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Essential Oil Extracted from *Plectranthus amboinicus* Induces Apoptosis in the Lung Cancer Cells via Mitochondrial Pathway

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ABSTRACT

The use of medicinal plants in the form of extracts and oils dates back to pre-historic times. The aim of the study was to determine the effect of Essential oil (EO) extracted from leaves of *Plectranthus amboinicus* on lung cancer cell line, A549. MTT assay was performed to check the efficacy of Essential oil to induce death in cancerous A549 cells and normal HEK293 cells. Acridine Orange/Ethidium Bromide staining, Hoechst staining and DNA fragmentation assays were used to confirm the apoptosis inducing ability of EO on A549 cells. RT-PCR and western blotting was performed to check the expression of pro- and anti-apoptotic factors. Calorimetric assay was done to check caspase-3 and caspase-9 activities. Essential oil at lower concentrations was found to induce cell death selectively in the lung cancer cells (IC_{50} = 10.74 μg/mL). The transcriptional expression studies of the pro-apoptotic Bax and anti-apoptotic Bcl-2; and the western blot analysis of pro-apoptotic BAK and BAD showed that EO upregulated the expression of pro-apoptotic factors and down regulated the expression of anti-apoptotic factors in A549 cells. Caspase-9 and 3 activities were found to be upregulated in the calorimetric studies. The down regulation of anti-apoptotic factors and upregulation of pro-apoptotic factors show that the Essential oil induced apoptosis in the lung cancer cells via the intrinsic or the mitochondrial pathway.

Keywords: Lamiaceae, Growth inhibition assay, Anti-apoptotic, intrinsic pathway.

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INTRODUCTION

Plants offer a huge number of natural compounds belonging to different classes of phytochemicals that tend to produce physiological action on human system. Bioactive principles have gained importance recently due to the effect they are found to have on epigenetics. The use of medicinal plants in the form of extracts and oils dates back to pre-historic times.

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Most aromatic plants produce volatile essential oils through secondary metabolism. These essential oils constitute a major group of cosmetics and pharmaceuticals. Secondary metabolites or plants based natural products are generally classified into 3 major groups viz., terpenoids, alkaloids and phenol acid related compounds. Essential oils are generally found to be rich in terpenes. A large number of terpenes have been reported for their cytotoxic effect on various cancer types both *in vitro* and *in vivo.* [1] Terpenoids are reported to possess various bioactivites ranging from anti-oxidant to anti-cancer activity the best example being Taxol, a diterpene. *Plectranthus amboinicus* belongs to the family Lamiaceae and is

reported to be rich in aromatic secondary metabolites. The Plectranthus genus is reported to be rich in terpenes. [2] Carcinogenesis has multiple stages and terpenes have been reported to block or inhibit the process. [3] A plethora of terpenes including taxol, geraniol, andrographolide, d-limonene, perillyl alcohol, farnesol, lupeol, ursolic acid, lycopene, and a-bisabolol have been found to induce apoptosis in various cancer cells. [4] The objective of this study was to extract Essential oil (EO) from the leaves of *Plectranthus amboinicus* and to determine the effect of the EO on the lung cancer cell line, A549 and normal HEK293 cell line. The apoptosis inducing ability of EO was studied using Acridine Orange/Ethidium Bromide staining, Hoechst staining and DNA fragmentation assays. The study also tried to elucidate the mechanism by which EO induced apoptosis in the lung cancer cells by studying the expression of pro- and anti-apoptotic factors.

MATERIALS AND METHODS

Extraction of Essential oil

The plant *Plectranthus amboinicus* was collected from Palakkad district in Kerala and authenticated at Botanical Survey of India, Coimbatore and the specimen (BSI/SRC/2/83/2014-15/Tech-326) was deposited at the Institute. Essential oil was extracted using the Clevenger apparatus from freshly collected leaves. The hydrodistillation was performed for 3 h. The essential oil was dried over anhydrous sodium sulphate and was stored at 4°C. All chemicals and reagents used including the solvents were of analytical grade obtained from Hi-media (Mumbai, India).

GC-MS analysis

The extracted Essential oil was subjected to gas chromatography-mass spectrometry analysis. The fraction was analyzed using a HP 6890 GC/HP 5973 MSD system at 70 eV and 250°C. H5-5 (DB5) fused silica capillary – 0.32 mm x 30 m with film thickness 0.25µm column was used. Helium was carrier gas with a flow rate of 1.3 mL/min. The column temperature was initially 50°C for 1 min., followed by an increase of 3°C/minute to 250°C. Run time was 40 minutes. The components were analyzed and ascertained with the help of Wiley NIST library data and literature data.

Cell culture

The Human lung cancer (A549) and normal (HEK293) human embryonic kidney cells were obtained from NCCS, Pune. The cells were maintained in DMEM supplemented with 10 % FBS, 75 mg/L streptomycin and 100 U/L penicillin. Cells were maintained at 37°C in a humidified atmosphere of 5 % CO_2 and 95 % air. All cell culture reagents were purchased from Hi-media (Mumbai, India).

Cell Viability Assay

The A549 and HEK293 cells were grown in 12-well culture plates. MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess cell viability according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). The assay was quantitated by measuring the changes in absorbance at 570 nm. [5] HEK293 cells were used as control to check the influence of EO on normal cells. After measuring the absorbance at 570 nm at the end of the MTT assay, the % growth inhibition was determined using the formula:

$$\% \text{ growth Inhibition} = 100 - (\text{Abs (sample)} / \text{Abs (control)} \times 100)$$

Apoptosis studies

Hoechst Staining

The A549 cells (1×10^4 cells/mL) were incubated with EO for 24 h. Apoptotic nuclear morphology was visualized using Hoechst staining. [3] p-formaldehyde (4%) was used to fix the cells and then stained with Hoechst dye 33258 (working concentration of 10µg/mL, stock 1 mg/mL), incubated for 15 min and examined under fluorescent microscope (Olympus IX71) with excitation/emission at 300/380 nm, which was connected to a digital imaging system.

Acridine Orange/Ethidium Bromide Staining

The A549 cells, treated with Essential oil for 24 h were washed with Phosphate buffered saline (PBS) and trypsinized. From this cell suspension, 25µL (1×10^4 cells/mL) was mixed with 1µL of acridine orange/ethidium bromide solution (one part each of 100µg/mL of acridine orange and 100µg/ml of ethidium bromide in PBS) just before microscopic examination. [3] Fluorescent microscope (Olympus 1X71) connected to a digital imaging system was used to examine the slide.

DNA Fragmentation Assay

A549 cells were grown in 60 mm culture plates and subsequently treated with different concentrations (10, 20, and 50µg/mL) of EO for 24 h. [6] Cells without addition of EO were taken as control. The cells were harvested after 24 h and washed with PBS and pelleted by centrifugation at 300 rpm. To the cell pellet, 100µL lysis buffer (50 mM Tris-HCl pH 8, 20 mM EDTA, 10 mM NaCl, 1% SDS) was added for 1 min and centrifuged for 5 min at 2000 rpm. The supernatant was collected and incubated with RNase A (5µg/mL) for 1 h followed by digestion of proteins with proteinase K (100µg/mL) for 5 h. Aliquots of lysate (20µL) were loaded to 1.5% agarose prepared in TBE buffer (Tris-Borate 45 mM, 1 mM EDTA, pH 8) containing 3 mg/mL EtBr, and DNA was separated by electrophoresis. The separated DNA fragments in the gel were visualized using ChemiDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA).

Reverse Transcriptase-PCR

The expression levels of B-cell lymphoma-2 (Bcl-2), COX-2 (Cyclooxygenase-2) and Bcl-2-associated X (BAX) mRNA were studied using reverse-transcriptase PCR. [7-8] The A549 cells were grown in 60 mm culture plates. The first set of A549 cells was untreated and was taken as control. The second set of cells was treated with Phorbol 12-myristate 13-acetate (PMA) at a concentration of 50 ng/mL. The third set of cells was

treated with EO alone at 10µg/mL concentration. The last, fourth set of cells, was treated with both EO and PMA at 10µg/mL and 50 ng/mL concentrations respectively. After incubation, total RNA was isolated from the treated and untreated cells using manufacturer's protocol (Chromous Biotech Ltd, Bangalore, India). Total RNA (1µg) was converted to cDNA with M-MLV reverse transcriptase (Promega, Madison, WI) at 37°C for 30 min and denatured at 90°C for 15 minutes. The PCR was performed using GeneAmp RNA PCR kit (Perkin-Elmer Corp., Branchburg, NJ). The forward and reverse primers used for Bcl-2, COX-2, BAX and beta-actin amplification are specified in Table 1. The PCR products were separated on 1.5 % agarose gels and analyzed using a ChemiDoc XRS (Bio-Rad Laboratories Inc., Hercules, CA).

Table 1: Forward and reverse primers for the genes

Genes	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)
Bcl-2	ATGGACGGGTCCGGGGAG	TCAGCCCATCTTCTTCCA
BAX	CAGCTGCACCTGACG	ATGCACCTACCCAGC
COX-2	TTCAAATGAGATTGTGGGA AAATTGCT	AGATCATCTCTGCCTGA GTATCTT
β-actin	GTTTGAGACCTTCAACACC CC	GTGGCCATCTCCTGCTCG AAGTC

Table 2: Compounds separated from the Essential oil extracted from the leaves of *P. amboinicus* after GC-MS analysis

Peak No	Retention Time	Chemical Compound	Percentage
1	5.38	ρ-Cymene	8.4
2	8.84	γ-Terpinene	1.6
3	13.83	Myrcene	0.5
4	14.29	Methyl octanoate	0.8
5	15.47	Carvacrol	38.1
6	9.37	Methyl chavicol	2.6
7	20.46	α-Humulene	20.8
8	25.92	Borneol	0.4
9	28.42	Thymol	23.5
10	29.51	Tetradecanol	1.2
11	30.63	Caryophyllene oxide	1.8

Western blot analysis

Western blotting was performed to study the expression levels of Bcl-2 homologous antagonist/killer (BAK) and Bcl-2-associated death promoter (BAD) proteins. Culture plates of 60 mm were used to culture the A549 cells. The first set of A549 cells was untreated and was kept as control. The second set of cells was treated with EO at 10µg/mL. The last and third set of cells was treated with EO at 20µg/mL. The protein was separated by SDS-PAGE on a 10 % separating gel and transferred to nitrocellulose membrane. [9-11] Addition of primary rabbit monoclonal anti-BAK and rabbit monoclonal anti-BAD antibodies were done after the transfer of protein on to the membrane. The nitrocellulose membrane was blocked with 5% non-fat dry milk in 0.1% Tween-20 in PBS (pH 7.4) at 4°C overnight to avoid nonspecific protein binding. The membrane was incubated with monoclonal antibodies for BAK, BAD and β-actin at the dilution of 1:1000 (in 3% BSA), 1:2000 (in 3% BSA) and 1:2000 (in 3% BSA) respectively at 4°C overnight with gentle shaking. The membrane after incubation was washed thrice with PBS-Tween-20 (5 minutes each) and incubated with goat anti-rabbit HRP antibody for BAK and goat anti-rabbit HRP antibody for BAD (in 5% non-fat milk solution) (abcam, Cambridge, USA) at RT for 1 hour. After incubation, the membrane was washed twice with PBS-Tween-20 (5 minutes each) and for 10 minutes with the same. The protein bands were quantified by autoluminographs. The membrane was then stripped with stripping buffer (at 70°C for 1 hour) and reprobed with monoclonal antibody against β-actin to confirm the amount of protein in each well by following the manufacturer's instruction (Santa Cruz Biotechnology, Inc., CA). ECL prime western blotting detection kit (Amersham Biosciences, Piscataway, NJ) was used for detection of protein expression and was visualized using a ChemiDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA).

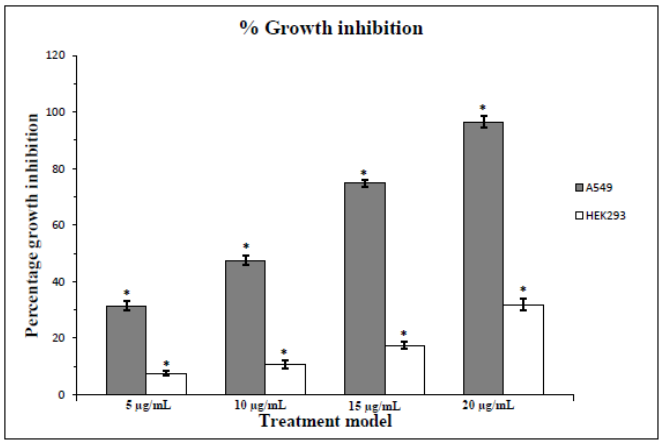

Fig. 1: Dose dependent response of Essential oil on the cell viability of A549 and normal HEK293 cells.

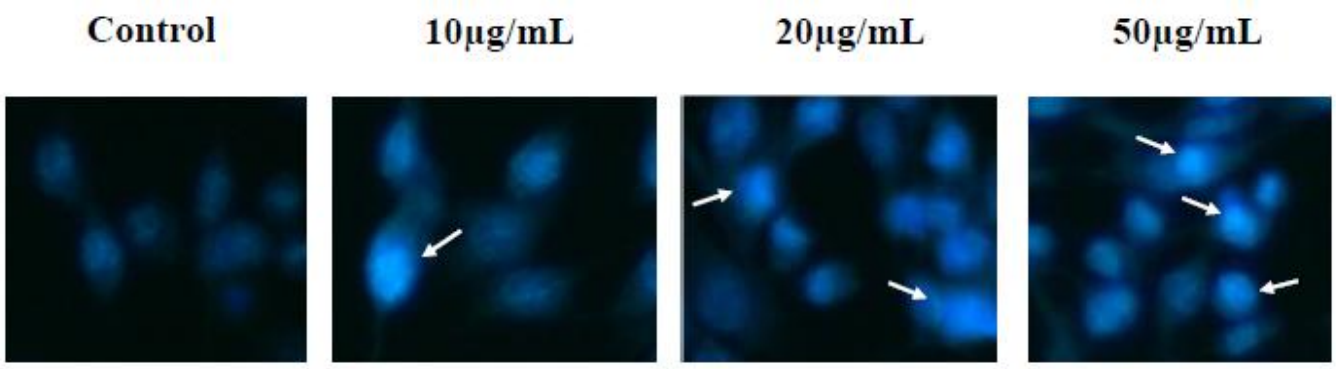

Fig. 2: Hoechst staining stained A549 cells after treatment with Essential oil

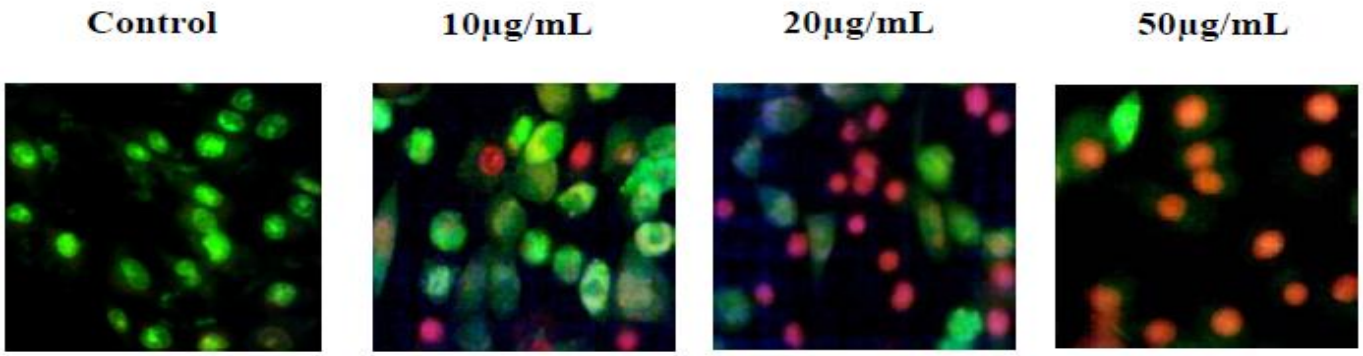

Fig. 3: AO-EtBr stained A549 cells after treatment with Essential oil

Caspases Activity Assay

The activities of Caspase-9 and 3 in the cell lysates were measured using the colorimetric assay with commercial kits according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Cells were incubated with different concentrations of EO and without EO as control for 24 h. The cells were collected by trypsinization and lysed using the lysis buffer (250 mM HEPES, pH 7.4 containing 25 mM CHAPS and 25 mM DTT) after incubation. The assay was based on the hydrolysis of the peptide substrates Leu-Glu-His-Asp p-nitroaniline (LEHD-pNA) by caspase-9 and acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac- DEVD-pNA) by caspase-3 resulting in the release of p-nitroaniline (pNA) moiety. The cell lysates were mixed with the substrate and absorbance read at 405 nm in a microplate reader. [12-13] The results were expressed as micromoles of pNA released per minute per mL.

Statistical Analysis

Results refer to mean ± standard deviation and are average of three values per experiment; each experiment was repeated at least three times. Statistical evaluations were assessed using the Student's *t* test, and $p < 0.05$ was considered significant.

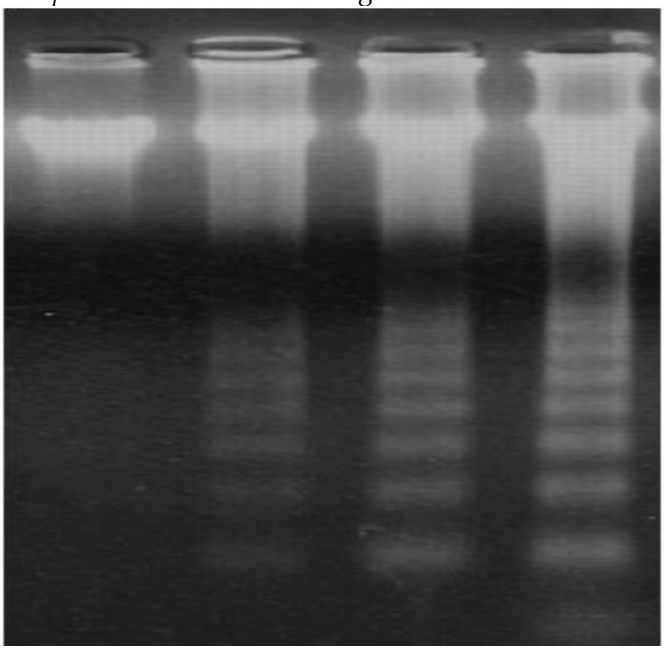

Fig. 4: DNA fragmentation assay gel after treatment with Essential oil on A549 cells.
Lane 1: Control, Lane 2: 10µg/mL, Lane 3: 20µg/mL, Lane 4: 50µg/mL

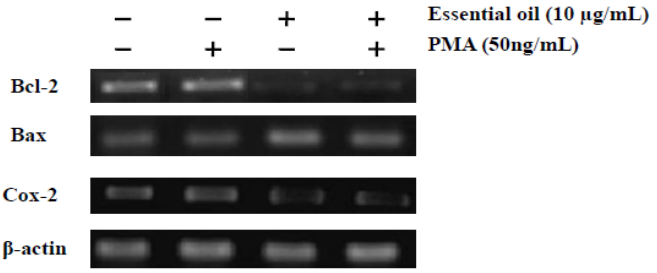

Fig. 5: RT-PCR gel showing mRNA expression of pro-apoptotic BAX and anti-apoptotic Bcl-2 and COX-2

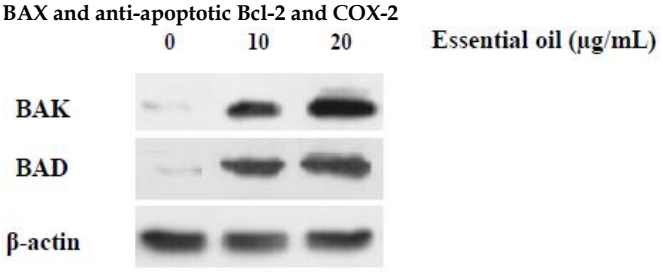

Fig. 6: Western blot of BAK and BAD

RESULTS

GC-MS analysis of the essential oil (EO) showed the presence of 11 compounds (Table 2). The constituents isolated were found to be rich in terpenoid compounds. The major components identified in the essential oil were Carvacrol, Thymol, γ-Terpinene, α-Humulene and ρ-Cymene.

MTT assay was used to check the effect of Essential oil on the cell viability of normal HEK293 cells and A549 lung cancer cells. The results showed that an increase in concentration of EO (0, 10 and 20µg/mL) selectively reduced the cell viability significantly in the cancer cells than normal cells (Figure 1). The IC_{50} value of the Essential oil was found to be 10.74 ± 0.83µg/mL on A549 cancer cells. Total (100%) growth inhibition was observed at a concentration of 20µg/mL of EO on the A549 cancer cells whereas this concentration produced only 40% cell death in the normal HEK293 cells which showed that EO has reduced cytotoxic effect on non-cancerous normal cells.

The nuclear morphology of the A549 cells were evaluated after treatment with different concentrations of Essential oil using Hoechst staining and compared with the nuclear morphology in untreated cells. The staining showed that lower concentrations of the Essential oil induced nuclear degradation and the degradation was found to increase significantly at

higher concentrations (Figure 2). This pattern clearly indicated that the EO degraded the nuclear morphology in A549 cells and these changes indicated features of apoptosis in the A549 cell line after treatment with essential oil. The acridine orange/ethidium bromide staining showed that the EO treated A549 cells took up the Ethidium bromide stain due to membrane damage and emitted orange/red fluorescence (Figure 3). In contrast, the untreated cells gave green fluorescence which indicated an intact cell membrane. It was also found that as the concentration of EO was increased, the degree of membrane damage also increased in the A549 cells indicated higher proportion of membrane damage. Treatment of A549 cells with different concentrations of EO showed considerable DNA fragmentation and as the concentration of EO was increased from 10µg/mL to 50µg//mL, the DNA fragmentation in the cells also increased confirming induction of apoptosis in the cancer cells (Figure 4).

The mRNA expression level of pro-apoptotic BAX from the control and Essential oil treated lung cancer cells were found to increase whereas the expression of anti-apoptotic Bcl-2 and COX-2 mRNAs were found to decrease in EO treated A549 cells (Figure 5). So, it was evident that as the concentration of Essential oil increased, the expression of pro-apoptotic BAX increased and the expression of anti-apoptotic Bcl-2 and COX-2 decreased, which confirmed the ability of EO to disturb the integrity of mitochondrial membrane thereby leading to release of cytochrome c into the cytoplasm which would form the apoptosome complex by binding with pro-caspase-9 and Apaf-1 triggering the downstream steps of Caspase cascade in the intrinsic pathway of apoptosis.

The protein expression studies of BAK and BAD, showed that the Essential oil induced increased expression of pro-apoptotic BAK and BAD (Figure 6); which can be compared to the expression of house-keeping protein beta-actin providing a relative expression pattern (Figure 7). As these factors play a very important role in the mitochondria facilitated apoptosis pathway, the increase in the expression of BAK and BAD could be vital in the induction of intrinsic pathway of apoptosis.

Finally, a significant increase in the caspase-9 and 3 activities was observed in Essential oil treated cells compared to control, which indicated the activation of caspase-9 and 3 in Essential oil induced apoptosis in the A549 lung cancer cells. The activity of the Essential oil at 10µg/mL was 113.63 ± 2.49µmols of pNA released/min/mL in caspase-9 assay and 103.94 ± 4.86µmols of pNA released/min/mL in caspase-3 assay which decreased when the concentration of the Essential oil was reduced (Figure 8).

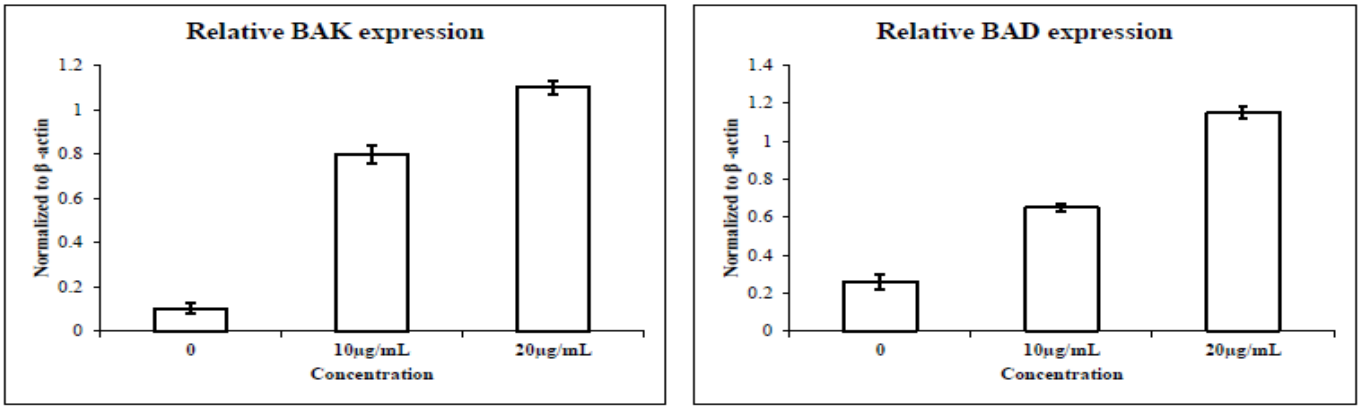

Fig. 7: Relative protein expression of BAK and BAD compared to β-actin.

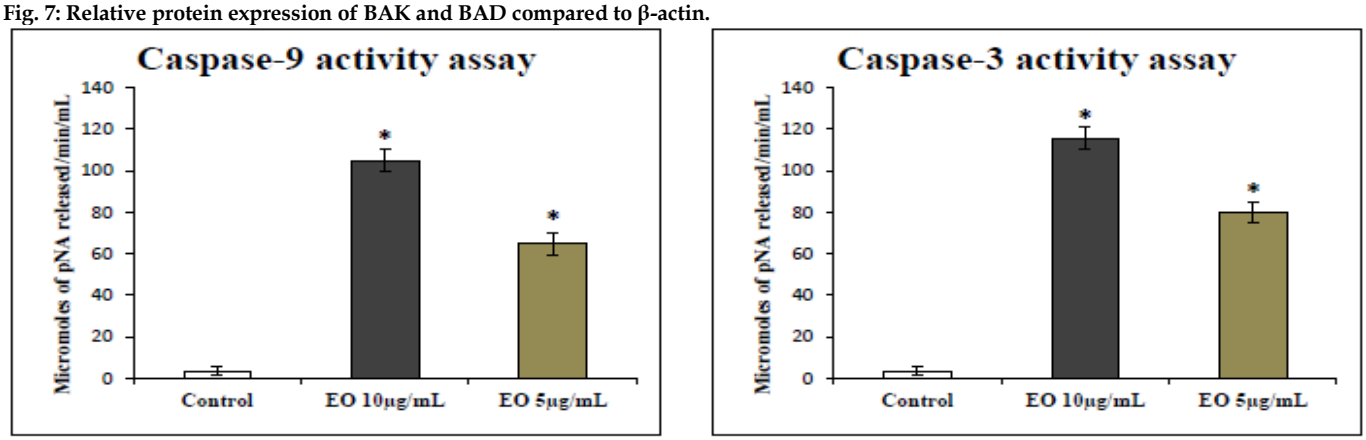

Fig. 8: Effect of Essential oil on the Caspase-9 and 3 releases in A549 cells when treated with different concentrations.

DISCUSSION

In India, the earliest mention of the use of medicinal plants is to be found in Rigveda which was written between 4500-1600 BC. [14] Lamiaceae is an important family of plants with regard to its pharmacological activity. The number of described products, the

structural diversity and pharmacological activities reported for various species of Lamiaceae demonstrate this family to be a promising source of new bioactive substances, which may give rise to new products as active molecules. Many of the plants belonging to Lamiaceae have widespread use in folk medicine and some showed anti-inflammatory, analgesic, antibacterial, mutagenic, antiviral, antioxidant, effect on vascular diseases as well as activity on the central nervous system. [15] Terpenes are among the most cited phytochemical constituent to possess anti-proliferative activity. Essential oil extracted from the leaves of *Plectranthus amboinicus* was found to be rich in mono and sesquiterpenes mainly carvacrol, thymol, p-cymene etc. [16]

The MTT assay indicated that the Essential oil induced growth inhibition in A549 cells with an IC_{50} value of 10.74 ± 0.83µg/mL. MTT assay is generally used as a preliminary assay to measure the growth inhibitory activity of any chemical on cells. The result showed that EO almost completely inhibited the growth of A549 cells at 20µg/mL. Further, EO was found to induce lower growth inhibition in normal HEK293 cells. At 20µg/mL, EO was found to induce only 30% growth inhibition in the HEK293 cells which shows that EO acts on the cancer and normal cells differentially.

Hoechst staining assay used to study apoptosis induction, revealed that the nuclear condensation, which is a feature of apoptosis, was visible prominently and increased in a dose dependent manner in the A549 cells. [17] The results showed that the nuclei of untreated control cells emitted lower fluorescence intensity compared to EO treated cells which showed typical features of apoptosis like chromatin condensation which leads to the increase in emission of intense fluorescence from the nucleus. The Acridine orange-Ethidium bromide staining showed that the Essential oil altered the membrane stability of EO treated cells, whereas the membranes of untreated cells were unaffected. The untreated cells fluoresced green due to the binding of Acridine orange to the intact cell membrane. But as the concentration of EO increased from 10µg/mL to 50µg/mL, the cells were found to take up more Ethidium bromide and fluoresce red due to the disturbance in the membrane integrity caused by the action of EO. Finally when the DNA fragmentation assay was performed, apoptosis induction in EO treated cells was confirmed when the DNA isolated from the treated cells formed a ladder like pattern in the gel. The DNA which gets cut into approximately 180-200 base pairs or its multiples forms a ladder like pattern when electrophoresed. The DNA gets cut at the internucleosomal linker sites by the action of Caspase activated DNase (CAD) and forms the ladder pattern which is a classic feature of apoptosis. [18-19]

Molecular studies of pro-apoptotic BAX and anti-apoptotic BCL-2 and COX-2 mRNA showed that EO regulated their expressions. The expression of BAX was found to be upregulated in EO treated cells whereas the expression of BCL-2 and COX-2 was found to be down-regulated in the treated cells. Mitochondrial membrane permeability changes when the ratio of BCL-2 family members changes. [20] When the pro-apoptotic members are up-regulated and anti-apoptotic members are down-regulated, this leads to the release of cytochrome-c from mitochondria, which activates the caspase cascade. [21] COX-2 is an anti-apoptotic factor, which is found to be up-regulated in various carcinomas and is reported to have a central part in tumorigenesis. [3] Overexpression of COX-2 in rat intestinal epithelial cells was found to reduce the rate of apoptosis and increased the expression of the antiapoptotic protooncogene BCL-2. [22] Here, EO was found to decrease the expression of anti-apoptotic BCL-2 and COX-2 mRNA and also increase the expression of pro-apoptotic BAX mRNA thus shifting the ratio towards the pro-apoptotic events which induces apoptosis in the A549 cells.

The mitochondria-anchored BAK and BAD proteins are found to be pro-apoptotic in nature and induce apoptosis in the cancer cells. [23-24] The BAK and BAD proteins induce apoptosis by destabilizing the mitochondrial membrane and creating pores in the mitochondrial outer membrane thereby initiating release of cytochrome-c into the cytosol which triggers caspase cascade. [25-26] The BAK and BAD protein expression was found to increase in EO treated A549 cells. This increase in expression in BAK and BAD proteins initiates apoptosis by forming pores in the mitochondrial outer membrane.

Finally, the activities of both caspase-9 and 3 were found to increase in the A549 cells when treated with EO. Caspase-9 and 3 are the major enzymes involved in the mitochondrial mediated or intrinsic pathway of apoptosis. [20, 26] The activity of both these enzymes which was studied calorimetrically proved categorically that EO has the ability to activate these enzymes and thus induce apoptosis in the A549 lung cancer cells.

Members of *Lamiaceae* family and Genus Plectranthus is reported to possess anti-inflammatory and anti-proliferative activity but no study has been conducted till date on Essential oil extracted from the leaves of *Plectranthus amboinicus* on lung cancer cells. The essential oil induced considerable lower percentage of cell death in normal HEK293 cells compared to the cancerous A549 cells, thus showing its ability to differentiate between normal cells and rapidly dividing cancerous cells. Our study shows that the essential oil induces apoptosis in the lung cancer cells via the intrinsic or the mitochondria mediated pathway by down regulating the anti-apoptotic factors and up regulating the pro-apoptotic factors.

The growing interest in biological activities of phytochemicals is a consequence of, among others, an increasingly high incidence of various cancers and a

need to find safe and effective method for prophylaxis and therapy. In this study, essential oil extracted from the leaves of *Plectranthus amboinicus* was found to induce apoptosis in the A549 lung cancer cells via the mitochondria-dependent pathway. However, the essential oil induced lower apoptotic activity on the normal HEK293 cells showing that EO acted on different cells differentially. This is the first report on the mechanism of apoptosis induction by essential oil extracted from the leaves of *Plectranthus amboinicus* and these findings would provide yet another method of targeting cancer cells using phytochemicals.

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