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Antiproliferative and Antioxidant effect of Ocimum tenuiflorum leaves extract on Breast cancer cell lines: In vitro study

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Abstract :

Plant extracts are complicated mixtures of phytochemicals. Plant derived products used in therapy continue to be a major source of beneficial drugs as most of the medicinal plants have pharmacological activity.This study aimed to assess the cytotoxic effect of *Ocimum tenuiflorum* on different cell lines (MCF-7, MDA-MB-468 and MDA-MB 231). For achieving this different doses of leaves extract were taken and introduced into cancer cells lines were recorded at 72 hrs respectively. The experiments are done in triplicates and values are expressed as mean ±SEM. The value of IC_{50} for different cell lines are as follows, be 1119 ± 102 nM, 1379 ± 167 nM, 1719 ± 120 nM and 3919 ± 602 nM respectively. *O.tenuiflorum* leaf extract alone is cytotoxic to MCF 7, MDA-MB-231 and MDA-MB-453 cancerous cell lines and reduces the CNVs, suggesting the very significant correlation with cell viability. Interestingly, we observed significant decrease in cell survival in these cancer cell lines when treated with the extracts of leaves.

Key words:*Ocimum tenuiflorum, Cytotoxicity, anti proliferative, cell lines.*

Introduction

Breast cancer is the most recurrent malignancy in women and is a heterogeneous disease on the molecular level. Over the last two decades, advancements in treatment have progressed, with prominence being positioned on more biologically-directed remedies and treatment phase down to decrease the antagonistic effects of action. An estimated 2.1 million women were diagnosed anew in 2019, with breast cancer, which impart roughly one new case analysed every 18 seconds; in addition, 626,679 women with breast cancer had been deceased[1].The global frequency of breast cancer has been growing with annual increases of 3.1%, beginning with 641,000 cases in 1980 and increasing to >1.6 million in 2010[2]. On a

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global scale, the incidence is found higher in high-income regions (92 per 100,000 in North America) than in lower income regions (27 per 100,000 in middle Africa as well as eastern Asia)[3]. Several studies have also shown that breast cancer presents earlier in Asian women (typically 40–50 years of age) than in their western counterparts (typically 60–70 years of age)[4].The aetiology of the breast cancer also fluctuates by ethnicity, which has consequences for the mortality variance[5]. For example, African and African-American women had the highest rates of TNBC compared with any other ethnic group. Nearly 10 percent of breast cancers are inherited and associated with a family history[6], even though this differs by the ethnicity *in milieu* of early-onset, consensual and/or TNBC. It has been estimated that about twenty percent of breast cancers around the globe can be attributed to modifiable risk factors, including obesity, physical inactivity and alcohol use, offering the potential for reduction in the disease burden by promoting a healthy lifestyle[7].

Herbs and various plant products are considered asone of the important source of medicine[8].These herbs arementioned in ayurveda for treatment of various tumorsincluding cancer therapy resulting in complete healingand reducing the side effects associated with cancer.In ayurveda (Rigveda) *Ocimum tenuiflorum* has been welldocumented for its therapeutic potential[9]. *Ocimum tenuiflorum* also known as holy basil or tulsi, belonging to mint family (*Labiateae*) has been one of the most popular herbs used in European and Asian countries for the treatment of various ailments since ancient times[10]. Tulsi is cultivated for religious and traditional medicinal and culinary purposes, and for its essential oil for at least 3000 years. The plant species is native to India, and is reportedly thrives in cultivation in areas at low altitudes up to 900 m with relatively high rainfall and humid temperatures[11]. Tulsi has been used since time immemorial to treat common colds, bronchitis, skin infections, earaches, urinary tract infections, headaches, stomach disorders, inflammation, heart disease, various forms of poisoning like snake bites and scorpion stings in Ayurveda and various folk systems of medicine in Southeast Asia[12]. The *Ocimum tenuiflorum* are richin secondary metabolites (which are potential sourcesof drugs) and essential oils of therapeutic importance.Phytochemicals in tulsi prevent chemical-induced lung, liver, and oral cancers because they increase antioxidant activity, induce cancer cell death, prevent blood vessel growth contributing to cell growth, and stop metastasis, which is the spread of cancer fromone organ to another[13].Hence in the present study, we would like to evaluate the effectiveness of anticancer properties of *O.tenuiflorum* (Tulsi) on commercially available breast cancer cell lines.In this study we used *O. tenuiflorum* leaves extract to observe its

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efficacy as an anti-cancer agent on breast cell lines. To interpret the effectiveness of leaves extracts, we analyzed cell motility and survival condition to measure the phenotypic changes in MCF-7, MDA MB231 and MDA-MB-453 breast cancer cell lines. The rationale behind this study and selection of this cell lines are; easy to access and availability also more importantly, prevalence of this cancer in a major population of the Indian. The present study based on analysis of *O. tenuiflorum* leaves extract therapeutic mechanisms to identify the DNA copy number variation of TNFα ,GST and SOD of treatment as well as control.

MATERIALS AND METHODS:

Cell lines and culture medium:

Human breast cancer cell lineMCF-7, MDA-MB-231, and MDA-MB-453 was obtainedfrom National Center for Cell Sciences (NCCS), Pune andgrown in Eagles minimum essential medium (EMEM)containing 10% fetal bovine serum (FBS) at 37°C, 6.5%humidified CO_2 incubator, 95% air and 100% relativehumidity. The MCF-10A cell line was cultured andsupplemented with 10% FBS. Maintenance of cultures waschanged twice a week.

Extract preparation:

The leaves were separately washed, shade-dried, broken into small pieces, mechanically reduced to moderate coarse powder and sieved. The powder obtained was used for further extraction with solvents. The extractions were performed using Soxhlet apparatus. About 50g of dried leaves, were grinded into coarse powder. The coarsely powdered specimens were taken in round bottom flask and 600 ml of ethanol was added. The extraction was continued for 6–8 h until all the soluble constituents dissolved in the solvent. The soluble extracts were filtered and evaporated in rotary evaporator to yield semi solid masses. Extracts thus obtained, were collected and stored at 4°C until further use. For this study 250 mg of extracts were dissolved in 1.0 ml of ethanol and filtered through a 0.22 μM filter.

Plant extract treatment:

A stock solution of extracts was prepared by dissolving 50 mg of extract in 1ml of ethanol and 4ml of cell culture medium to a final stock concentration of 10 mg/ml and then diluted with complete culture medium to reach the desired concentrations. The test cell lines were grown in 25 cm^2 flat bottles and to a density of approximately 75% confluency. The cells have been trypsinized with Trypsin-EDTA solution and then detached cells were counted with a neobar lam. Then, cells were seeded (400 cells per well) into 96-well microtitre plates, a density that allowed the untreated control to grow exponentially for 72 h. Twenty-four hours after seeding, cells were treated with different concentrations of plants

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extracts including 0, 25, 50, 100, 250 and 500 µg/ml at the specified times of 48 h and 72 h. The medium was changed every 48 h. The control cell lines were incubated with DMSO without plant extract at the same final concentration. For MTT test, control samples were incubated with equivalent amount of DMSO as a solvent of plant extracts. There were three replicates for each concentration of plants extracts.

MTT assay:

The effect of ethanol extract of the plants on theviability of cells was determined by using MTT assay. ForMTT assay, at the end of incubation period (48 and 72 hrs), 20µL of (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazoliumbromide (MTT) solution (Atocel) (5 mg/ml) wasadded into each well. After 3 hrs. of incubation at 37 °C, mediawas removed and 100 µL of DMSO was added to each well.Further, optical densities (ODs) were carried out by anenzyme-linked immunosorbent assay (ELISA) reader (BioTekELx800,USA) at 490 nm. ODs were used to calculate theviability of cells, percentage of viable cells and dividing themean absorbance of treated cells with mean absorbance of itscontrol cells.

Determination of IC50:

Inhibition concentration 50 (IC50)were calculated by using the concentration of compoundrequired to inhibit 50 % cell growth by plotting a graph of Log(concentration of Extract) in compare with % cell inhibition. Aline drawn from the 50 % value on the Y axis meets the curveand incorporate to the X axis. The X axis value gives the Log(concentration of the compound). The antilog of that valuegives the IC50 value. Percentage inhibition of novelcompounds against all cell lines was calculated using the
following formula:

$$GrowthInhibition\ (\%) = \frac{(Controlabsorbance - Testabsorbance)}{(Controlabsorbance)} \times 100$$

Trypan blue exclusion assay

Cell viability was measured using the trypan blue exclusion assay. In this assay, live cells with intact cell membranes are not coloured, so have a clear cytoplasm whereas; trypan blue can be absorbed by dead cells, so they have a blue cytoplasm. In each set of experiments, the cancerous cells were plated at a concentration of 6×10^4 (48h) and 3×10^4 (72h) cells per well in 6-well. After plating for 24 h, fresh medium containing half maximal inhibitory concentration (IC_{50}) of extract for each time 48h, 72h was added. After exposing

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cells to extract for defined time, the medium was transferred to a falcon tube. Attached cells were trypsinized and pooled with cells in tube. This mixture was then centrifuged for a short time and then suspended in 2 ml PBS. A cell suspension was mixed with equal parts trypan blue solution, 0.4% (Merck) and placed in a haematocytometer. Numbers of viable and dead cells were counted separately in two different samples for each plate. Finally, by dividing the number of viable cells by the total number of cells, percentage of viable cells was calculated.

DNA Isolation & Real Time PCR:

DNA isolation kit(ReliaPrep Blood gDNA Miniprep System) was acquired fromPromega (Promega Corporation, USA) and SYBR Green PCRMaster Mix (Bio-Rad USA).To determine the DNA copynumber variation of TNFα for cell signalling, SOD & GSTtreated as oxidative stress marker and GAPDH was used ashousekeeping gene, primer were selected and checked from primer blast software[3]. The PCR assay will be carried out byusing specific forward and reverse primers of gene ofinterest in total volume of 20 µl which contain 50 ng ofDNA, 10 picomole of each primer, sybergreen green mastermix (2.5mM MgCl2 2.5mMdNTand 5 Unit of Taq DNApolymerase).

Results:

The effects of*O. tenuiflorum*on proliferation of Cell Lines: Effect of anticancer property of *O. tenuiflorum*on MCF 7, MDA MB 231 and MDA MB 453 was evaluated through MTT assay. The different concentration of ethanolic extract were used and effective doses were calculated from dose response curve. Results of the cytotoxicity evaluation against MCF 7 cell lines of the *O. tenuiflorum*extract are shown in figure (1 (A & B), MDA MB 231 in figure 2 (A & B) and MDA MB 453 shown in figure 3 (A &A-C). Interestingly, we observed significant decrease in cell survival in these cancer cell lines when treated with the extracts of leaves. A graphical presentation was constructed and summarizing the effect of extracts on growth of these three cancer cell lines mentioned in figures 1-3.

IC50 value determination of plants extract:

The Cytotoxicity of ethanolic extract of *O.tenuiflorum*were determinedusing MTT assay cell lines 0-500 µg/ml of extracts at twoincubation period of 48 and 72 and IC50 values were determined. On the treatment with *O. tenuiflorum*extract, the MCF-7,MDA MB 231 and MDA MB 453 cell lines showed anincreased rate of cell death at a higher concentration of

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theplant extract. Graphs were plotted with concentration of extracts (µg/ml) on the X-axis and cell death (%) observed on the Y-axis. The IC_{50} values (concentration at which 50% cell death was observed) were determined from the graph and expressed as µg/ml. The IC_{50} values (concentration at which 50% cell death was observed) were determined from the graph and expressed as µg/ml. The experiments are done in triplicates and values are expressed as mean ±SEM. The value of IC_{50} for different cell lines are as follows,MCF-7-29.3± 2.1 ,MDA-MB-231-37.2 ± 2.8,MDA-MB-453-33.4 ± 2.2.

Cell Viability using Trypan Blue Exclusion.

The MCF-7, MDA-MB-231,and MDA-MB-453 breast cancer cells were treated with various concentrations of the ethanolic extracts of the selected plant leaves for 2 days. A cell suspension was mixed with trypan blue dye and then visually examined to determine whether cells take up or exclude dye. The number of live cells (excluded dye) was quantified and IC_{50} value for each of the leaf's extracts were determined.The trypan blue exclusion assay provides a rapid and effective means in screening multiple drugs. Assays for each of the extract have been conducted in triplicate and the statistical significances are shown along with the IC_{50} values are MCF-7, MDA-MB-231, and MDA-MB-453 which have been found to be 1119 ± 102 nM, 1379 ± 167 nM, 1719 ± 120 nM and 3919 ± 602 nM respectively.

Quantification of DNA copy number variation (CNV):

Results showed that cells treated with leaf extracts have been presented significant decreases in TNF-α, GST and SOD levels with respect to housekeeping gene (GAPDH) shown in 4 A-C. The results indicates that *O.tenuiflorum* extract is a good antioxidant scavenger, as it decreases cell viability. However, *O.tenuiflorum* leaf extract alone is cytotoxic to MCF 7, MDA-MB-231 and MDA-MB-453 cancerous cell lines and reduces the CNVs, suggesting the very significant correlation with cell viability. In addition, the appearance of TNF-α, GST and SOD gene were significantly reduces the efficiency of cell lines. Whereas statistical analysis was calculated using comparative Ct value analyses considered as statistically significant shown in Table1. Interestingly, we observed significant decrease in cell survival in these cancer cell lines when treated with the extracts of leaves. A graphical presentation was constructed summarizing the effect of *O.tenuiflorum* extracts on growth of these three cancer cell lines mentioned in figures 1-3.

Discussion:

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The pharmacological evaluation of various plants used in traditional system of medicine may be helpful in identifying the principle responsible for their action[15]. In the traditional system, a large number of plants are also reported to possess anticancer properties which are still extensively used by the tribal people worldwide[16]. *O. tenuiflorum*has been one of the most valued and holistic herbs used over years in traditional medicine in India and almost every part of the plant has been found to possess therapeutic properties[17]. Traditional uses of Tulsi aqueous extracts include the treatment of different types of poisoning, stomach-ache, common colds, headaches, malaria, inflammation, and heart disease[18].All these results are in resonance with earlier documented results reported that aqueous extracts of leaves of *Ocimum sanctum* herb inhibited chemotaxis of breast cancer cell lines MDA-MB-435, MDA-MB-231 and endothelial cell line HUVEC towards specific chemo attractants[19]. When *Ocimum sanctum* aqueous extracts of leaves were fed to nude mice injected with MDA-MB-435 cells, there was a significant reduction in the rate of tumour growth in the treated mice.On the similar note[20], earlier showed that crude extract of *Ocimum gratissimum* and its hydrophobic and hydrophilic fractions differentially inhibit breast cancer cell chemotaxis and chemo-invasion *in vitro* and retard tumour growth and temporal progression of MCF10ADCIS.com xenografts, a model of human breast comedo-ductal carcinoma in situ (comedo-DCIS). Similar results have also been demonstrated the antiproliferative effect in a dose dependent cytotoxic activity of aqueous extract of *O. sanctum* leaves on KB cell line (Mouth Epidermal Carcinoma Cells)[21,22]. The aqueous extract of *O. sanctum* leaves exhibited significant cytotoxic effect against oral cancer cell line as an antiproliferative agent which caused apoptosis in oral cancer cell line.

Conclusion:

We have endeavoured to education novel anticancer agents with a potential for more selective and safer treatment. This study focuses on screening the leaves of indigenous plants for cytotoxic potential and isolating an anticancer principle from sources with selective toxicity against cancer cells and not against normal cells.The results suggest, leaves extract exhibited anti cancerous and cytotoxic activity against breast cancer cells and lower toxicity.The cytotoxic effect of this fraction inhibited cell growth and appears to have induced apoptosis in cells. Further studies are warranted in progress with bioactivity guided isolation and purification and to delineate the specific molecular mechanism of anticancer properties of the active phytochemicals.

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Conflicts of Interest:

The authors declare no conflicts of interest.

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Figures:

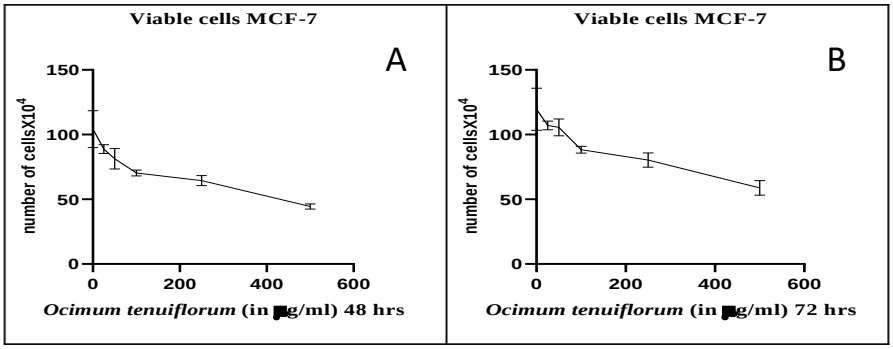

Fig.1A&B: Effect of ethanolic leaf extracts of *Ocimum tenuiflorum* (OT) on MCF-7 breast cancer cell lines. Concentration of dry extract tested at 00 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml at the specified times of 48 h and 72 h.

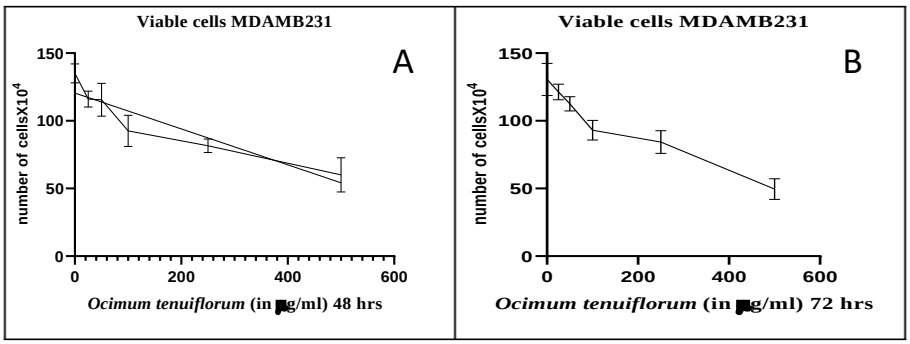

Fig.2A&B: Effect of ethanolic leaf extracts of *Ocimum tenuiflorum* (OT) on MDA-MB-231 breast cancer cell lines. Concentration of dry extract tested at 00 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml at the specified times of 48 h and 72 h.

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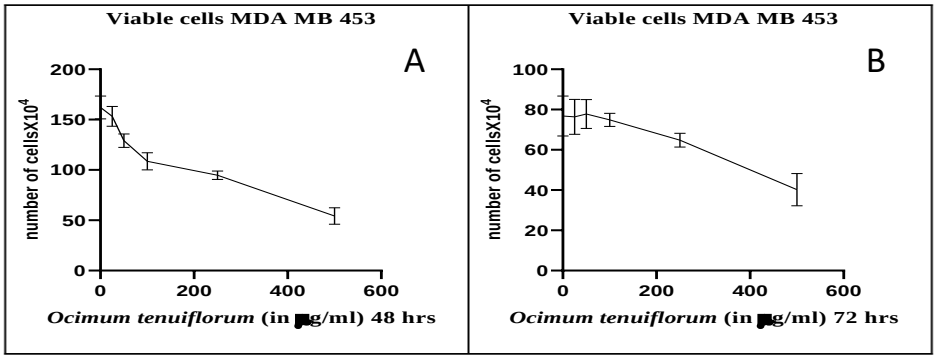

Fig.3A&B: Effect of ethanolic leaf extracts of *Ocimum tenuiflorum* (OT) on MDA-MB-453 breast cancer cell lines. Concentration of dry extract tested at 00 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml at the specified times of 48 h and 72 h.

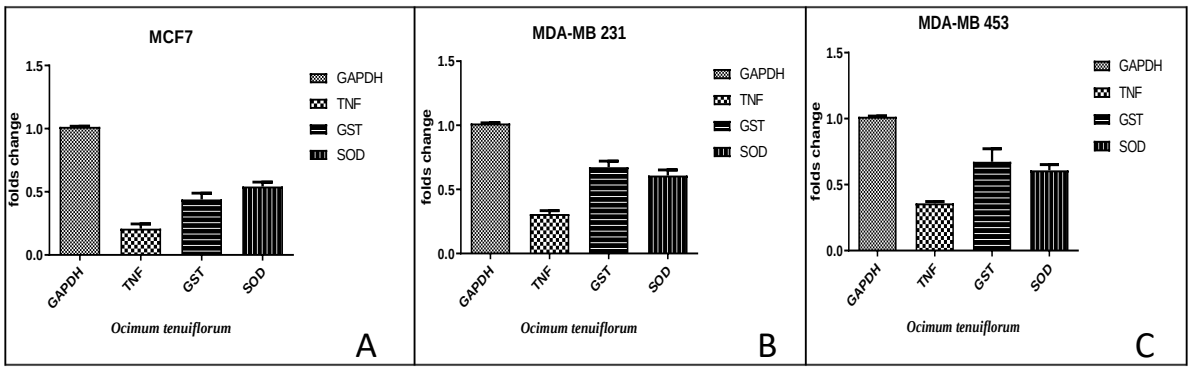

Fig.4: Real time PCR analysis of expression of receptors in human breast carcinoma cells when treated with OT extract. Relative DNA copy number levels of various receptors were calculated as a value of the cycle threshold (Ct), which was normalized to GAPDH DNA copy number (Ct values) by calculating the 2[Ct(GAPDH) − Ct(ER)] value, simply termed 2−ΔCt.

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