibition of combined extracts of polluted area 81.2% and natural area 84% having IC<sub>50</sub> values 82.400 µg/mL, 91.980 µg/mL respectively. As such the plants that grown in industrial polluted area showed the better activity against MCF – 7 cell lines, further it could be recommended to study the pure components and their anti-cancer affectivity for the substitution of synthetic drugs.

**Keywords:** *Eucalyptus globulus*, *Tinospora cordifolia*, MCF-7 breast cancer cell lines

### INTRODUCTION

Cancer is a class of diseases characterized by out of control cell growth, invasion and sometimes metastasis. There are over 100 different types of cancers are found. Cancer may affect people at all ages, even fetus, but the risk for most varieties increases with age. Cancer causes about 13% of all human deaths. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007 (1, 11).

Cancer is the second leading cause of death in the world, so scientists over the entire world do their best to discover safe cancer therapy. Cancer is considered as a major public health problem either in the developed and developing countries over the world.

In the world it is estimated 9 million new cancers are diagnosed every year and over 4.5 million people die of cancer. In India per year 7lakh cases and over 3.5 lakhs people die of cancer and 2.3 lakhs new cases are of tobacco related. The leading sites of cancer in male are pharyngeal cancer. The most common and accounts for 14.1% of total cancer and the leading sites of cancer in female is cervix uteri accounted for 26.7% and second most common site is Breast accounted for 16.6% (28). As per the statistics news of WHO the

Number of people being diagnosed with cancer in the world each year has leaped to more than 14million.

India is the first of the emerging economics to join IARC in 2006, and is active participating state of the global cancer research agency. In India around 555000 people died of cancer in 2010. According to estimates published in the lancet (38) of which 45% of Breast cancer death. The Breast cancer in Urban Indian women is 25-30 and the age is adjusted to 30-35 and the new cases 100000-125000, Breast cancer cases every year in India (36).

A Number of undecided side effects that occur during Chemotherapy can be reduced by using plant derived products in cancer treatments. Various active compounds derived from medicinal plants have been assisting for their efficacy and tolerability and treatment of Breast Cancer. The present work is an attempt to find out cytotoxicity of crude extracts of *Eucalyptus globulus* and *Tinospora cordifolia* on MCF-7 breast cancer cell lines.

### Cytotoxicity of crude extracts of Medicinal plants:

Measurement of cell viability and proliferation comprise the underlying basis for numerous in vitro

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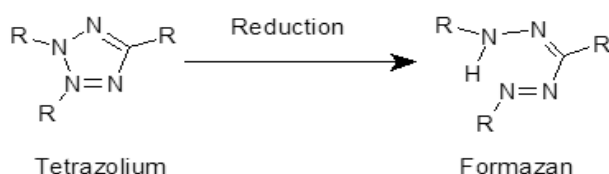
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assays directed towards the quantitation of a cell population's response to external factors. The use of tetrazolium salts, like MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) and XTT (2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide), to assay cell proliferation, cell viability, and/or cytotoxicity is a widespread, established practice.

According to Mosmann and Tada *et al.*, (22, 35) One weakness inherent in the use of MTT is that the resulting colored formazan product is insoluble, precluding direct spectrophotometric absorbance measurements without first dissolving the crystals. While a number of useful procedures have been devised to handle this problem, they all require additional sample processing steps and increase the length of time required to complete the assay.

Therefore Tetrazolium salt, XTT, has been synthesized by Paull and colleagues (24) Bioreduction of XTT yields a highly colored formazan product, which in contrast to other tetrazolium salts like MTT, is water soluble. Studies by Scudiero *et al.*, (31), evaluated the use of XTT with human tumor cells and demonstrated the ability of electron coupling agents to potentiate bio-reduction of XTT. Advantages of XTT are Sensitive, No radioactivity, Rapid (no solubilization step as in an MTT assay) (29, 34) Ideal for high throughput assays (no washing or other steps that can cause cell loss and variability).



Marjaneh (20) explained the 50% inhibitory concentration (IC<sub>50</sub>) value determined using the proliferation assay was 25µM thymoquinone. Late apoptotic cell percentage increased rapidly when treatment duration was increased to 24 h with 25 and 100µM thymoquinone. Further analysis using cell cycle assay showed thymoquinone inhibition of breast cancer cell proliferation at minimal dose 25 µM and led to S phase arrest significantly at 72 h treatment ( $P = 0.009$ ). It was also noted elevation sub-G<sub>1</sub> peak following treatment with 25µM thymoquinone for 12 h. Increase in thymoquinone to 50µM caused G<sub>2</sub> phase arrest at each time-point studied. The works many authors proved anticancer activity of plant derivative drugs (10, 25).

Lambertin *et al.*, (18) determined the activity of extracts from Bangladeshi medicinal plants on human breast tumor cell lines. Extracts displayed anti-

proliferative activity on MCF7 and MDA-MB-231 breast cancer cell lines. Supportingly Marvin *et al.*, (21) works reported chemo preventive agent that affects cell proliferation by arresting the cell cycle in G<sub>2</sub> and modulating the wnt signaling pathway.

Shoib (32) works with Thymoquinone TQ-induced cytotoxicity was investigated using canine human breast adenocarcinoma (MCF7), cytotoxicity was determined using a proliferation assay (MTT assay) and apoptosis assays. These results suggest that TQ kills cancer cells by a process that involves apoptosis and cell cycle arrest. Non-cancerous cells are relatively resistant to TQ.

Also there were reports from Sandhya and Mishra (30) about the cytotoxic effects of Triphala (TPL), an Indian Ayurvedic formulation with known anti-cancer properties, has been investigated on two human breast cancer cell lines differing in their p53 status. In vitro studies showed that MCF 7 with wild type p53 was more sensitive. The results have demonstrated that MCF 7 and T 47 D cells exhibited differential sensitivity to TPL, which seems to be dependent on their p53 status. Inhibition of anti-proliferative ability of TPL by antioxidants suggests a role for TPL induced ROS in the induction of apoptosis. It is concluded that p53 status of cancer cells formed an important factor in predicting the response of cancer cells to pro-oxidant drugs.

Investigations of Yosonet *et al.*, (40) growth-inhibitory effect on MCF-7 human breast cancer cells, resulted from proliferation assay using tritium uptake showed that the proliferative capacity of MCF-7 cells was strongly suppressed in the presence of plant ethanol extract. This was further confirmed through MTT assay.

#### Cytotoxicity of *Tinospora cordifolia*:

The aqueous extract of *T. cordifolia* stem has shown to produce immunological activity due to the presence of arabinogalactan. The plant is known for its antispasmodic, antipyretic, anti-neoplastic, hypolipidemic, hypoglycemic, immune potentiating and hepatoprotective properties. It is also used in general debility, digestive disturbances, loss of appetite and fever in children, dysentery, gonorrhoea, urinary diseases, viral hepatitis and anaemia (1,12,33). Present communication reports the scientific evaluation of medicinal efficacy of *T. cordifolia* as antibacterial, antioxidant and anticancer agents.

*T. cordifolia* has immune-modulating (16), antidiabetic (7) and antineoplastic (11, 22) activities. It can also reduce the metastatic potential of melanoma cell in mice (19), decrease symptoms of allergic rhinitis (3).

*Tinospora cordifolia* have been used in the traditional system of medicine in India (Ayurveda) for the treatment of cancer. The current study investigated the cytotoxic and apoptotic effects of extracts of the *Tinospora cordifolia* on human breast cancer cells. MTT-based Assay revealed dose-dependent cytotoxic effects of the ethanol extracts of *Tinospora cordifolia* in human breast cancer cells.

*Tinospora cordifolia* have been used in the traditional system of medicine in India (Ayurveda) for the treatment of cancer (26,39,6).

More recently, extracts of *Tinospora cordifolia* have been reported to inhibit skin carcinogenesis (4) and experimental metastasis in mouse model (19), to induce cytotoxic effects in cultural HeLa cells (11,12), and antineoplastic effects in Ehrlich ascites carcinoma bearing mice (13).

Induction of apoptosis in cancer cells is recognized as a valuable tool for breast cancer treatment (5). Therefore, the current study investigated the cytotoxic and apoptotic effects of *Tinospora cordifolia* extracts against human breast cancer cell lines such as MCF7 was used as experimental control in the study since previously TC extract was shown to have cytotoxic effects against HeLa (4, 11). Agents that are proficient to induce apoptosis in cancer cells without harming normal cells have drawn considerable attention for the development of novel anticancer drugs (5). Hence, the current study also investigated the effects of *Tinospora cordifolia* extracts on human immortalized but 'non-cancerous' cell line (HaCaT).

#### **Cytotoxicity of *Eucalyptus globulus*:**

Cell culture and treatment: Plant extracts were evaluated for their in vitro cytotoxicity towards two human breast adenocarcinoma cell lines: MCF7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative). Cell culturing and experimental procedure were conducted as previously described (14,17).

The half-maximal inhibitory concentration values (IC<sub>50</sub>), defined as the concentration that inhibits 50% of cell growth, were calculated from concentration-response curves. IC<sub>50</sub> values were expressed as the mean of a minimum of three repeated experiments performed for each plant extract.

All *M. communis* and *E. camaldulensis* extracts exhibited cytotoxic effects on MCF 7 and MDA MB-231 cell lines, and the results were confirmed both by MTT and SRB assay. At the highest tested concentration (50µg/ml), cytotoxicity of organic extracts/fractions was in the range from 40% to as high as 98%, while for water fraction it was in the range of 30-70%.

Aqueous acetone extract of *E. camaldulensis* exhibited a dose-dependent growth inhibitory effect on MCF 7 cell line, with recorded IC<sub>50</sub> value of 36.5µg/ml (33).

The Anticancer works done (8) by using as a sulphorhodamine B assay analyzed the in vitro cytotoxic activities of extracts against Human breast adenocarcinoma cell line (MCF7) exhibited cytotoxic action on MCF-7.

#### **Experimental Investigations:**

**Cell culture:** Human cancer cell lines used in this study were procured from National Centre for Cell Science, Pune. All cells were grown in Minimal essential medium (MEM, GIBCO) supplemented with 4.5g/L glucose, antibiotics (BenzylPencillin-50units/mL, Streptomycin -50µg/ml and Amphotericin -B -50 µg/ml), 2 mM L-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37°C in 5% CO<sub>2</sub> incubator.

**XTT assay:** The biochemical procedure is based on the activity of mitochondrial enzymes which are inactivated shortly after cell death. This method was found to be very efficient in assessing the viability of cells. A colorimetric method based on the tetrazolium salt, XTT, was first described by P. A. Scudiero in 1988. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5x10<sup>3</sup> cells/well in growth medium and cultured at 37°C in 5% CO<sub>2</sub> to adhere. After 24hr incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (12.5,25,50,100 and 200µg/ml) in triplicates to achieve a final volume of 100µl and then cultured for 48hr. The compound was prepared as 1.0 mg/ml concentration stock solutions in DMSO. Culture medium and solvent are used as controls. Each well then received 50 µl of fresh XTT (0.9mg/ml in RPMI along with XTT activator reagent) followed by incubation for 2hr at 37°C. At the end of the incubation shacked the 96 micro well plate for 15sec. The Optical Density (OD) of the culture plate was read at a wavelength of 490 nm (reference absorbance at a wavelength of 630 nm) on an ELISA reader, Anthos 2020 spectrophotometer.

- **% cell survival:**  $100 - \{(At-Ab) / (Ac-Ab)\} \times 100$   
Whereas, At = Absorbance of test  
Ab = Absorbance of blank  
Ac = Absorbance of control
- **% cell inhibition:** 100 - % cell survival

**RESULTS**

The Cytotoxic Effects of the Methanolic crude extracts of *Eucalyptus globulus* and *Tinospora cordifolia* were evaluated on MCF-7 breast cancer cell lines by micro culture XTT Assay. The multiple concentration of methanolic extract from *Eucalyptus globulus* and *Tinospora cordifolia* were use and effective doses were calculated from dose response curve. The Results of

cytotoxicity evaluated against MCF-7 using the extracts at different concentrations (6.25, 1.5, 25, 50, 100 and 200ug/ml) (table 1). The Methanol extracts of *Eucalyptus globulus* and *Tinospora cordifolia* exhibited cytotoxic effect on MCF-7 cell lines were confirmed by XTT Assay.

**Table 1:** Dose Response of E.g (N), E.g (P), T.c (N), T.c (P), E.g (N)+T.c (N) and E.g (P)+T.c (P) on MCF -7 Cell line.

Blank = 0.040  
Control = 0.497

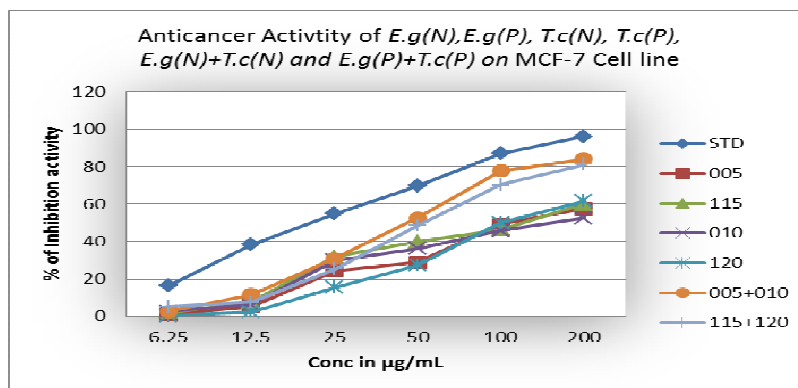
Conc (ug/ml)	OD of STD (Tamoxifen) at 490 nm			OD of E.g (n) at 490nm			OD of E.g (p) at 490nm			OD of T.c (n) at 490nm		
	% CS	% CI		% CS	% CI		% CS	% CI		% CS	% CI	
6.25	0.423	83.8	16.2	0.492	98.9	1.1	0.488	98	2	0.485	97.4	2.6
12.5	0.322	61.7	38.3	0.473	94.7	5.3	0.462	92.3	7.7	0.468	93.7	6.3
25	0.246	45.1	54.9	0.386	75.7	24.3	0.352	68.3	31.7	0.361	70.2	29.8
50	0.179	30.4	69.6	0.365	71.1	28.9	0.315	60.2	39.8	0.331	63.7	36.3
100	0.095	12	87	0.272	50.8	49.2	0.285	53.6	46.4	0.288	54.3	45.7
200	0.058	3.9	96.1	0.234	42.5	57.5	0.222	39.8	60.2	0.257	47.5	52.5

Conc (ug/ml)	OD of 120 STD at 490 nm		OD of E.g + T.c (n) at 490nm		OD of E.g (p)+T.c (p) at 490nm		% CS	% CI	
	% CS	% CI	% CS	% CI	% CS	% CI			
6.25	0.495	99.6	0.4	0.485	97.4	2.6	0.474	95	5
12.5	0.486	97.6	2.4	0.444	88.4	11.6	0.462	92.3	7.6
25	0.426	84.5	15.5	0.356	69.1	30.9	0.382	74.8	25.2
50	0.372	72.6	27.4	0.257	47.5	52.5	0.276	51.6	48.4
100	0.269	50.1	49.9	0.142	22.3	77.7	0.176	29.8	70.2
200	0.215	38.3	61.7	0.113	16	84	0.126	18.8	81.2

E.g=*Eucalyptus globulus*  
T.c=*Tinospora cardifolia*  
N=natural  
p=polluted

**Table 2:** showing the IC50 values of plant extracts used against MCF-7 cell lines

Plant	E.g(N)	E.g(P)	T.c(N)	T.c(P)	E.g(N)+T.c(N)	E.g(P)+T.c(P)
IC50	145.85µg/mL	136.53µg/mL	156.95µg/mL	139.91µg/mL	82.400µg/mL	91.980µg/mL
Slope	0.27773134	0.26371002	0.23139161	0.32012509	0.40436674	0.39459844
Corrélation coefficient	0.907716280	0.86551911	0.8376033	0.9425528	0.88480409	0.90949691
Intercept	9.49054726	13.9940299	13.681592	5.20845771	16.6800995	13.7044776



E.g=*Eucalyptus globulus*  
T.c=*Tinospora cardifolia*  
N=Natura  
P=Polluted

### The cytotoxic effect of Methanolic extracts of *Eucalyptus globulus* on MCF-7 breast cancer cell line by XTT Assay:

At the final tested concentration (200ug/ml) the cytotoxicity of *Eucalyptus globulus* grown in natural and polluted areas was 57.5% and 60.2% respectively. The effects were dose dependent and based on dose response curve IC<sub>50</sub> values were determined (Table 2). The methanol extract of both extracts of *Eucalyptus globulus* exhibited the anti-proliferative effect on both cell lines with IC<sub>50</sub> values 145.85ug/ml and 136.53ug/ml respectively. From the result it is evident that effect of extract *Eucalyptus globulus* of natural area showed less cytotoxicity than the *Eucalyptus globulus* polluted. The cytotoxicity of the crude extract of both are half the way ranging between 50% to 60% to reach the results of control Tamoxifen where it has 96.1%.

### The cytotoxic effect of Methanolic extracts of *Tinospora cordifolia* on MCF-7 breast cancer cell line by XTT Assay:

At the final concentration of 200ug/ml the cytotoxicity of *Tinospora cordifolia* grown in natural and polluted areas were 52.5% and 61.7% respectively based on the dose response curve IC<sub>50</sub> values were determined (Table 2). The plant extracts exhibited the high antiproliferative effect on MCF-7 cell lines with IC<sub>50</sub> values 156.95ug/ml and 139.91ug/ml respectively. And like in the case of *Eucalyptus globulus* the cytotoxicity is less in *Tinospora cordifolia* natural area than that of polluted area.

### The cytotoxic effect of combined extracts of E.g and T.c from natural and polluted sources on MCF-7 cell lines:

The cyto-toxic effect of combined samples proved better result in comparison with the individual samples. The combined samples of *Eucalyptus globulus* & *Tinospora cordifolia* natural area showed at final concentration of 200ug/ml, 84% of cell inhibition having IC<sub>50</sub> value 81.400ug/ml and the *Eucalyptus globulus* & *Tinospora cordifolia* Polluted area showed 81.2% of cell inhibition IC<sub>50</sub> value 91.980ug/ml.

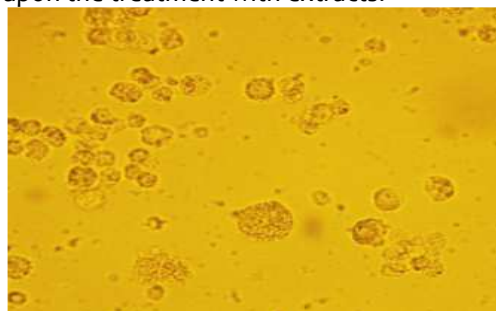
Here in contrary to the individual samples the cytotoxicity exhibited by the combined extract of plants grown in polluted area is less than that of natural extracts. Cytotoxicity results also promising as the cytotoxicity of the combined extract almost nearing to substitute the control tamoxifen results 96.1% at final concentration.

### Evaluation of Morphological changes of MC (F) – 7 cell lines upon the treatment with extracts.

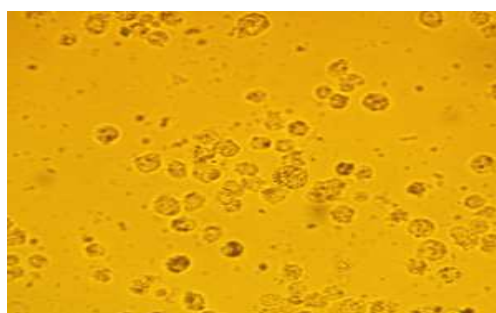
Morphological changes of MC (F) – 7 cell lines on treating with extracts of the plants grown both polluted and natural resources were observed under

phase contract microscope. The cells indicated most prominent effect after exposure to the extracts of *Eucalyptus globulus* & *Tinospora cordifolia* of Polluted area and their combined extracts than that of individual extracts of *Eucalyptus globulus* & *Tinospora cordifolia* of natural area (photos 1 – 8). In the combined sample extracts 40% to 50% of the cells showed membrane blebbing (demonstrated with small protrusions of the membrane) and ballooning were apparent in the cells.

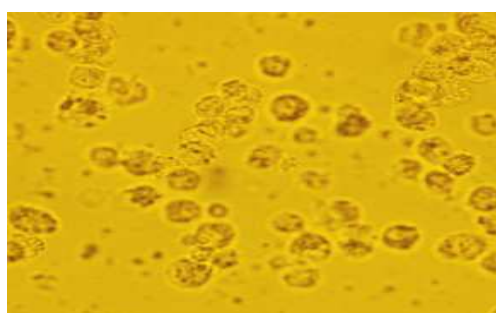
### Photos 1-8: Morphological changes of MC (F) – 7 cell lines upon the treatment with extracts.



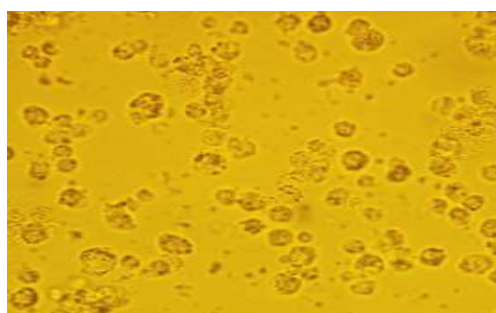
1. E. g (N) at final concentration



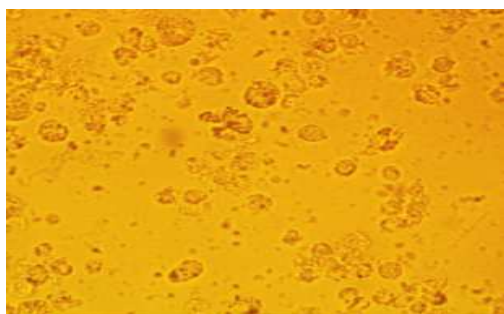
2. E.g (P) at final concentration



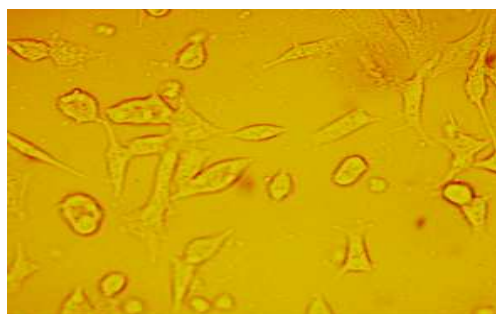
3. T.c (N) at final concentration



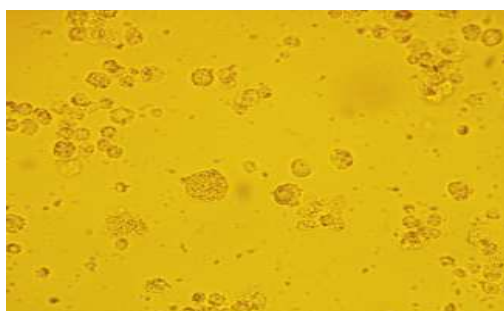
4. T.c (P) at final concentration



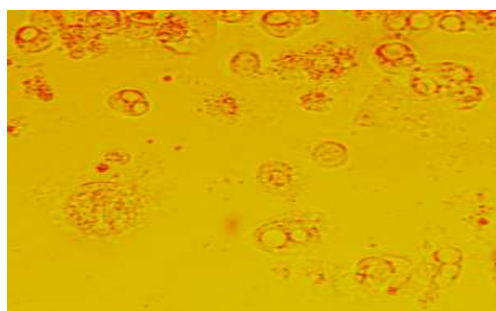
5. E.g (P)+T.c (P) at final concentration



7. MCF-7 Untreated



6. E.g (N)+T.c (N) at final concentration



8. Tamoxifen treated MCF-7

E.g=*Eucalyptus globulus*

T.c=*Tinospora cordifolia*

N=Natural

P=Polluted

The presence of apoptotic bodies could also be seen in the treated cells (photos 1-8). Cells also showed extensive vacuolation in the cells cytoplasm, indicating autophagy like mechanism of cell death. Autophagosome like structures were clearly seen in the cells treated with extract. At highest concentration (200ug/ml) the cells became rounder, shrunken and showed signs of detachment from the surface of the wells denoting cell death.

### DISCUSSION

Plants contain almost unlimited capacity to generate compounds that fascinates researchers in the quest for new and novel chemotherapeutics. The persistency search for new anticancer compounds in plants medicines and traditional foods is a realistic and promising strategy for its prevention (Yan-Wei et al., 2009). Numerous compounds found in plants with anticancer properties are such as alkaloids, phenylpropanoids and terpenoid.

In the present study anticancer activity of extracts of indigenous medicinal plants *Eucalyptus globulus* & *Tinospora cordifolia* grown in polluted and natural sources were investigated against human breast cancer cells MC (F)-7 and Tamoxifen was used as experimental control. A methodical evaluation of cytotoxicity effects revealed that the individual methanolic extract of *Eucalyptus globulus* & *Tinospora cordifolia* Polluted and natural areas and their combined samples and

tamoxifen showed dose dependent cytotoxicity against MC (F)-7 cell lines. The IC<sub>50</sub> values of combined plant extracts were found to be less than 100ug/ml indicating potent cytotoxic effects on breast cancer cell lines and further potential of these extracts for the isolation of biologically active phytochemicals. Most anti-cancer drugs are designed to eliminate rapidly proliferating cancerous cells and therefore they show cytotoxicity and induce apoptosis in cancer cells.

Several studies have evaluated the relations between anticancer activities of plant extracts and their phenolic content (33). Previous studies suggest that a correlation exists between the structural oxidation state and the position, number, and nature of substituents of the polyphenolic compounds and their anticancer effects (9). The effect of phenolics on the cell cycle could probably contribute to the tumor cell killing. Phenolic compounds are reported to be exerting a direct anticancer action, evident at low concentrations even, comparable with those found in biological fluids after ingestion of foods rich in phenolic compounds. Furthermore, the direct interaction with the aryl hydrocarbon receptor, the nitric oxide synthase inhibition and their pro-apoptotic effect provides some insights into their biological modes of action (15).

The phytochemical analysis of my study observed the presence of a large number of bio active

compounds in the methanolic extract of these plants including Terpenoids, alkaloids, phenols and flavonoids which exhibit various biological activities. These compounds hold great potential as drugs and wide accepted among the public. The investigation provides evidence for cytotoxicity in MG (F)-7 which may be due to existing phytochemicals in the extract as mention previously. The sensitivities of cancer cells to cell death by flavonoids are accordance with this finding from previous reports in literature. In another study, the presence of alkaloids with flavonoids in *Onobishirta* was reported expressing superior activity against cancer cells.

Our results have shown in par with the results of Mishra (2) that the phytochemicals present in *T. cordifolia* have potent cytotoxic and anti-cancer potential against MCF-7 cell line. Cancer cell lines used in the study exhibited differential sensitivity towards different plant extracts. The differential behavior of cell lines may be due to different molecular characteristics of these cells. The present study clearly indicates that *T. cordifolia* extracts are very active against human breast cancer cell lines.

Polyphenols have been shown to possess antimutagenic and antimalignant effects. Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis. The cytotoxic and antitumor properties of the extract may be due to presence of these compounds. Adhvaryu(1) have shown very high efficacy in *T. cordifolia* extracts against Dalton Lymphoma ascites (DLA) tumor model in Swiss Albino mice in terms of survival as well as tumor volume control. However, the exact mechanism is not clear. Available evidences suggest that DNA damage, inhibition of topoisomerase II, decline in clonogenecity and glutathione-S-transferase activity, activation of tumor associated macrophage, increase in lipid peroxidation and LDH release to be probable mechanisms behind the cytotoxic activity (12,13). The arabinogalactan present in aqueous extract of guduchi stem has also been shown to produce immunological activity. Many of the compounds mentioned above have been reported to be cytotoxic.

### CONCLUSION

In this study the findings show there is much relationship between anti-cancer activity and phenolic composition. The extract of polluted plants show high anti-cancer activity also showed high phenolic composition. The finding suggests the plants grown under polluted conditions reduced the number of viable cells than that of plants grown under natural conditions. As the geochemical analysis ruled out the toxicity of heavy metals in these plants as their concentration fall below permissible (27) limits and the

scope of using plants under abiotic stress and taboo of avoiding these medicinal plants could be altered by further studies in these areas.

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