INTRODUCTION

Cancer, the second major cause of human death, spares neither men nor women. The American Cancer Society statistics estimated that about 1,665,540 new cases of cancer were expected to be diagnosed in 2014. Though, different groups of drugs work in different ways to fight cancer cells and shrink tumors, nowadays, herbs are used for cancer therapy [1, 2]. Chemotherapy may be used alone for some types of cancer cells and shrink tumors, nowadays, herbs are used for cancer therapy [3]. Recent researches revolve around the urgency to evolve suitable chemotherapy consistent with new discoveries in cell biology and mitochondrial membrane potential.

MATERIALS AND METHODS

Plant material
Fresh and healthy roots of *Ocimum sanctum* were collected from Chidambaram. The plant was identified (No-754) by Dr. N. Karmegam, Associate Professor, Department of Botany, Government Arts and Science College, Salem-636007, Tamil Nadu, India.

Preparation of root extract
The fresh roots of *Ocimum sanctum* were finely minced and ground with ethanol 95% v/v with the help of homogenizer. The ground material was filtered using white filter cloth. Then the solvent present in the filtrate was completely dried using boiling water bath at 50 °C under reduced pressure. The required material of *Ocimum sanctum* was dissolved in 0.2% dimethyl sulfoxide (DMSO). The stock solution was diluted with sterile DMEM medium to arrive at 5, 10, 25, 50, 75, 100, 125 and 150 µg/ml of *Ocimum sanctum* and was used for further studies.

Preparation of drug
The anticancer activity of *Ocimum sanctum* extract has been proved against various cancer cells (both *in vitro* and *in vivo*) like human fibrosarcoma cells culture, Swiss albino mice bearing Ehrlich ascites carcinoma (EAC), hamster buccal pouch carcinogenesis, papillomas, etc. In the present study, we investigated the cytotoxic effect of *Ocimum sanctum* root extract in non-small cell lung carcinoma cell line.

Conclusion: The present study suggests that *Ocimum sanctum* extract act by increasing oxidative damage in NCI-H460 cells.

Keywords: *Ocimum sanctum*, NCI-H460 lung carcinoma cells, MTT assay, Apoptosis, Oxidative damage
Treatment procedure
The NCI-H460 cells were treated with *O. sanctum* extract in different concentration and incubated at 37 °C in 5% CO₂ incubator. After 24 h incubation, the cells were harvested by trypsinization for further experiments.

Cell line
The present work was carried out in human non-small cell lung cancer cell line (table 1). This cell line was obtained from National Centre for Cell Science (NCCS), Pune, India.

**Table 1: Characteristics of cell line used in this study**

<table>
<thead>
<tr>
<th>Designation</th>
<th>NCI-H460</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Human non-small cell lung cancer</td>
</tr>
<tr>
<td>Gene transfers vehicle</td>
<td>T25-Flask</td>
</tr>
<tr>
<td>Cell doubling time</td>
<td>12-hours</td>
</tr>
<tr>
<td>Recommended media</td>
<td>RPM with 10%FBS</td>
</tr>
<tr>
<td>Nature</td>
<td>Radiosensitive</td>
</tr>
<tr>
<td>Other Recommended</td>
<td>Cells are ready to expand next day</td>
</tr>
</tbody>
</table>

Culturing cells
The NCI-H460 cells were grown as a monolayer in RPMI medium supplemented with 10% FBS, 1% glutamine, and 100 U/ml penicillin-streptomycin in at 37 °C in 5% CO₂ atmosphere. Stocks were maintained in 25 cm² tissue culture flasks. After cell numbers are counted, cells were seeded at 5 x 10⁴/cells per well in 24-well plates. Cells were harvested by trypsinization.

Study groups
Cells were divided into five groups (table 2).

**Table 2: Different groups of cells used in the study**

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (Untreated cancer cells)</td>
</tr>
<tr>
<td>II</td>
<td><em>O. sanctum</em> with (25 μg/ml)</td>
</tr>
<tr>
<td>III</td>
<td><em>O. sanctum</em> (50 μg/ml)</td>
</tr>
<tr>
<td>IV</td>
<td><em>O. sanctum</em> (75 μg/ml)</td>
</tr>
<tr>
<td>V</td>
<td><em>O. sanctum</em> (100 μg/ml)</td>
</tr>
</tbody>
</table>

Measurement of cell proliferation (MTT assay)
The proliferation activity of cell populations-untreated and treated with *O. sanctum* extract in a different concentration such as 10, 25, 50, 75, 100 μg/ml was determined by the MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells [16].

Measurement of intracellular ROS in cells by spectrofluorimetric method
ROS was measured by using a non-fluorescent probe, 2, 7-diacetyl dichlorofluorescein diacetate (DCFH-DA) that can penetrate into the intracellular matrix of cells where it is oxidized by ROS to 2, 7-dichlorofluorescein (DCF) that can penetrate into the cell. ROS was measured by using a non-fluorescent probe, 2, 7,-diacetyl dichlorofluorescein (DCFH-DA) that can penetrate into the cell.

Apoptotic morphological changes by acridine orange/ethidium bromide dual staining method
Apoptotic nuclei exhibiting typical changes such as nuclear condensation and segmentation were stained by AO/EBBr [18]. *O. sanctum* extract treated and untreated cells (2 x 10⁴/well) were seeded into the 6-well plate and incubated in CO₂ incubator for 24 h and then the apoptotic morphological changes were observed using a fluorescence microscope with the blue filter.

Changes in mitochondrial transmembrane potential
Alteration in mitochondrial membrane potential (Depolarization) is an indication of early stages of apoptosis. Rhodamine 123 (Rh 123) is a lipophilic cationic dye, highly specific for mitochondria. Polarized mitochondria are marked by orange-red fluorescence and depolarized mitochondria are marked by green fluorescence.

Statistical analysis
All quantitative measurements were expressed as means ±SD for untreated and *O. sanctum* extract-treated cells. The data were analyzed using one-way analysis of variance (ANOVA) on SPSS/PC (statistical package for social sciences, a personal computer) and the results were considered statistically significant if the *P* value is less than 0.05.

**RESULTS**

Effect of *O. sanctum* extract on cell proliferation in NCI-H460 cells (MTT Assay)
The growth inhibitory effect of *O. sanctum* extract in NCI-H460 cells was measured by MTT assay. MT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay, in which the yellow tetrazolium salt is metabolized by NAD-dependent dehydrogenase (inactive mitochondria) to form a dark blue formazan product and the absorbance is directly proportional to the number of viable cells. Effect of *O. sanctum* extract on cell proliferation was determined by MTT assay. NCI-H460 cells proliferation was significantly inhibited by *O. sanctum* extract. The inhibitory effect was observed after 24 h incubation. 25, 50, 75 and 100 μg/ml of *O. sanctum* extract treatment showed only 15% cell viability. Hence, for further experiment we have chosen 25, 50, 75 and 100 μg/ml of *O. sanctum* extract.

**Effect of *O. sanctum* extracts in generating ROS level in NCI-H460 cells**
Cytotoxic drugs are known to induce oxidative stress through the generation of reactive oxygen species (ROS) resulting in the imbalance of prooxidants and antioxidants in the cells. Intracellular generation and accumulation of ROS such as superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl radical and peroxyl radical in the stressed cells overcome natural antioxidant defense causing damage to biological macromolecules including nucleic acids, proteins, and lipids.

The levels of ROS were measured by using a non-fluorescent probe, 2, 7-diacetyl dichlorofluorescein (DCFH-DA) that can penetrate into the cell.
the intracellular matrix of cells where it is hydrolyzed by cellular esterases to form dichlorofluorescein (DCFH). The non-fluorescent DCFH is oxidized by intracellular ROS and forms the highly fluorescent DCF which is measured spectrofluorimetrically at emission filters set at 485±10 nm and 530±12.5 nm respectively.

Levels of ROS in control and *O. sanctum* extract treated cells were depicted in fig. 2 and 3. *O. sanctum* extract treatment significantly increased ROS level in NCI-H460 cells. Among all the doses tested 100 μg/ml of *O. sanctum* extract showed the maximum generation of ROS in NCI-H460 cells.

Effect of *O. sanctum* on apoptotic morphological changes in NCI-H460 cells

To confirm whether the cytotoxic effect induced by *O. sanctum* extract involves apoptosis, we observed morphological changes in the *O. sanctum* extract treated and untreated cells. The initial structural changes of apoptosis are condensation of the cytoplasm and nucleus, loss of microvilli and disruption of intracellular junctions. *O. sanctum* extract treated cancer cells were stained with acridine orange-ethidium bromide and incubated in CO₂ incubator for 24 h at room temperature.

Untreated control cells appeared green in color (acridine orange stained) whereas the *O. sanctum* treated cells appeared orange in color (ethidium bromide stained). Acridine orange is a cationic dye that enters only live cells and stain DNA and hence the live cells observed as green under blue emission. On the contrary, ethidium bromide stains DNA in the cells undergoing apoptosis and hence apoptotic cells appeared orange in color.

Fig. 4 & 5 shows the effect of *O. sanctum* extract on apoptotic morphological changes. We observed 92% apoptotic cells in 100 μg/ml of *O. sanctum* extract treated cells; 85% apoptotic cells in 75 μg/ml of *O. sanctum* extract treated cells; 50 μg/ml of *O. sanctum* extract treatment showed 65% apoptotic cells and 25 μg/ml of *O. sanctum* extract treatment showed 45% apoptotic cells.

**O. sanctum** extract modulates mitochondrial membrane potential in NCI-H460 cells

The uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. Many of these probes can be classified as lipophilic cations or "redistribution dyes." These compounds accumulate in the mitochondrial matrix because of their charge and solubility in both the inner mitochondrial membrane and matrix space. Alteration in mitochondrial membrane potential (ψm) is an indication of early stages of apoptosis. Rhodamine 123 (Rh 123) is a lipophilic cationic dye, highly specific for mitochondria. Polarized mitochondria are marked by orange-red fluorescence and depolarized mitochondria are marked by green fluorescence. Control cells appeared orange-red in color whereas the *O. sanctum* extract treated cells appeared green in color. *O. sanctum* extract treated cells appeared green in color. Rhodamine 123 is a lipophilic cationic dye that enters only live cells and stain mitochondrial DNA and hence the live cell mitochondria appeared orange-red in color under blue emission.

Changes in mitochondrial membrane potential in control and *O. sanctum* extract treated cells were depicted in fig. 6 & 7. *O. sanctum* extract treatment significantly increased mitochondrial depolarization in NCI-H460 cells. Among all the doses tested 100 μg/ml of *O. sanctum* extract showed high level of mitochondrial depolarization in NCI-H460 cells.
Increased ROS levels are thought to constitute an essential step in cell death induction by many different cytotoxic drugs. ROS levels were assessed after 24 h of incubation with *O. sanctum* extract. DCF fluorescence was measured by spectrophuorometer/fluorescence microscope. Changes in the mean fluorescence intensity (MFI) relative to untreated control cultures were interpreted as increase or decrease of the amount of internal ROS.

*O. sanctum* extract treatment caused a rapid increase of intracellular ROS in NCI-H460 cells (fig. 4). We further noticed that a significant increase in the ROS levels in 100 µg/ml *O. sanctum* treated cells. The increased ROS levels during *O. sanctum* extract treatment might be due to its prooxidant property.

Previous studies suggest the protective effect of basil against oxidative DNA damage and mutagenesis [26]. In addition to scavenging of reactive oxygen species (ROS), chelation of metal ions (such as iron and copper) which initiate radical reactions and inhibition of enzymes responsible for free radical generation [27], antioxidants can interfere with xenobiotic metabolizing enzymes, block activated mutagens/carcinogens, modulate DNA repair and even regulate gene expression [28, 29]. Based on these mechanisms they may be important for their antimutagenic and anticarcinogenic properties [30].

The mitochondrion is one of the most important organelles in regulating cell death as well as a marker in apoptosis. Increased ROS formation followed by mitochondrial membrane depolarization in cell lines has been reported [31]. Mitochondrial dysfunction is also an early indicator of apoptosis in cell lines [32]. Rh was served to determine the alteration of mitochondrial membrane potential. We observed accumulation of Rh in the mitochondria of control cells (fig. 6) and the *O. sanctum* extract treated cells showed no uptake of Rh showing membrane potential change. Similar changes have been observed in adriamycin treated human non-small lung cancer cell lines [33]. Also, cytotoxic chemotherapeutic drugs are targeted to produce ROS in cells or tissues [34].

We have observed *O. sanctum* extract pretreatment significantly increased apoptotic morphological changes in NCI-H460 cells. The microscopical observation showed a typical morphology of apoptosis, i.e., cell pyknosis, chromosomal condensation and nuclear fragmentation in *O. sanctum* extract-treated cells. Apoptosis has been shown to play an important role in determining cellular cytotoxicity [35]. Apoptosis has been shown to be a significant mode of cell death after cytotoxic drug treatment [36]. The increased ROS levels and subsequent oxidative DNA damage might be the reason for increased apoptotic morphological changes in the *O. sanctum* extract-treated cells. Sun-Chae Kim et al., 2010, has also reported the inhibitory effect of ethanol leaf extract of *O. sanctum* extract on lung metastasis using the mouse Lewis lung carcinoma (LLC) cells. It prevented cell adhesion and invasion of LLC cells to extracellular matrix (ECM) [10].

Furthermore, there is evidence that *O. sanctum* extract has proven to be useful and effective in sensitizing conventional agents, prolonging survival time and preventing side effects of chemotherapy. It has also been used in polyherbal therapy with various important Ayurvedic herbs viz. *Azadirachta indica*, *Curcuma longa*, *Emblica officinalis*, *Ocimum sanctum*, *Semecarpus anacardium*, *Tinospora cordifolia* etc. Over the years, the herbal usage has been increased many folds and the conventional medical usage also shows the positive results.

**CONCLUSION**

Our results summarize that *O. sanctum* extract shows anticancer activity by decreasing cell proliferation, increasing intracellular ROS, alteration in mitochondrial membrane potential and apoptosis in NCI-H460 cell line. The results obtained from our investigation confirmed the therapeutic potency of the plants. Further research is warranted to isolate the phytochemicals (s) that is responsible for cancer cell apoptosis.

**CONFLICT OF INTERESTS**

Declare none
REFERENCES


Sridevi et al.