Cytotoxic Effects of Lemon Grass and Mint Formulation Mediated Zinc Oxide Nanoparticles Against Lung Cancer Cell Line.

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Abstract

Background: For thousands of years natural products, especially plants and vegetables, have been used to fight against various diseases such as cancer, microbial infections and even neurodegenerative diseases. It has been shown that consumption of plants and vegetables have a direct influence on the proliferation, angiogenesis and metastasis of cancer cells.

Materials and methods: The cell viability of lemon grass and mint ZnO NPs treated A549 cells was assessed by MTT assay. The A-549 cells were treated with different concentrations of lemon grass and mint ZnO-NPs (10-100µg) for 24 h. Analysis of changes in the cell morphology is examined by phase contrast microscope. For the nuclear morphological changes analysis, AO/EtBr dual staining was used in lung cancer cells. The apoptotic nuclei were viewed under a fluorescent microscope.

Result: The cytotoxic potential of lemongrass and mint extract in lung cancer cells was assessed by an MTT assay. The cells were treated with different concentrations (10-100µg/ml) of lemongrass and mint extract for 24h. Lemon grass and mint ZnO NPs treatment significantly decreased the viability of A549 cancer cells compared to control at 24 h time point.

Conclusion: The present data demonstrate that extracts of lemon grass and mint ZnO NPs may inhibit the proliferation of cancer cells and induce apoptosis and could provide protection from oxidative stress diseases due to their high antioxidant molecules content.

Keywords: Lung cancer, herbal plants, Lemon grass, Mint, Apoptosis

INTRODUCTION

Cancer is a challenging problem for global health, and its increasing burden seeking novel and alternative therapies. Cancer hallmarks include uncontrolled proliferation, refractoriness to proliferation blockers, escaping apoptosis, unlimited proliferation, enhanced angiogenesis, and metastatic spread(1). Therefore, targeting more than one molecule in apoptotic pathways can be an efficient approach for identifying new anticancer therapeutics and preventing resistance to therapy(2). Lung cancer is a disease with high morbidity and mortality rates. It is often associated with a significant amount of suffering and a general decrease in the quality of life(3).

Herbal medicines are recognized as an attractive approach to lung cancer therapy with little side effects and are a major source of new drugs. Our team has extensive knowledge and research experience that has translate into high quality publications (4–13). Nowadays plants and its products are said to be effective against various diseases including cancer. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects. Currently, a few plant products are being used to treat cancer (14,15). The anticancer properties of several plants have been identified several years ago. The anticancer properties of plants have still been actively researched (16–26). Lung cancer is currently the malignant tumor with the highest mortality rate, often because it is not detected until there has been substantial progression of the illness, which leads to a reduction in quality of life of the patient (27). Different factors are pointed out as possible causes of lung cancer,which includes cigarette smoking, exposure to secondhand cigarette smoke, pipe and cigar smoking, exposure to indoor and outdoor air pollution, exposure to radiation, and occupational exposure to agents such as asbestos, nickel, chromium, and arsenic(28). Smoking causes the majority of lung cancers both in smokers and in people exposed to secondhand smoke. s of breath if cancer grows to block the major airways(29). Lung cancer can also cause fluid to accumulate around the lungs, making it harder for the affected lung to expand fully when you inhale(30).

The p53 is described as “the guardian of the genome” due to taking part in the cell cycle controlling apoptosis, repair of DNA and preserving the whole genome integrity(31) . In lung carcinomas, the p53 converted from active to inactive protein due to missense mutations in the region of coding p53 gene which play an substantial role in oncogenic transformation of lung epithelial cells as well as development of lung carcinoma(31,32). Therefore, searching for other anticancer medicines with lower or no side effect for treating lung cancer is still the main goal. Aim of the present study is to demonstrate the cytotoxic effects of kemon grass and mint formulation mediated zinc oxide nanoparticles against lung cancer cell line.

MATERIAL AND METHODS

Reagents

DMEM (Dulbecco's Modified Eagle Medium), Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS), were purchased from Gibco, Canada. Acridine orange (AO), ethidium bromide (EtBr), Dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), DAPI, AO/EtBr were purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were extra pure of molecular grade and were purchased from SRL, India.

Cell line maintenance

A549 lung cancer cell lines were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. Upon reaching confluency, the cells were trypsinized and passaged.

Lemongrass and mint (Zinc Oxide) Nanoparticle preparation

1g of lemongrass and 1g of mint was added to 100 mL distilled water. The mixture was boiled for 15-20 minutes using a heating mantle at 70ºC for 15-20 minutes. The boiled extract was filtered through Whatman No:1 filter paper. The filtered extract was stored and used for nanoparticle synthesis. 20 mM of zinc oxide was dissolved in 50 mL of distilled water. To that, 50 mL of lemongrass and mint extract was added. The reaction mixture was kept in an orbital shaker for 48-72 hours. UV-visible readings were recorded to preliminarily confirm the synthesis of nanoparticles at specific intervals of time. After that, centrifugation was done to collect the pellect the nanoparticle solution. The centrifugation was done at 8000 rpm for 10 minutes and the pellet was washed thrice with distilled water and supernatant was discarded.

Cell viability (MTT) assay

The cell viability of lemon grass and mint extract treated A549 cells was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. A549 cells were plated in 96 well plates at a concentration of 5x103 cells/well 24 hours after plating, cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with lemongrass and mint at different concentrations for 24 hours. At the end of treatment, the medium from control and lemongrass and mint treated cells were discarded and 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4 h at 37ºC in the CO2 incubator.

The MTT containing medium was then discarded and the cells were washed with 1x PBS. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as percentage of control cells cultured in serum-free medium. Cell viability in control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of

treated cells/A570 nm of control cells]×100.

Morphology study

Based on MTT assay we selected the optimal doses (IC-50: 40µg/ml) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 2×105 cells were seeded in 6 well plates and treated with lemon grass and mint ZnO NPs (40µg/ml concentration for A-549 cells) for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

Determination of mode of cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining

The effects of lemon grass and mint ZnO NPs (40µg/ml) in A549 cell death were also determined by AO/EtBr dual staining as described previously. The cells were treated with lemongrass and mint for 24 h and then the cells were harvested, washed with ice-cold PBS. The pellets were resuspended in 5 µl of acridine orange (1 mg/mL) and 5 µl of EtBr (1 mg/mL). The apoptotic changes of the stained cells were then observed by using a fluorescence microscope.

Cell migration analyzed by scratch wound healing assay

Human lung cancer cell lines (2×10^5 cells/well) were seeded onto six-well culture plates. The cell monolayer was scratched using a 200µl tip to create a wound. The detached cells were removed by washing with 1X PBS and add fresh culture medium with (lemon grass and mint ZnO NPs (40µg/ml) for 24 h along with control group for 24 h. After incubation, the wells were washed and fixed in 4% paraformaldehyde. Photographs were taken using an inverted microscope (Euromex, The Netherlands).

Statistical analysis

All data obtained were analyzed by One way ANOVA followed by Students-t-test using SPSS, represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$.

RESULT

Effect of lemongrass and mint extract on cell viability of lung cancer cell line

The cytotoxic potential of lemon grass and mint ZnO NPs in lung cancer cells was assessed by an MTT assay. The cells were treated with different concentrations (10-100µg/ml) of lemongrass and mint extract for 24 h. Lemongrass and mint extract treatment significantly decreased the viability of A549 cancer cells compared to control at 24 h time point (Fig. 1). The percentage of cell viability gradually decreased with increase in the concentration. We observed the 50% growth inhibition at (40µg/ml) concentration. Hence, IC-50 dose (40µg/ml) was considered for the further experiments.

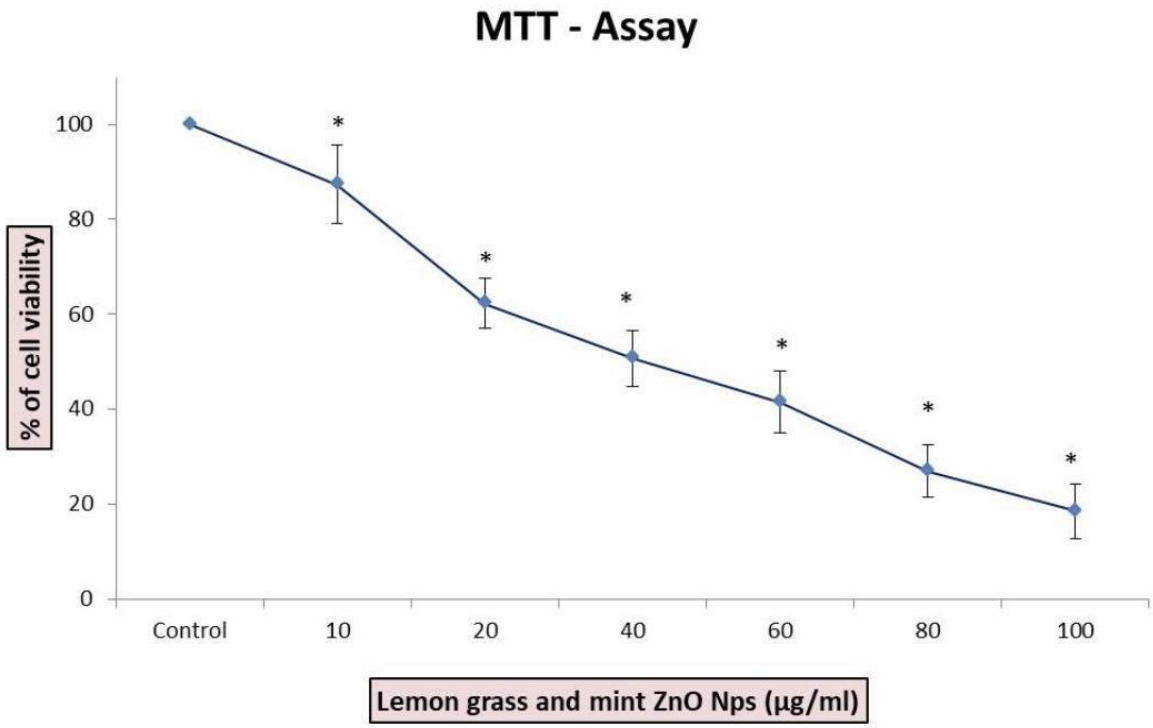


Figure 1: Effect of lemongrass and mint extract on cell viability of A549 cell lines showed IC-50 dose 40µg/ml.

Effect of Lippia nodiflora on cell morphology

The cell morphological analysis of lemongrass and mint extract treated lung cancer cells was observed in an inverted phase contrast microscope. The A549 cells were treated with lemongrass and mint(40 µg/ml) for 24 h, compared with the untreated cells, treated cells showed significant morphological changes, which are characteristic of apoptotic cells, such as cell shrinkage and reduced cell density were observed in the lemongrass and mint extract treated cells (Fig. 2). Cells undergoing apoptosis also displayed other types of morphological changes such as rounded up cells that shrink and lose contact with neighboring cells. Some sensitive cells were even detached from the surface of the plates.

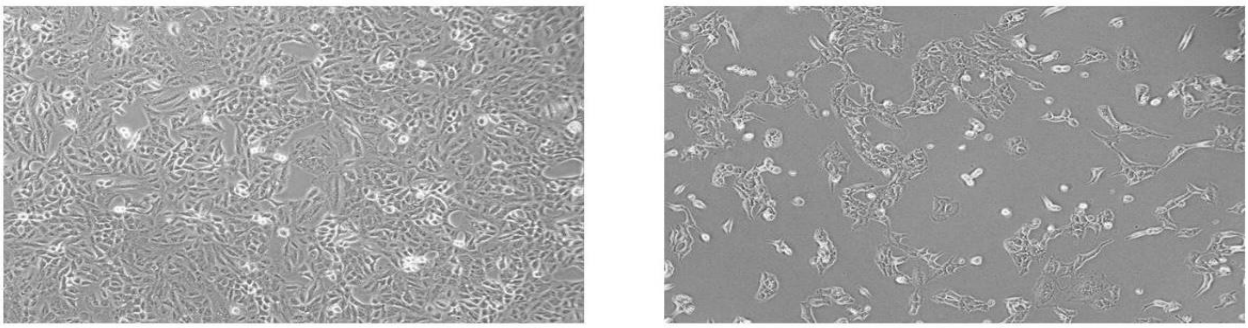


Figure 2: The figure represents the morphological changes in A549 cell lung cell line upon, without and with the treatment of lemongrass and mint at 40 µg/ml for 24 hrs by phase contrast microscope at 20x magnification. The number of cells decreased after the treatment and the arrow indicates the cells

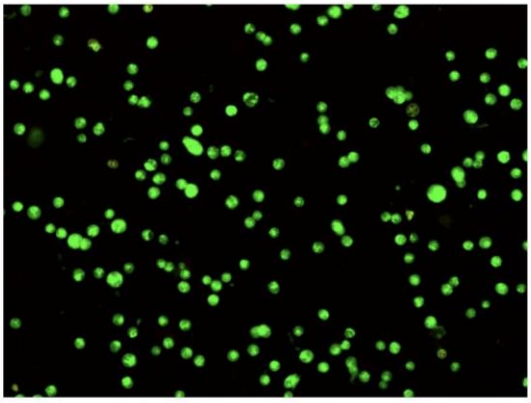
exhibited shrinkage and cytoplasmic membrane blebbing

Pro-apoptotic effect of lemongrass and mint

The induction of apoptosis lemongrass and mint extract (40 µg/ml) treated cells was analyzed by AO/EtBr staining. After a 24 h treatment period, the cells were stained with nuclear staining (AO/EtBr) and observed in fluorescence microscopy. The treated cells clearly showed condensed chromatin and nuclear fragmentation, which are characteristics of apoptosis compared to the control which showed clear round nuclei.(Fig. 3).

Control

Lemon grass and mint ZnO NPs (40 µg/ml)



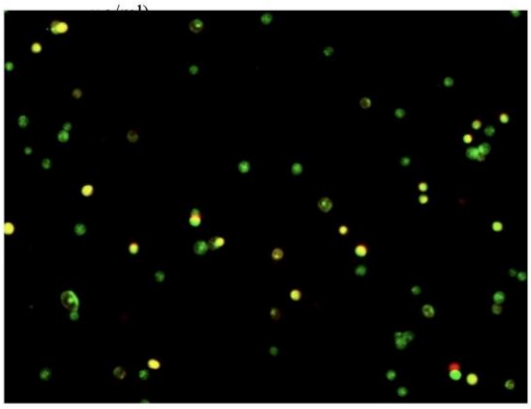


Fig.3. This figure represents the AO/EtBr staining of A549 lung cancer cell line upon, without and with treatment of lemongrass and mint at 40 µg/ml for 24 hrs, viewed under a fluorescence microscope at 20x magnification. The viable cells possess a uniform green color nucleus, and cells treated at 40 µg/ml possessing condensed chromatin and nuclear fragmentation.

DISCUSSION

Lemongrass and mint is a herb used in traditional medicine. Aqueous extracts of dried lemongrass leaves are used in traditional medicine for treating digestive disorders, diabetes, nervous disorders and cancer(33). However, previous studies had not investigated their anti-inflammatory and anticancer properties. The present study demonstrates the cytotoxic potential of lemongrass and mint extract in lung cancer cells was assessed by an MTT assay. Lemongrass and mint extract treatment significantly decreased the viability of A549 cancer cells compared to control at 24 h time point.The IC-50 dose (40µg/ml) was considered for the further experiments.

Cell death process can be subdivided into two different fates, apoptosis or necrosis. On morphological evaluation, we noted a major increase of necrotic cells than apoptotic underlining a genotoxic mechanism, but this assumption needs to be further addressed(31). Mint and lemongrass can induce a strong cell death mechanism by cell cycle analysis, cell morphology assay and by viability assays(31,34). Previous studies Extracts were found to contain components that inhibit cell proliferation and display cytotoxic activity on cancer cells but not on normal cells. The cytotoxicity of the studied extracts was also evaluated on young cerebellar granule cells and found to be non-significant(35). The anti-proliferative activity appeared to be mediated by apoptotic mechanisms, as suggested by activation of caspases 3/7

following cell exposure to the extracts. In the present study lemongrass and mint extract induced apoptosis (40 µg/ml) treated cells were analyzed by AO/EtBr staining.

One effective technique for anticancer drug production is to induce apoptosis in cancer cells. Many substances derived from plants have been studied for their ability to induce apoptosis (19,36,37). To determine if the extract's anticancer activity depended on an apoptotic pathway, AO/EtBr dual staining experiments confirmed the apoptosis-inducing impact of the lemongrass and mint extract NPs. The lemongrass and mint extract NPs treated cells clearly displayed apoptotic morphous in double sequential AO/EtBr staining, such as bright orange areas of condensed or fragmented chromatin in the nucleus indicating the presence of early apoptotic cells, while the late apoptotic cells having a uniform bright red nucleus were observed using a fluorescent microscope. The treated cells showed condensed chromatin and nuclear fragmentation, which are important characteristics of apoptosis compared to the control which showed clear round nuclei.

CONCLUSION

The present data demonstrate that extracts of Lemon grass and mint may inhibit the proliferation of cancer cells and induce apoptosis and could provide protection from oxidative stress diseases due to their high antioxidant molecules content. However more research is needed to understand the mechanisms of anti-cancer potential of this lemongrass and mint.

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