



Research articles

Anticancer activity of natural compound from lemon grass leaves extract on lung carcinoma cell line A549: an in vitro study

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ABSTRACT

Lemon grass is a widely cultivated plant, whose extracts are known to possess anti-cancer properties. However, studies related to the effect of different solvent extracts of lemon grass on lung cancer cell lines are scarce. This study was conducted to study the effect of various extracts on the viability of the A549 lung carcinoma cell line. Four solvents (hexane, chloroform, ethyl acetate and ethanol) were used for extraction of lemon grass. The effect of these solvent extracts on A549 cell line was studied. Cell viability was studied by 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) method. The effect of apoptosis on exposure to aforementioned extracts was observed with help of nuclear staining assays and western blotting techniques. Apoptosis was confirmed by nuclear staining using acridine orange ethidium bromide (Ao/EtBr), while pro- and anti-apoptotic cells expressions analysis was conducted using western blotting. All extracts showed cytotoxicity on lung carcinoma cell line, wherein, ethyl acetate and hexane extracts showed up to 48.6±3.8% and 51.7±1.7% viability at 250µg/mL concentration. Up regulation of pro-apoptotic gene expressions like Caspase-3 and inhibition of anti-apoptotic gene expression like Bcl-2 was observed after 24h. An inhibitory concentration (IC₅₀) of 220.01 µg/mL was obtained for the ethyl acetate extract. When observed under fluorescence microscope, stained cells showed an orange red color in the nuclei, indicating the ethyl acetate extract had induced apoptosis potentially for 24 h exposure cells expression, when compared with control cells. The results show that the ethyl acetate extracts were efficient in inhibiting the A549 cell line under in vitro condition, suggesting this extract can be further used as a potential chemotherapy drug.

Keywords: Lemon grass, A549cell line, Apoptosis, Ao/ EtBr, Leaf extracts.

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INTRODUCTION

Cancer, the second major cause of death globally with nearly 1 in every 6-death attributed to it, was responsible for 8.8 million deaths in 2015, with 70% of these deaths occurring in low- and middle-income countries [1]. Around one third of deaths from cancer are due to the five leading behavioral and dietary risks such as high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use and alcohol use. Amongst different cancers, lung cancer alone causes about 1.69 million deaths globally [2]. Amongst them, tobacco use is a highly important risk factor contributing to about 22% of deaths, mostly due to lung cancer [3,4]. This kind of cancer is due to the rapid proliferation of cells, which grow beyond their usual capacities, and which then invade the adjoining parts of the body and spread to other organs, by a process referred to as metastasizing. Cancer can also spread regionally, to nearby lymph nodes, tissues, organs, or to even distant parts of the body and when this happens, it is called metastatic cancer [5].

Existing anticancer drugs have been revealed to show various side effects. To overcome this issue, many works have been developed to synthesize anti-cancer drugs from natural resources. Plants contain active metabolites in form of bio-active compounds such as phenolic, flavonoids, tannins and saponins etc. [6-8], some of which might inhibit the cell proliferation of abnormal growth of cells [9]. Many plant derived metabolites have an anti-proliferative activity towards different cancer types [10,11]. In this regard, this study highlights the anti-proliferative activity on lung cancer cell line after exposure to Lemon grass extracts.

Lemon grass is a widely cultivated soft woody tree with pharmacological properties, and is found all over tropical and subtropical regions. The origin of this plant is in West Indies and America, but it is now widely found in India, Thailand, Indonesia, Ghana, and Australia. Traditionally, different parts of the plant material were used as cure against cardiac problem, parasite,

Haemorrhage, bacterial infection, dysuria, fever, ulcer and also as an insecticide [12]. Moreover, there are reports that this plant root extract has potent anti-proliferative advantageous activity towards lung cancer cell lines like HeLa, MDA-MBetc. Additionally, the root extract has been shown to have anticancer activity against melanoma cells in mice and in vitro inhibitory activity on MDA-MB-435 human melanoma cells [13-15]. Another study on anti-proliferative activity against colon cancer, human lung cancer (Hop65) and hepatoma cell line using Lemon grass leaves single solvent methanol extract showed a formidable growth inhibition [16].

Even though there have been studies against various cancer cell lines, only a limited study combine the extraction of active compounds from Lemon grass and use them directly on lung cancer cell lines [17,18]. Accordingly, the present study was aimed to screen the cytotoxicity against lung carcinoma cell line using different solvent extracts. The effect of apoptosis was observed with help of such nuclear staining assays and western blotting techniques, which are helpful to interpret the cytotoxicity level between different solvent extract used beyond the cell viability analysis using MTT assay and pro- and anti-apoptotic cells expressions analysis by western blot.

MATERIAL AND METHOD

Plant collection and processing

Plant was collected in the month of January 2018 from Vellore district, Vanniyambadi, Tamil Nadu, India (12.6858° N, 78.6178° E). The collected plant leaves were washed with 0.9% NaCl for cleaning the leaves surface and kept in dark under the room temperature to avoid any degradation of plant bio actives^[19] until further use.

Extraction procedure

Extraction was carried out by following the works of Mehra and Hiradhar^[20]. In brief, dried plant leaves were chopped into small size and grounded using electrical mixer until a fine powder was obtained. The powder was weighed to about 10 % w/v (10 g in 100 mL solvent) for extraction. The extraction was carried out by stirring the mixture using magnetic stirrer. The fractions were extracted sequentially by addition of 100 mL of different solvents from low polarity to high polarity. All solvent fractions (hexane, chloroform, ethyl acetate and ethanol) were evaporated under room temperature until powder like paste was obtained, which was stored at 4°C until use.

Cell culture and maintenance

Cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. The obtained cell was brought to animal tissue culture lab at once, the attached cell was transferred to fresh DMEM complete medium (10 % fetal Bovine Serum along with 100 µL of penicillin) after detachment using Trypsin-EDTA. Then cells were maintained in 5 % CO₂ incubator at 37 °C until multiplication of cell

growth for cytotoxic assay.

MTT cell viability

The cell viability analysis with different solvent extracts was carried out followed by method of Qaiser et al. ^[21]. For the cell viability analysis, the cells were detached using Trypsin-EDTA. The cells were gently washed with phosphate buffered saline (PBS) at pH 7.2 and after detachment of cells were centrifuged at 1000 rpm for 5 minutes. After centrifugation, the supernatant containing old medium was discarded. Then appropriate fresh medium was added and cells were pipetted into 96- well plate with 90 % cells confluence. The seeded plate was incubated in 5% CO₂ incubator with relative humidity of 37°C for 24 h and next day the supernatant medium was discarded from the 96-well plate. Each solvent extract stock solution was prepared by weighing 1mg of dry weight, dissolved in 1 mL of 0.5% dimethyl sulphoxide (DMSO). From these stock solutions, various concentrations of 50, 100, 150, 200 and 250µg/mL were prepared using DMEM medium and added the sample to each well and incubated as usual for 24 h. After 24 h exposure, the supernatant medium from well was discarded and 0.5% of 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide was added into each well with required volume and incubated for 4h. Following this incubation, the MTT was removed and formazon crystal was allowed to form by viable cells, which was dissolved in DMSO containing DMEM medium and solubilized sample was read at 540nm. The MTT assay was performed in three replicates & percentage of cell viability was calculated using the Eq. 1.

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treated}}{\text{Absorbance of control}} \times 100$$

Apoptosis analysis

The dual staining assay was performed according to method followed by Zainnal Ariffin et al. ^[22]. The apoptosis was analyzed using dual staining Acridine orange/Ethidium bromide (Ao/EtBr), which is able to detect early and late apoptosis cells precisely. The cell was seeded as described in MTT assay, but here the cells were seeded on the top of the cover slip which were then placed into the 12 well plates with 90% cells confluence for 24h monolayer formation. After monolayer formation the supernatant medium discarded the sample (ethyl acetate extract) were treated at their IC₅₀ concentration 220.01µg/mL and incubated in 5% CO₂ incubator for 24 h. After 24h treatment the cells were washed slightly with PBS pH 7.2 and cells were stained using dual stain Ao/EtBr prepared by separately by 100µg/mL each stain powder dissolved in PBS buffer, the prepared stains were mixed together, then dual stain 100µL was added into 12 well plate containing cells monolayer. The plate was incubated for 12 to 15 minutes in a dark room at room temperature, following which, the remaining stain was removed by washing with PBS buffer. The

stained cells were placed under fluorescence microscope (Nikon H550L, 80i, Japan) to detect the cell apoptosis.

Western blotting

Approximately, 50 µg protein of the total cell lysate was mixed with an equal volume of 2x sample buffer, heated for 5 minutes at 95°C, cooled, loaded onto each lane of 8-15% polyacrylamide gel, and separated by SDS-PAGE at room temperature. The resolved proteins were transferred to PVDF membranes. The membranes were then blocked with 5% BSA in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature, and probed with the following primary antibodies: Bcl-2, [rabbit polyclonal antibody at a dilution of 1:500]; active caspase-3 [goat polyclonal antibody at a dilution of 1:250]; β-actin [mouse monoclonal antibody at a dilution of 1:2000] overnight at 4°C. The blots were then washed thrice with Tris-buffered saline with 0.1% Tween 20 and then incubated with respective HRP labeled secondary antibody (Genei, Bangalore, India) at dilution of 1:1000 for 1h at room temperature. Further the membrane was washed in TBS-T,

and the bands were visualized by treating the membranes with ECL kit (Millipore, USA). The band intensity was quantified by Quantity One software (Bio Rad, USA). Quantification of expression levels was performed by densitometric analysis using Image J [23].

Statistical analysis

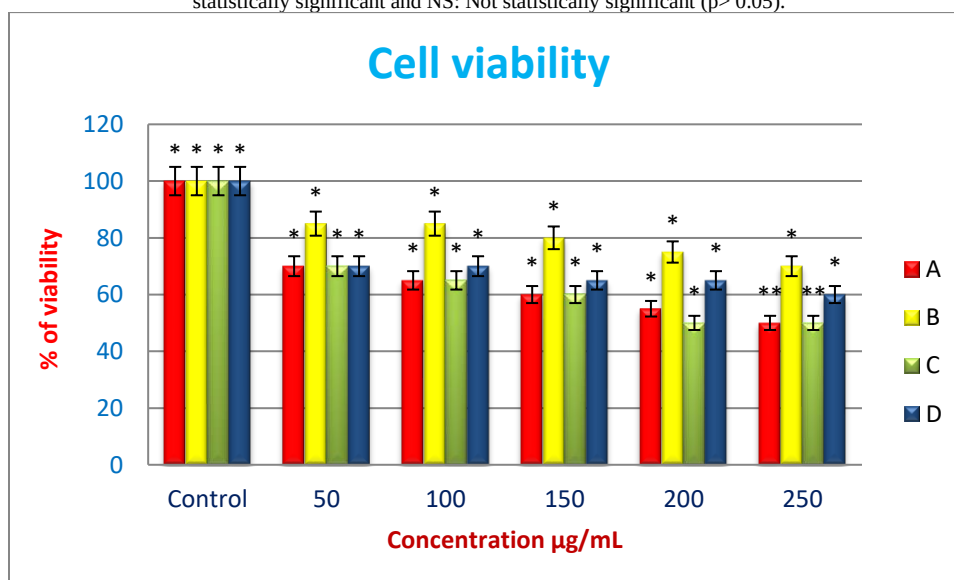
All results were expressed in mean and standard deviation obtained using Excel. The control and treated values were subjected into student t-test to found significant between these groups. The probit analysis were performed using Statplus V.5.0

RESULT

Cell viability

Treated cells showed cytotoxicity to A549 cell under *in vitro* conditions to all the solvent extracts and viability of this cell were reduced significantly ($p > 0.001$) in hexane and ethyl acetate extract compared to other solvent extracts as showed in Figure 1A-D, with the ethyl acetate extract showing more than 50 % cell reduction as shown in Figure 1C.

Figure 1. The percentage cell viability obtained after treated with hexane, chloroform, ethyl acetate and ethanol (A, B, C and D) solvent extract for 24h exposure. Each vertical bar represents mean ± SD of three replicates. Bullet icons indicates significance difference between control and treated group. (* $p < 0.01$, ** $p < 0.001$) statistically significant and NS: Not statistically significant ($p > 0.05$).



The IC₅₀ value of ethyl acetate extract was obtained as 220.01 µg/mL for 24 h treated extract. Based on the results obtained the ethyl acetate extract was further tested for dual fluorescent staining Ao/EtBr for further confirmation whether it precisely elicits the induction of apoptosis or not by treated the cells with their IC₅₀ concentration.

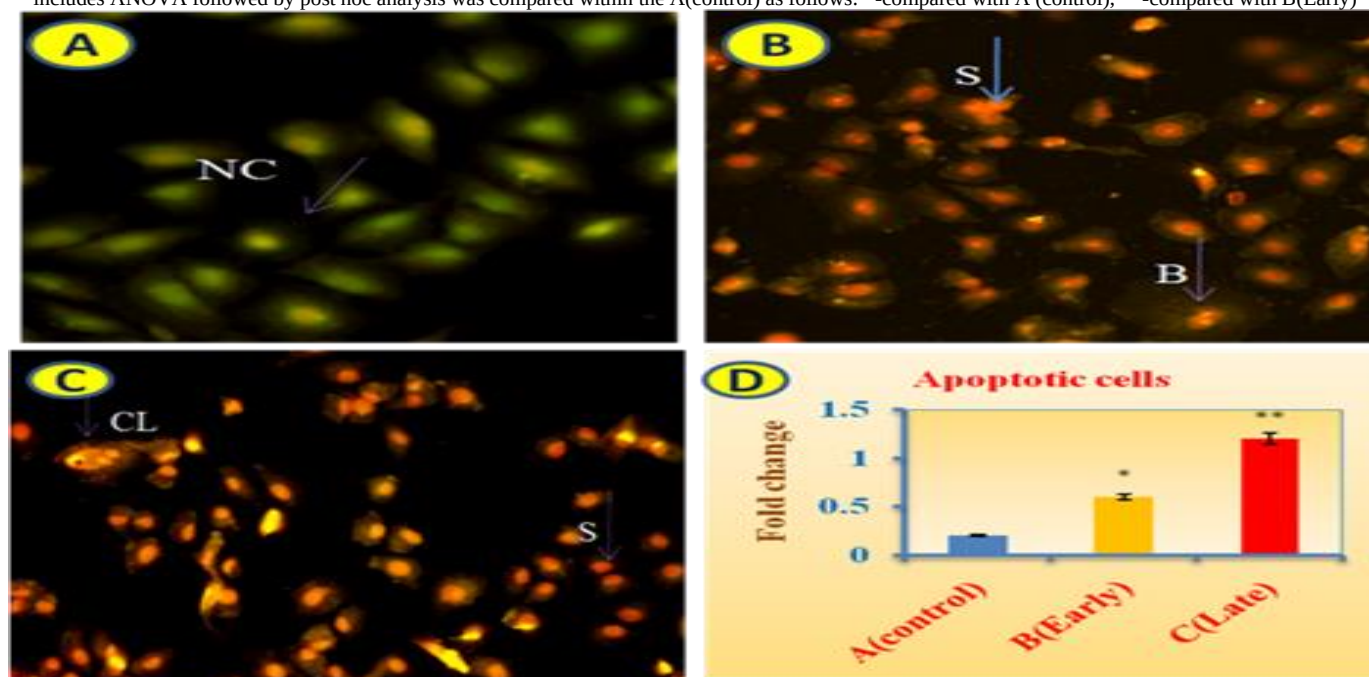
Nuclear staining of apoptotic cells

Apoptosis analysis was conducted for both control and ethyl acetate treated cells using Ao/EtBr staining. The reason to combine two staining is that, the AO bind to both viable and dead cells, but EtBr only enters into dead cells. This dual staining helps to distinguish between apoptotic and non-apoptotic cells by producing a fluorescence

color, where the viable cells seems to be green with retention of their cell morphology, while apoptotic (dead) cells resemble yellowish and orange color.

In this study, as shown in Figure 2, the cells appeared yellow orange, which indicates that the cells had already undergone apoptosis (Figure 2B) after 24h exposure to ethyl acetate extract in an *in vitro* condition at their IC₅₀ concentration. As shown in Figure 2, the cells showed shrinkage, loss of membrane integrity by releasing organelles from inside of the cells and membrane blobbing as shown in Figure 2B and 2C. The apoptosis happens when anti-apoptosis protein family such as Bcl-2, is unable to inhibited the mitochondrial membrane permeability.

Figure 2. The apoptosis was induced by ethyl acetate extract at their IC_{50} concentration for 24h treatment under in-vitro condition. Cell morphologically changed by shrinkage and ruptured as determined by Ao/EtBr dual staining. A: control showing an inviolate condition with green fluorescence display. B: cells showed morphologically violated by ethyl acetate-induced increased membrane bound apoptotic bodies with yellowish orange nuclei. The different arrows indicated the state of cells under in-vitro condition. NC: Normal cell morphology; S: shrinkage of cells; B: Membrane blebbing; CL: Cell leakage. The all experiments were repeated in three replicates. Values represent the mean $\pm$ SD for each group, (n=3) * denotes the values are statistically significance at $p < 0.05$ by hypothesis testing method which includes ANOVA followed by post hoc analysis was compared within the A(control) as follows: *-compared with A (control), **=compared with B(Early)



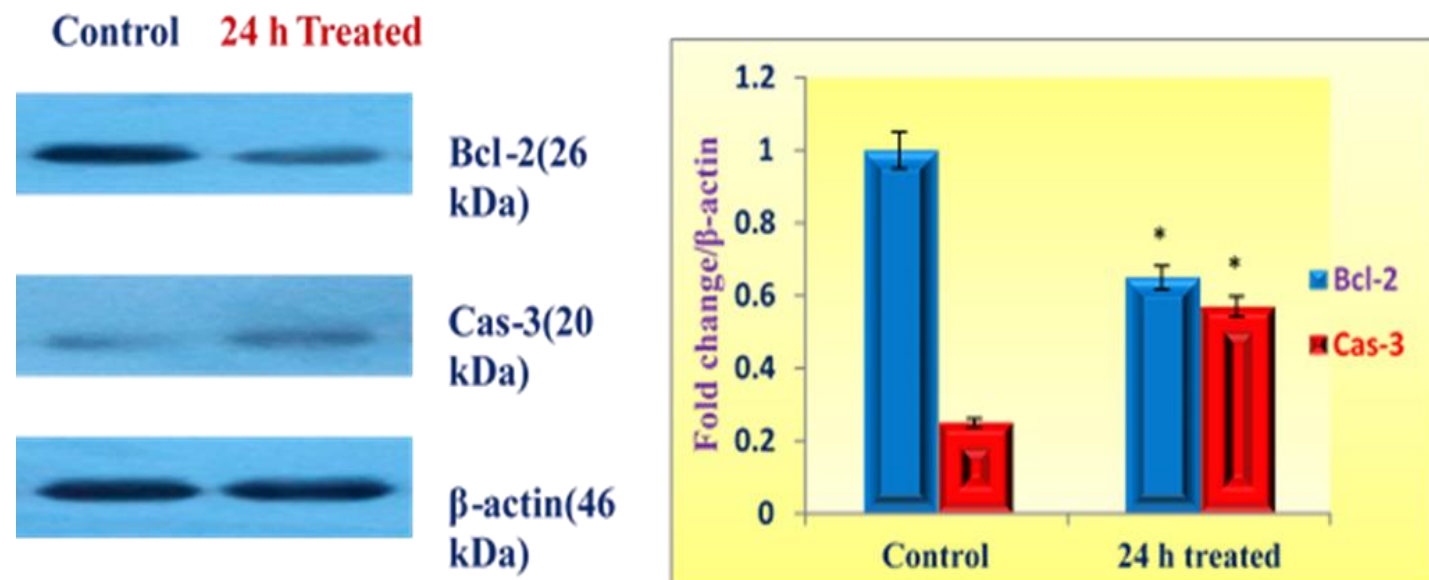
Western Blotting

Apoptosis psychiatry was analyzed for both control and ethyl acetate treated A549 cells using western blotting. Results in Figure 3

demonstrate a down regulation of anti-apoptotic molecules like Bcl-2 and up regulation of Caspase-3 expression in group-II treated cells in

Figure 3

Figure 3. The apoptosis was induced by ethyl acetate extract at their IC_{50} concentration for 24 h treatment an in-vitro condition. Western blot and Densitometric analysis of Bcl-2 and Caspase-3 and β -actin. Group I control, Group II S. Lemon grass treated. Each Values represent the mean $\pm$ SD for each group, (n=3) * denotes the values are statistically significance at $p < 0.05$ by hypothesis testing method which includes ANOVA followed by post hoc analysis was compared within the A(control) as follows: *-compared with A(control).



DISCUSSION

The plant derived metabolites contain diverse bio-active compounds such as alkaloids, phenolic and tannin etc. These natural compounds and its' derived synthesized compounds have potential roles in anti-tumor, antimicrobial and anti-oxidant activities [24]. Accordingly, the present study aimed to evaluate anti-proliferative

activity of lemon grass extract mediated apoptosis against A549 cell line with help of MTT assay, which helps to determine the cell viability by reduction of MTT by viable cell mitochondrial dehydrogenase enzyme forming purple formazan crystal after the drug or compound of mixture treated at certain period [25]. Using different solvent extract

from this plant leaves on lung cancer cells, it was found that ethyl acetate extract showed most significant reduction in cell viability. The efficient toxicity can be attributed to an active compound present in the ethyl acetate extract.

The apoptosis was determined using Ao/EtBr staining, which helps to distinguish a apoptotic and non-apoptotic cells by producing a fluorescence color. In our studies, the apoptosis cells clearly showed that cells underwent shrinkage and loss of membrane integrity by releasing organelles from inside of the cells and membrane blebbing as shown in Figure 2B. The apoptosis happens when pro-apoptosis protein family such as Caspase-3 increased expression in Lemon grass is treated group II and anti-apoptotic molecules like Bcl-2, decreased expression [26,27] in group II. Lemon grass was capable to inhibit the mitochondrial membrane permeability. Hence it leads to elicit the deregulation of cancer cells and finally the cells undergo apoptosis, consequentially, cells stop proliferation and die [28]. Even number of studies have been reported in Annonareticulata plant extract and isolated compounds against different cancer cell line, there is only a limited study were only carried out. Our studies confirm studies the exact cytotoxicity at apoptosis level using staining assay and gene expression levels, and thus indicates that lemon grass is efficient to reduce cell viability in potent manner.

CONCLUSION

The ethyl acetate extract exhibited significant cytotoxicity against lung cancer cells as the extract induced apoptosis at their 50% inhibitory concentration of 220.01 µg/mL for 24 h treated extract. From this observation, we suggest that Lemon grass leaves extract should have an emerging role as a novel therapeutic drug. Further studies are needed to explore the isolation of active compound from the extract and study the protein and mRNA expression level to reveal the exact mechanisms of ethyl acetate extract induced apoptosis.

LIST OF ABBREVIATIONS

Ao: Acridine orange

EtBr: Ethidium bromide

DMEM: Dulbecco's Modified Eagle's medium

SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis

PVDF: Polyvinylidenedifluoride

Bcl-2: B-cell lymphoma 2

caspase- 3: cysteine-aspartic acid protease - 3

HRP: Horseradish Peroxidase

TBS-T: a mixture of tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20)

ECL: Enhanced chemiluminescent

IC50: half maximal inhibitory concentration

MTT:3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide

NCCS: National Centre for Cell Science

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