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Original Article

ANTI-CANCER EFFECT OF *OCIMUM-SANCTUM* ETHANOLIC EXTRACT IN NON-SMALL CELL LUNG CARCINOMA CELL LINE

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ABSTRACT

Objective: The present study was aimed to investigate the effects of alcoholic root extract of *Ocimum sanctum*, in human non-small cell lung carcinoma cell (NCI-H460).

Methods: The effect of ethanolic extract of *O. sanctum* in NCI-H460 cell was investigated by the cell viability assay, generation of ROS in a cancer cell, apoptotic morphological changes and by mitochondrial membrane potential.

Results: The cytotoxicity was observed by MMT assay. NCI-H460 cell was treated with various concentrations (10-150 µg/ml) of extract for 24 hr and 150 µg/ml showed a maximum decrease in cell viability. The extract (25-100µg/ml) showed significant increase ROS production in NCI-H460 cell. It greatly inhibits cell viability and colony forming capacity of NCI-H460 cell, possibly because of increased oxidative stress. An increased apoptotic cell in *Ocimum sanctum* further shows its anticancer nature. Loss of mitochondrial membrane potential is an early stage of apoptosis. Our results showed that extract treatment caused serve loss of in NCI-H460 cell.

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

Keywords: *Ocimum sanctum,* NCI-H460 lung carcinoma cells, MTT assay, Apoptosis, Oxidative damage

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 remedy [1, 2]. Chemotherapy may be used alone for some types of cancer or in conjunction with other therapy such as radiation or surgery [3]. Recent researches revolve round the urgency to evolve suitable chemotherapy consistent with new discoveries in cell biology for the treatment of cancer with less or no toxic effect [4, 5].

Ocimum sanctum (*O. sanctum*) is a tropical annual herb and is a member of the family Lamiaceae (Labiatae). It shows various biological activities such as immunomodulation, anti-ulcer, anti-inflammation, antimicrobial, antihypertensive, cardioprotective, hepatoprotective, antidiabetic, antifertility, radio protective and anti-carcinogenesis, etc [6-9]. The findings of Kim *et al.*, (2010) supports that ethanolic extract of *O. sanctum* (EEOS)can be a potent anti-metastatic candidate which inactivate matrix metallo-proteinase-9 (MMP-9) and enhance antioxidant enzymes [10]. Magesh *et al.*, (2009) demonstrated that EEOS induces apoptosis in A549 cells via a mitochondrial caspase-dependent pathway and inhibits the *in vivo* growth of Lewis lung carcinoma animal model, suggesting that EEOS can be applied to lung carcinoma as a chemo preventive candidate [8]. Tae-kyung Kwak *et al.*, 2014 suggested that anti-metastatic mechanism of EEOS is mediated by inhibition of PI3K/Akt in Osteopontin (OPN) treated NCI-H460 non-small cell lung cancer cells [11]. Extracts from Ocimum Sp. and Phytochemicals from *O. sanctum* like eugenol, rosmarinic acid, apigenin, my retinal, luteolin, β–sitosterol, and carnosic acid are reported to prevent chemical-induced skin, liver, oral, and lung cancers and to mediate these effects by increasing the antioxidant activity, altering the gene expressions, inducing apoptosis, and inhibiting angiogenesis and metastasis [12-15].

The anticancer activity of *O. 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.

MATERIALS AND METHODS

Plant material

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

Preparation of root extract

The fresh roots of *O. sanctum* were finely minced and ground with ethanol 95% v/v with the help of homogenizer. The grounded 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 *O. sanctum* was separated, and that crude ethanoic extract was investigated for anticancer studies.

Drugs and chemicals

3-(4, 5-dimethyl-2-thiaozolyl)-2, 5-diphenyl-tetrazolium bromide (MTT), 2-7-diacetyl dichloro fluorescein (DCFH-DH), Rhodamine 123 (Rh 123), ethidium bromide, acridine orange, cell culture chemicals such as heat-inactivated fetal calf serum (FBS), RPMI-1640, glutamine, penicillin-streptomycin, EDTA, trypsin, Ethyl alcohol and phosphate buffered saline (PBS) were purchased from Sigma chemical Co., St. Louis, USA.

Preparation of drug

O. sanctum extract 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 *O. sanctum* and was used for further studies.

Treatment procedure

The NCI-H460 cells were treated with *O. sanctum* extract in different concentration and incubated at 37 °C in 5% CO_2 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

Designation	NCI-H460
Tissue	Human nonsmall cell lung cancer
Gene transfers vehicle	T25-Flask
Recommended media	RPMI with 10%FBS
Cell doubling time	12-hours
Nature	Radioresistant
Other Recommended	Cells are ready to expand next day

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 at 37 °C in 5% CO_2 atmosphere. Stocks were maintained in 25 cm^2 tissue culture flasks. After cell numbers are counted, cells were seeded at 5 x 10^4cells 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

Group I	Control (Untreated cancer cells)
Group II	*O. sanctum* with (25 µg/ml)
Group III	*O. sanctum* (50 µg/ml)
Group IV	*O. sanctum* (75 µg/ml)
Group IV	*O. sanctum* (100 µg/ml)

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, 125 and 150 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 fluorescent dichlorofluorescein (DCF). The non-fluorescent DCFH-DA is oxidized by intracellular ROS and forms the highly fluorescent DCF [17] which is measured spectrofluorimetrically at emission filters set at 485±10 nm and 530±12.5 nm respectively.

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/EtBr [18]. *O. sanctum* extract treated and untreated cells (2 x 10^4/well) were seeded into the 6-well plate and incubated in CO_2 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 group means were compared by Duncan's Multiple Range Test (DMRT). 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. MTT (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.

Fig. 1 shows the changes in the levels % of cell viability in the untreated and *O. sanctum* extract-treated cells. 10 µg/ml of *O. sanctum* extract treatment has not showed significant (P<0.05) proliferation inhibition. 25, 50, 75 and 100 µg/ml of *O. sanctum* extract treatment significantly inhibits NCI-H460 cells. 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.

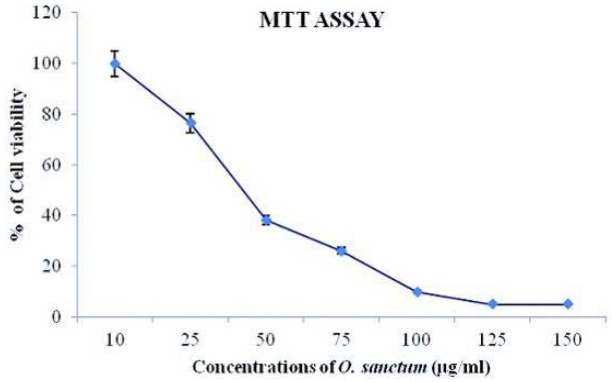

Fig. 1: Effect of *O. sanctum* extract on cell proliferation in NCI-H460 cells

Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P<0.05 (DMRT)

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 was measured by using a non-fluorescent probe, 2, 7,-diacetyl dichloro fluorescein (DCFH-DA) that can penetrate into

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.

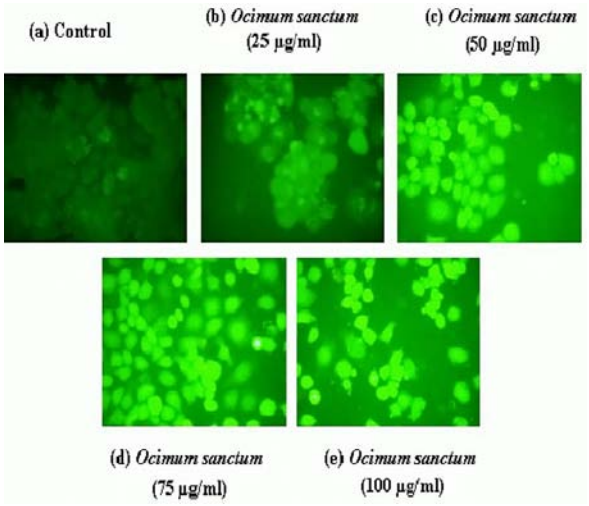

Fig. 2: *O. sanctum* generates ROS level in NCI-H460 cells

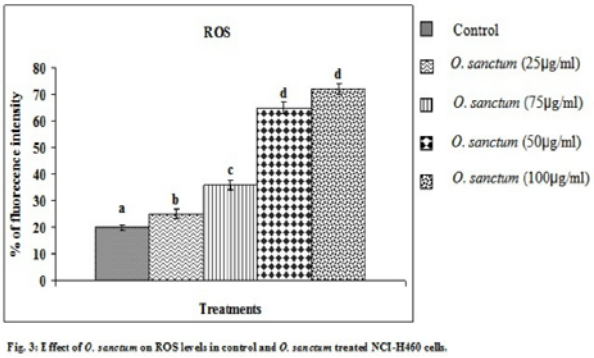

Fig. 3: Effect of *O. sanctum* on ROS levels in control and *O. sanctum* treated NCI-H460 cells.

Fig. 3: Effect of *O. sanctum* on ROS levels in control and *O. sanctum* treated NCI-H460 cells

Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P<0.05 (DMRT)

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 CO_2 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.

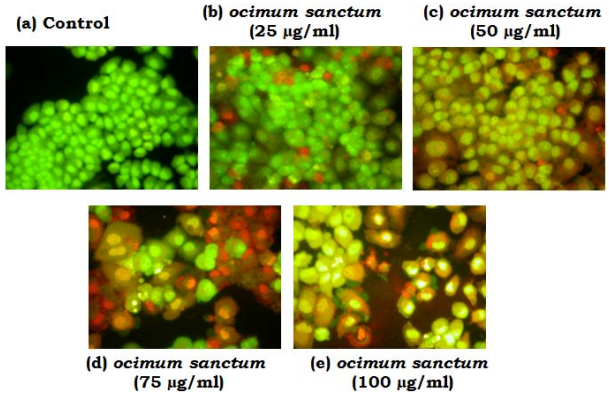

Fig. 4: Effect of *O. sanctum* on apoptotic morphological changes in NCI-H460 cells

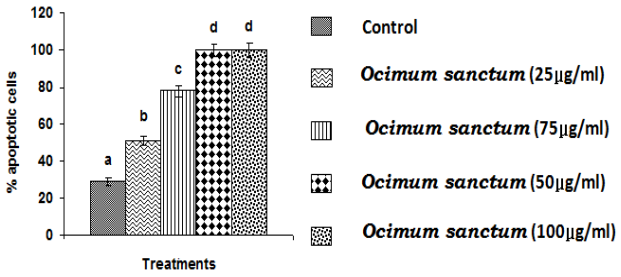

Fig. 5: Effect of *O. sanctum* (24 h) on apoptotic morphological changes in control and *O. sanctum* extract treated NCI-H460 cells

Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P<0.05 (DMRT)

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. 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.

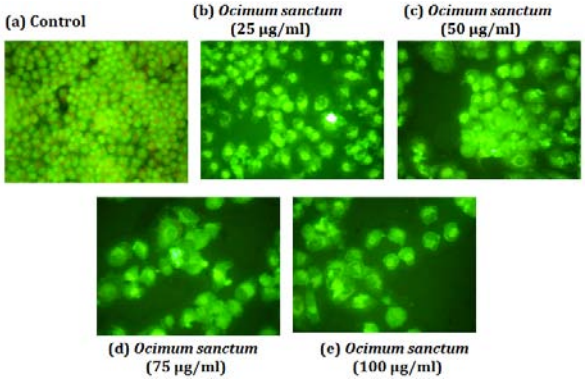

Fig. 6: *O. sanctum* modulates mitochondrial membrane potential in NCI-H460 cells

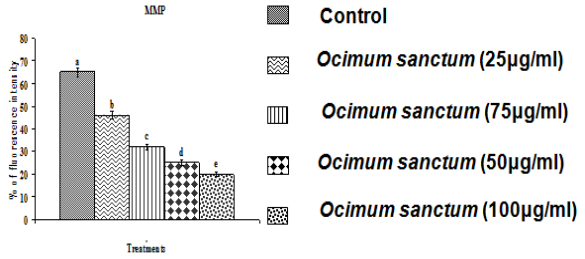

Fig. 7: Effect of *O. sanctum* (24 h) on MMP alterations in control and *O. sanctum* treated NCI-H460 cells

Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P<0.05 (DMRT)

DISCUSSION

Plant-derived polyphenolic compounds include flavonoids, tannins, curcuminoids, gallocatechin, stilbenes such as resveratrol; anthocyanidins such as delphinidin possess a wide range of pharmacological properties and are considered to possess chemopreventive and therapeutic properties against cancer. Moreover, phenolic phytochemicals manage oxidation stress-related diseases due to its direct involvement in quenching the free radicals [19]. Anticancer mechanism of plant polyphenols involves mobilization of endogenous copper possibly chromatin-bound copper and the consequent prooxidant action [20]. It has also been suggested that the cell killing activity of plant phenolics exhibit prooxidant activity [21] and cytotoxic properties [22-24].

In the present study, we evaluated the anticancer effect of *O. sanctum* extract in non-small cell lung cancer cell line (NCI-H460) *in vitro.* We have observed cytotoxicity of *O. sanctum* extract in NCI-H460 cells. Hence, our result indicates that concentration of phytochemicals plays a role for cytotoxicity. Probably, at higher concentration *O. sanctum* extract exhibits prooxidant property. This prooxidant property might disrupt mitochondrial dehydrogenase activity. This might be the reason for increased cytotoxicity at higher doses. The previous study shows that the *O. sanctum* extract shows that more than70% of growth inhibition activity against colon (HT-15 and HT-29), neuroblastoma (IMR-32) and lung cancer (A-159) cell lines and hence it has anticancer activity [25].

Alcoholic root extract showed more degree of inhibition against the cell lines. Our results along with previous reports suggest that mitochondrial activity of cancer cells may be influenced by *O. sanctum,* and that might be the reason for increased cytotoxicity observed in *O. sanctum* extract-treated cells.

Reactive oxygen species (ROS) are known to cause oxidative modification of DNA, proteins, lipids and cellular small molecules. 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 spectrofluorometer/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 cooper) 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 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 mitochondrion 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 aclarubicin 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, Embelica 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

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