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INHIBITORY EFFECT OF ETHANOL EXTRACT OF *OCIMUM SANCTUM* ON LUNG CANCER CELLS: AKT ACTIVITY, CELL PROLIFERATION, AND VIABILITY STUDIES

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Abstract

Chemopreventive activity of ethanolic extracts for *Ocimum sanctum* (Tulsi) (EEOS) over Non Small Cells Lung Cancer (NSCLC) A549 was evaluated with special consideration to its effect on Akt phosphorylation and Akt expression. EEOS was prepared using soxhlet apparatus and dried to powder which was further diluted to five different concentrations with DMSO. NSCLC A549 cells were treated with the prepared EEOS and total Akt mRNA transcription, Akt phosphorylation was quantified using RT-PCR and western blotting respectively. Cell proliferation, cell viability, cytotoxicity and alteration in mitochondrial membrane potential were also measured under DMSO (negative control), paclitaxel (positive control) and EEOS treatment. Akt phosphorylation was markedly affected by EEOS treatment as compared to DMSO treatment. No alterations were observed in amount to total Akt mRNA (Akt1, Akt2 and Akt3) transcribed under control and treated cells. Cell proliferation, cell viability was severely affected under EEOS treatment as compared to either control as suggested by a very *p* value after ANOVA. NSCLC cells experienced high toxicity by EEOS as compared to control. Mitochondrial membrane potential was highly reduced upon EEOS treatment. Results revealed high potential of EEOS for prospecting development of chemopreventive medicine and Akt phosphorylation was found to be altered suggesting a possible step in understanding the mechanism of its action.

Keywords: Ethanolic extract, *Ocimum sanctum*, Akt, A549.

Introduction

Plant extracts and other products have been since long in our society for treatment of several ailments. Many beneficial drugs have been isolated and produced from natural products (Bhat *et al*., 2017; Chugh *et al*., 2012; Gupta *et al*., 2019; Gupta *et al.*, 2013; Harsha & Aarti, 2015; Kelly *et al*., 2005; Koul *et al*., 2019). These products even in crude form are able to exhibit its effect owing to presence of one or more active ingredients. Several natural products and its derivatives have been shown to exert inhibitory effects on cancerous cells *invivo* and *in vitro* (Manjeshwar Shrinath Baliga *et al*., 2013; Baliga *et al*., 2016; Kausar *et al*., 2012; Patel & Goyal, 2012; Yesil-Celiktas *et al*., 2010; Zhang *et al*., 2017). Cancer is a general term for a state where cells loses its regulation and grow in undefined manner. Cancer is a leading cause of death all over the world and cancer of lung is reportedly the most common cause of cancer deaths as per World Cancer Report 2014 (Stewart & Wild, 2014). *Ocimum sanctum*, a traditional Indian herb is well prospected as a medicine for cure and prevention of several ailments (Choudhury *et al*., 2104; Kumar *et al*., 2018; Sethi *et al*., 2004; Sharma *et al*., 2017; Singh *et al*., 2013; Suanarunsawat *et al*., 2014) including cancers (Manjeshwar *et al*., 2013; Bhattacharyya & Bishayee, 2013; Singh *et al*., 2012). Therapeutic uses of *Ocimum sanctum* has been reviewed by Prakash (Prakash & Gupta, 2005) which suggested that the active constituent, eugenol (l-hydroxy-2-methoxy-4-allylbenzene), is responsible for therapeutic potentials of the herb. Although its widespread use in Indian traditional medicine system exhibits its importance, rational approaches of its mechanism are still under dark.

Kim *et al*. (Kim *et al*., 2010) have suggested ethanolic extract of *Ocimum sanctum* is potent anti-metastatic agent and exhibits its activity by inactivation of matrix MMP9 (metalloproteinase-9) and enhancement of anti-oxidantenzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). Another study highlighted that anti-metastatic mechanism of EEOS is mediated by inhibition of PI3K/Akt (Kwak *et al*., 2014). Gupta *et al*. evaluated chemopreventive activity of seed oil of *Ocimum sanctum* and suggested that this activity is partially attributed to its antioxidant properties (Gupta *et al*., 2002). *Ocimum sanctum* essential oil (OSEO) have been found to have both antimetastatic and anti-inflammatory potentials (Manaharan *et al*., 2014). OSEO are also suggested to exert its antifungal activity by inhibiting ergosterol biosynthesis and membrane integrity in standard laboratory isolates of *Candida sp* (Khan *et al*., 2010).

Extracts of *Ocimum sanctum* are reported to induce cytotoxicity, reduce cell proliferation and cell viability (Devi, 2001; Karthikeyan *et al*., 1999; Prashar *et al*., 1994). It has shown to induce apoptosis in *in vitro* cell culture and *in vivo* systems (Dhandayuthapani *et al*., 2015; Shivpuje *et al*., 2015; Venkataraman *et al*., 2009).

In the present study, ethanolic extracts of *Ocimum sanctum* (EEOS) is used to evaluate its effect on proliferation, viability, mitochondrial membrane potential of NSCLC A549 cells and serine/threonine protein kinase B (PKB, also known as Akt) expression and activity *in vitro*. Akt constitutes an important branch point in diverse signaling events. Akt is found to play an important role in cell growth, its migration, survival, proliferation, lipid and glucose metabolism, cell cycle progression, muscles contraction, angiogenesis, and self-renewal of stem cells (Liao & Hung, 2010; Mishra *et al*., 2018; Sharma *et al*., 2019). Alteration in Akt activity has been shown to trigger cancer, diabetes mellitus, neurodegenerative diseases, and muscle hypotrophy. Akt activity has been proposed as a crucial signal in development of cancers (Altomare & Testa, 2005; Bellacosa, 2005; Luo *et al*., 2003; Testa & Tsichlis, 2005). Reduction in its activity is postulated to be related to reduction in viability and proliferation of cancerous cells (Kumar *et al*., 2016; Sharma & Karnwal, 2018; Venkataraman *et al*., 2009).

Materials and Methods

1. Chemicals and reagents

Gibco RPMI 1640 medium, Fetal Bovine Serum (FBS), streptomycin and penicillin antibiotics were procured from Gibco (Grand Island, NY). DMSO, ethyl alcohol, trypsin-EDTA solution, Phosphate Buffered Saline (PBS), Bovine Serum Albumin (BSA), Paclitaxel were from Sigma-Aldrich (Sigma-Aldrich Corporation Bangalore, India), RIPA Lysis buffer was from ThermoFisher Scientific, Bangalore, India. Primary antibody against phosphor-Akt, β-actin and secondary antibody conjugated Horseradish Peroxidase (HRP) was purchased from Abcam, USA.

2. Preparation of extract

Plant samples were collected from medicinal plant garden at PAU Ludhiana. For preparation of extracts, 100 g (wet weight) of leaves were collected and extracted in ethanol in a soxhlet apparatus (Sigma-AldrichZ218979) at 60°C for 48 hrs, concentrated using a rotary vacuum evaporator (Buchi India Pvt. Ltd. Santacruz East, Mumbai) and subsequently freeze dried (Cole-Parmer, India) to obtain 17 g of ethanol extract of *Ocimum sanctum* (EEOS). 1g of dried powder was dissolved in 100 mL of DMSO and this stock was diluted to 5 different concentration(25, 50, 100, 150 and 200 µg/mL) for test studies.

3. Cell lines

Human NSCLC A549 cells were used in present study. The cells were procured from ATCC USA and were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, and streptomycin (100 µg mL^{-1})/penicillin(100 units mL^{-1}). The cultures were maintained at 37°C and 5% CO_2/95% air.Cells were routinely grown to logarithmic phase and harvested with trypsin-EDTA treatment and sub cultured for further experiments.

4. In vitro studies

Log phase NSCLC A549 cells were cultured and treated with5 different concentrations of EEOS (200, 150, 100, 50, 25 µg/mL) for 1 hour to evaluate its effect on Akt activity, Akt expression, cell proliferation, cell viability, cytotoxicity and mitochondrial membrane potential changes.

a) Western Blot Analysis: Akt activity

After the treatment of cells with mentioned experimental design, cells were washed with ice cold PBS and lysis buffer (Pierce RIPA Lysis and Extraction Buffer). The protein concentration in the lysate was measured with protein assay kit by Bio-Rad. Aliquot containing 50 µg proteins were separated by SDS-Polyacrylamide gel electrophoresis and transferred to PVDF membrane (Immun-Blot PVDF membrane, Bio-Rad, India). The membrane was further blocked with BSA for 1 hour at room temperature with gentle agitation on a laboratory shaker and incubated with primary antibody overnight at 4°C. Following this, membrane was incubated with secondary antibody conjugated to HRP (horseradish peroxidase) for detection of immunoreactive signals by enhanced chemiluminescence method.

b) RT PCR for Akt expression

RT-PCR: Reverse Transcription of Akt mRNA was performed to quantify expression of Akt under treatment and control. For this NSCLC cells were harvested and total RNA was extracted using PureLink RNA minikit (ThermoFisher Scientific) and quantified spectrophotometrically. PCR (Applied BiosystemsGeneAmp PCR System 9700) was performed with SuperScript III One-Step RT-PCR System (ThermoFisher Scientific) usingset of primers as per Table 2. Reaction was performed by adding Reaction mix (25µl), Template RNA (1µg), 1 µl of each primer (to reach concentration of 10µM), SuperScript III RT/Platinum Taq mix (2 µL). This mixture was made to 50 µl with autoclaved distilled water. cDNA synthesis and pre-denaturation was done at 50°C for 30 min and at 94°C for 2 min respectively. PCR amplification of cDNA was done by performing denaturation at 94°C for 15 s. Further annealing was done at 55°C for 30 s and extension at 68°C for 45 s. 40 cycles of PCR were performed and final extension was done at 68°C for 5 min. Agarose gel electrophoresis of PCR products were done on a 1% agarose gel and bands were visualized with ethidium bromide.

c) BrdUCell Proliferation Assay

In vitro proliferation of NSCLC A549 cells was determined by using a cell proliferation enzyme linked immunosorbentassaykit (SIGMA) which quantifies the cell proliferation based on amount of BrdU incorporated during DNA synthesis in replicating cells. For the assay, log phase A549 cells were taken and 10^4 cells/well were seeded into 96-well plate. BrdU (10µl/well) was added to each well and cells were fixed as per manufacturer's protocol. The labeled cells were incubated with peroxidase conjugated antibodies specific to BrdU. Finally, color was developed using substrate solution provided by manufacturer. The absorbance was measured in ELISA plate (uQuantmicroplate, Bio-Tek Instruments, Inc., Winooski, VT, USA) reader at 450 nm.

d) Cell Viability Assay

Alamar blue (AB) dye reduction assay:It determined proliferation of cells under control and treatments. Paclitaxel (1µM) was used as positive control.NSCLC A549 cells were seeded in triplicates at a density of 10^4 cells/well and incubated for 48 h. Further cells were incubated in dye diluted with DMEM for 4 h. Dye reduction was measured spectrophotometrically at 570 nm with Microplate reader (Molecular Devices Co., USA) and percent AB reduction was calculated as follows:

$$\% \text{ AB reduction} = [(\varepsilon_{ox}\lambda_2)(A\lambda_1) - (\varepsilon_{ox}\lambda_2)(A\lambda_2)]/[(\varepsilon_{red}\lambda_1)(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(A'\lambda_1)]$$

Where, $\varepsilon\lambda_1$ = molar extinction coefficient of alamar blue at 570 nm

$\varepsilon\lambda_2$ = the molar extinction coefficient of oxidized (ε_{ox}) and reduced (ε_{red}) forms of alamar blue at 600 nm. $A\lambda_1$ and $A\lambda_2$ denoted the absorbance of test wells.

$A'\lambda_1$ = absorbance of negative control well at 570 nm

e) Cytotoxicity Assay

Cell viability was assessed by Cell Proliferation Reagent Kit I (MTT; Roche Applied Science). 10^4cells/well were seeded in triplicates in 96 well plates and treated with EEOS, Paclitaxel and DMSO as per the experimental design. Plates were incubated for 48 h. MTT was added to each well and kept for 4 h at 37°C. Microplate reader (Molecular Devices Co., USA) was used to determine the absorbance at 570 nm.

f) Mitochondrial membrane potential assay

From several available parameters, mitochondrial membrane potential ($\Delta\Psi_M$) was used to indicate cell health using a dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazoylcarbocyanine iodide (JC-1) as per the supplier's protocol (JC-1 Mitochondrial Membrane Potential Assay Kit, Cayman Chemicals USA). Healthy cells, with high $\Delta\Psi_M$,exhibits intense red fluorescence when incubated with JC-1. In contrast to cells with low $\Delta\Psi_M$which gives green fluorescence. Higher ratio of red to green fluorescence indicates better growing cells. A549 cells were treated with controls (DMSO and paclitaxel) and indicated concentration of EEOS and at the end of treatment the cells were incubated with JC-1 at 37°C for 15 min in a CO_2 incubator. Further cells were harvested and analyzed with flow-cytometry (Flow Cytometer and Cell Sorter -Dako (now Beckman Coulter) MoFlo™ Cytomation).

4. Statistical analyses

All the data were expressed as mean±SD. The differences in treatments among the several groups under tests were analyzed with ANOVA using *Origin 6.0*. *p* value of 0.05 or less is considered as significant.

Results

1. Akt activity:

Immunostaining of western blotting membranes of electrophoresed cell lysate developed a pattern as shown in Fig. 1. It was observed from the chemiluminescence intensity that concentration of phosphorylated Akt decreases as the EEOS concentration increases. Maximum amount of phosphorylated Akt was determined in DMSO treated cells and it decreased as the concentration of EEOS is increased. β-actin was also probed with antibody to certain equal amount the protein was loaded on the membrane.

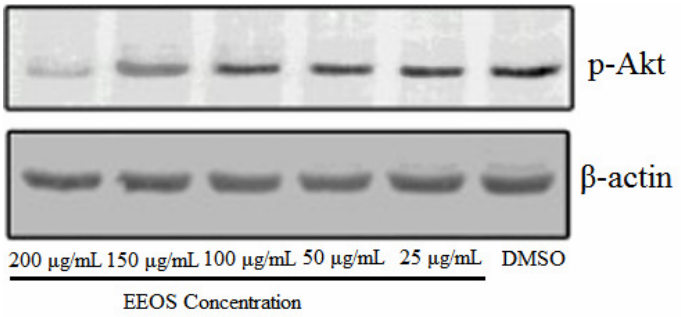

Fig. 1: Chemiluminescence pattern developed on western blot membrane for detection of phosphorylated Akt.Akt phosphorylation was found to reduce with increase in EEOS concentration. β-actin was loaded to ensure equal protein load in each well during SDS PAGE and further transfer to PVDF membrane.

2. Akt expression

Electrophoresis image of RT PCR products of Akt1, Akt2, Akt3 mRNA for quantifying respective mRNA content is shown in Fig. 2. There was no change in the concentration of mRNA of all the three types in the cell under treatment of EEOS as compared with blank (DMSO only). β actin mRNA was quantified along with to certain amount of sample loaded in each well. The expression of mRNA revealed that there was no effect of treatment over the *AKT* gene expression and amount of Akt produced will not be affected by EEOS treatments. It was deduced from above results that the phosphorylation of Akt rather than its expression is reduced upon EEOS treatment which may lead to loss in cell proliferation and viability.

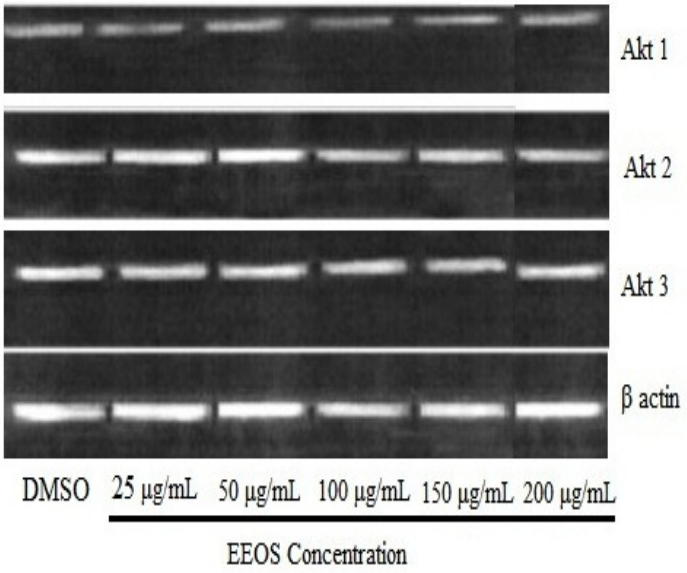

Fig. 2 : Agrose gel electrophoresis result of RT PCR products for quantitative measurement of expression of different isoforms of *Akt* under test conditions. β-actin is used as loading control.

3. Cell Proliferation:

Increased cell proliferationis one of the key indicators of cancers. Here we observed a contrasting proliferation of cells under treatment of EEOS as compared with control and with increasing dose of EEOS (Fig. 3). The proliferation of NSCLC cells, as measured with amount of BrdU incorporated in the replicating cells, is severely affected upon treatment with EEOS (p value=). ANOVA among the treatment group revealed significant effect of treatment of EEOS over cell proliferation. However when compared with paclitaxel, the proliferation results revealed that paclitaxel is much more effective then EEOS levels under study (ANOVA Table).

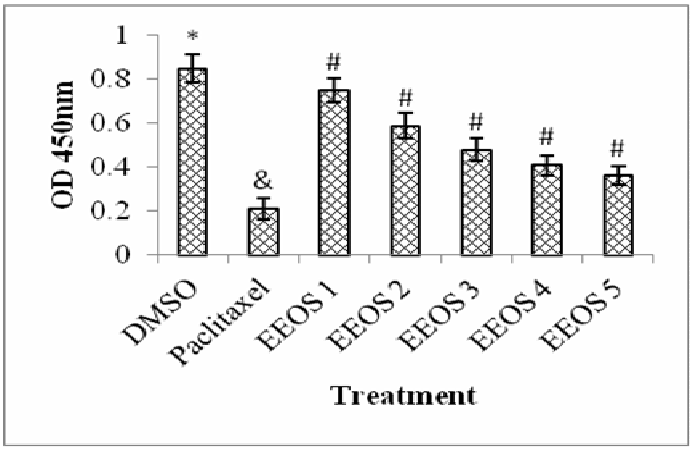

Fig. 3 : Cell proliferation as measured with BrdU incorporation into replicating DNA under controls and treatments. EEOS1= 25µg/mL, EEOS2=50µg/mL, EEOS3=100µg/mL, EEOS4=150µg/mL, EEOS5=200µg/mL. *p<0.05 DMSO versus EEOS groups, &p<0.05 paclitaxel versus EEOS groups, # p<0.05 among EEOS groups

4. Cell Viability:

To access degree of viability of cells upon treatment with EEOS, Alamar blue dye reduction assay was performed and the observation is depicted in Fig. 4. It is well interpreted from the plot and corresponding analysis of variance that EEOS caused severe loss in viability of NSCLC A549 cells

which is significantly different than control (Table 1). It is also suggested from results of table 1 that viability losses are significantly higher with increase in concentration of EEOS. Under the test conditions, viability losses are less but significant as compared to positive control i.e. paclitaxel.

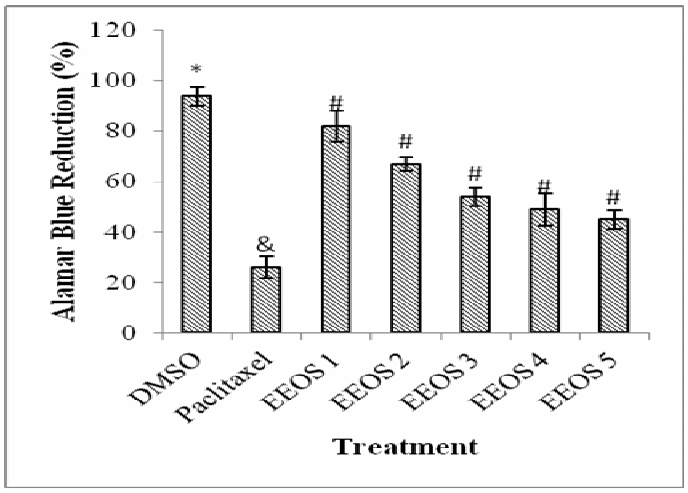

Fig. 4: Representation of cell viability as percentage of Alamar Blue dye reduced for different treatments under study. EEOS1= 25µg/mL, EEOS2=50µg/mL, EEOS3= 100µg/mL, EEOS4=150µg/mL, EEOS5=200µg/mL. *p<0.05 DMSO versus EEOS groups, &p<0.05 paclitaxel versus EEOS groups, # p<0.05 among EEOS groups

5. Cytotoxicity Assay:

NSCLC A549 cells were treated with EEOS (25-200 µg/mL) and cytotoxicity was measured with MTT assay. The results are represented in Fig. 5. It is evident from the histogram that EEOS exerted significant cytotoxicity over the cells in a concentration dependent manner but the effect is less as compared to paclitaxel. Cell viability was observed to be 100% in case of DMSO i.e. the negative control. Analysis of variance suggested a strong direct relation between concentration of EEOS and toxicity exerted over the cells under treatment (Table 1).

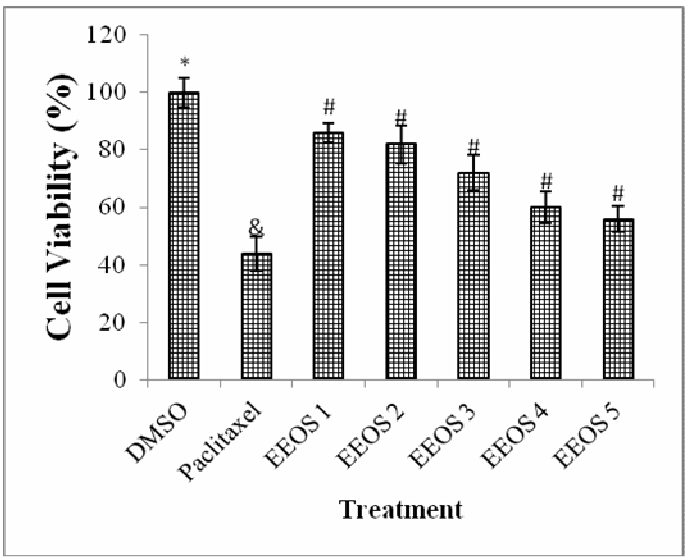

Fig. 5: Cell viability as a measure of cytotoxicity towards A549 cells under different treatments. EEOS1= 25µg/mL, EEOS2=50µg/mL, EEOS3=100µg/mL, EEOS4=150µg/mL, EEOS5=200µg/mL. *p<0.05 DMSO versus EEOS groups, &p<0.05 paclitaxel versus EEOS groups, # p<0.05 among EEOS groups

6. Mitochondrial membrane potential assay

Fluorescence remission shift of JC-1 dye due to $\Delta\Psi_M$change was used to determine the mitochondrial disruption in A549 cells under treatment. EEOS treated cells exhibited marked difference in its mitochondrial activity and higher the EEOS concentration higher the green/red fluorescence.Apoptosis of cells was significantly different between treated cells and DMSO (p *value* ≈ $1.77\text{x}10^{-6}$), treatment group and paclitaxel (p *value* ≈ $1.209\text{x}10^{-6}$), and among EEOS treatments (p *value* ≈ $8.329\text{x}10^{-5}$).

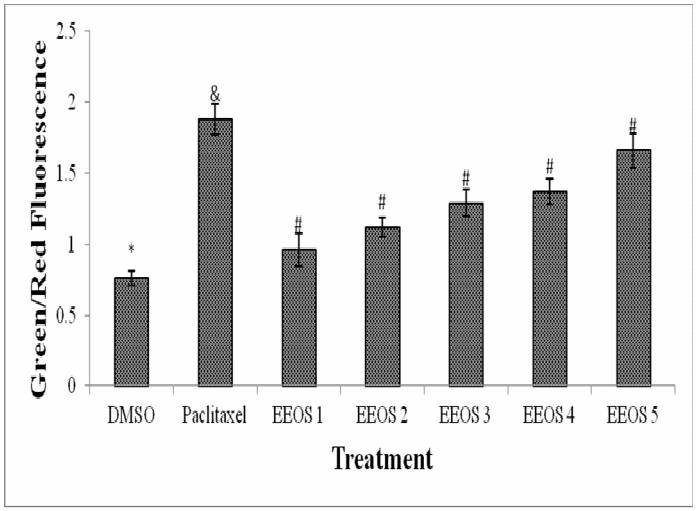

Fig. 6: Representation of Mitochondrial Membrane Potential as ratio of green/red fluorescence of JC-1 dye incubated cells after treatments. EEOS1= 25µg/mL, EEOS2=50µg/mL, EEOS3=100µg/mL, EEOS4=150µg/mL, EEOS5=200µg/mL. Higher ratio indicates higher apoptosis.*p<0.05 DMSO versus EEOS groups, &p<0.05 paclitaxel versus EEOS groups, # p<0.05 among EEOS groups

Discussions

It is well evident from available literature that extracts of plants possess suitable potential for treatment of various cancers including lung cancer. Extracts of plant material can act as anticarcinogenic agents with less or no side effects and this make it more potent for scientific research. Here we collected the evidences of role of ethanolic extracts of *Ocimum sanctum* in inhibiting viability and proliferation of NSCLC A549 under *in vitro* conditions and also discovered that it is mediated via loss in activation of Akt enzyme. Akt, as suggested, is a protein which plays a central role in progression of several functions of cell like protein synthesis, survival, proliferation, glucose metabolism via different pathways. To certain that the treatment is having any effect on concentration of total Akt in cells, we performed quantification of mRNA representing Akt1, Akt2, and Akt3. With observed results it was confirmed that although equal amount of mRNA is transcribed in all the cells under treatment, the amount of phosphorylated Akt is less which suggested loss in activation of Akt upon treatment. In a similar study, Kim et.al.(Venkataraman *et al*., 2009) used ethanolic extracts of *Ocimum sanctum* on Lewis Lung carcinoma cells and NSCLC A549 cells and demonstrated that the extract induced apoptosis in A549 cells and inhibited the *in vivo*growth of LLC, strengthening the hypothesis that EEOS can be applied in lung cancers as a chemopreventive substitute. Our study strengthens the findings of earlier studies where phosphorylation of Akt was inhibited by treatment with extracts of plants (An *et al*., 2015; Lu *et al*., 2015).

Table 1: ANOVA for cell proliferation assay, cell viability assay, MTT assay for cytotoxicity and Mitochondrial membrane potential assay. ANOVA is done among DMSO (negative control) and all EEOS concentrations, Paclitaxel (positive control) and all EEOS concentrations, All EEOS concentrations.

	DMSO to EEOS 1-5	Paclitaxel to EEOS1-5	Among EEOS1-5
Cell proliferation assay : *p* value			
	2.57429×10^{-6}	4.87227×10^{-6}	1.52727×10^{-4}
Cell Viability assay :*p* value			
	6.43698×10^{-8}	1.03964×10^{-7}	1.2252×10^{-5}
MTT Assay : *p* value			
	4.04668×10^{-7}	2.71582×10^{-7}	1.66851×10^{-5}
Mitochondrial membrane potential assay : *p* value			
	1.17772×10^{-6}	1.2095×10^{-6}	8.32892×10^{-6}

All the values suggest that the groups under analyses were significantly different

Table 2: Set of primers used for cDNA synthesis in RT-PCR based quantification of gene expression.

Gene	Primer
Ak1	5'-TGTTGAGGGTTGTCTCCGTG- 3' (sense)
	5'-CGAGTAGGAGAACTGGGGGA-3' (antisense)
Akt2	5'-GGCCGATGATCAGACTCTA-3' (sense)
	5'-TCCTCAGTGCTGGAGGTGT-3' (antisense)
Akt3	5'-GCTAGTCCACGAGAATTAGTCTC- 3' (sense)
	5'-ACAATGGTCGCCTCATGCTATCC-3' (antisense)

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