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Evaluation of Anticancer Effect of *Lippia nodiflora* Leaf Extract on Lung Cancer Cells

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Lung cancer, also known as bronchogenic carcinoma, is a kind of cancer that begins in the lung parenchyma or inside the bronchi. In the United States, it is one of the top causes of cancer-related fatalities. *Lippia nodiflora* is a flowering, broadleaf plant native to South America. *Lippia nodiflora* has been used as a natural medicine for a number of disorders due to its antioxidant, anti-inflammatory, anti-bacterial, and anti-tumor characteristics.
Aim of the Study: To evaluate the anticancer effect of *Lippia nodiflora* leaf extract on lung cancer.
Materials and methods: The cytotoxic effect of *Lippia nodiflora* leaf extract was assessed by an MTT assay. The A-549 cells were treated with different concentrations of *Lippia nodiflora* leaf extract (10-120 µg) for 24h. Analysis of changes in the cell morphology is examined by phase contrast microscope. For the nuclear morphological changes analysis, DAPI staining was used in lung cancer cells. The apoptotic nuclei were viewed under a fluorescent microscope.
Results: In our study, *Lippia nodiflora* leaf extract treated cells showed significant reduction of cell viability in lung cancer cells. 50% of the inhibition was observed in a 20 µg/ml concentration, which has been taken as the inhibitory concentration (IC-50) which was used for further analyses. The pro-apoptotic effect of *Lippia nodiflora* leaf extract in A549 cell line was determined by DAPI staining under a fluorescence microscope. *Lippia nodiflora* leaf extract treatment alters the nuclear

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morphology and condensed nuclei in lung cancer cells at 20µg/ml concentration.
Conclusion: Overall, it can be concluded that *Lippia nodiflora* presents anticancer activity at 20µg/ml concentration at 24 hrs againstthe lung cancer cell line. However, further research is warrented to find out the molecular mechanism of cytotoxicity of effect this plant extract against lung cancer.

Keywords: Lippia nodiflora; lung cancer; cell viability; Cytotoxicity; Apoptosis.

1. INTRODUCTION

Cancer is a disease which causes uncontrolled growth of cells in our body. Cancerous tumours spread into, or invade, nearby tissues and can also travel to distant places in the body to form new tumours [1]. This abnormal cell reproduction does not allow the regulation of the normal cell growth around it and hence shows invasion properties and changes in the surrounding tissues [2]. According to the WHO, 14 million people suffer from cancer and 8 million people die out of cancer. Lung cancer is the leading cancer in the mortality of both men and women in the U.S and worldwide. Lung cancer is mainly caused by smoking and use of tobacco products. There are other factors like air pollution exposures, asbestos and chronic infections which can lead to lung cancer [3]. It can be divided into small cell lung carcinoma and non-small cell lung carcinoma [4]. It mainly arises from the different sites of bronchial tree. Squamous cell lung cancers represent 20-30% of all lung cancers and they tend to arise from the main bronchi and main advance to the carina. Small cell lung cancers are from the hormonal cells of the lung and they are found to be extremely aggressive [5].

According to the severity and treatment to be done, it mainly depends on TNM staging. Lung cancer occurs due to the transformation of normal cells into malignant cells through a series of genetic alterations, leading to invasive cancer by clonal expansion. Adenocarcinoma is the most common lung cancer and it seems to be rapidly increasing [6]. Treatment for lung cancer includes surgery followed by chemotherapy or radiation therapy. Plants and plant products are said to be effective and versatile chemoprotective agents in various types of cancer. Traditional background of Indian medicine depicts the extensive use of herbal plants in preventing cancer. Natural products are always preferred due to higher effectiveness and less side effects. Medicinal plants are thus helpful in treating cancer due to the presence of various components in different parts of the plant. Plants provide a different range of compounds where each has its own properties. Anticancer effects are derived by suppressing cancer stimulating oncogenes, repairing DNA, stimulating the production of tumor suppressor proteins in the cell, increasing the body immunity and inducing antioxidant effects [7]. Herbs are used to reduce the toxicity and thereby it promotes the anticancer effects. Apoptosis is a process of cell death and this mechanism is used in developing the antitumour drugs using the herbal products [8]. *Lippia nodiflora*, also called Phyla nodiflora, belongs to the family Verbenaceae. The antipyretic, antiinflammatory, antioxidant properties of *Lippia nodiflora* are exhibited by the leaves of this plant. This plant in Indian medicine is considered to be a Siddha remedy for alopecia also. It contains a variety of phytochemical constituents such as alkaloids, glycosides, flavonoids, tannins, phenolic compounds, steroids, terpenoids, carbohydrates, proteins, amino acids, gums and mucilage [9]. Flavonoids are the major component present in this plant. These are considered as the secondary metabolites found in the herbal plants which are said to treat chronic diseases [10]. Flavonoids can be divided into flavones, flavonols, isoflavones, flavanones, flavanols and anthocyanidins.This plant has its own medicinal properties as it could be used for treatment of anorexia, colic, diarrhea, ulcer, asthma, bronchitis, knee joint pain, gonorrhea, hepatis, fever, etc. In Taiwan, it is used as a herbal drink, antiinflammatory agent and immunomodulator inorder to protect against various diseases [11].

Cytotoxicity of this plant is exhibited by the Halleridone and Hallerson which had been isolated from dichloromethane and methanol extract of the leaves of this plant [12]. This extract was found to reduce the tumour cell volume, cell content and packed cell volume. The apoptotic effect was noticed by the methanol and ethyl acetate extracts of leaves and stem of *Lippia nodiflora* [13]. The tender stalks and leaves are found to be bitter and are used to give the children suffering from indigestion and also to pregnant women after delivery. A study revealed

that 90-120 µg/ml of extract concentration was capable enough to inhibit cancer cell growth. Previous studies revealed that some of *Lippia nodiflora* leaf extracts caused the formation of DNA laddering in MCF-7 cells (breast cancer cells). Thus, the aim of this study is to evaluate the anticancer effect of the *Lippia nodiflora* leaf extract on lung cancer cell line.

2. MATERIALS AND METHODS

2.1 Reagents

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

2.2 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 37◦C in a humidified atmosphere containing 5% CO_2. Upon reaching confluency, the cells were trypsinized and passaged.

2.3 Preparation of the Herbal Extract

Lippia nodiflora leaf extract obtained from IMPCOPS (Chennai, India) was used for the present study. About 50g of *Lippia nodiflora* powder was soaked in 500 mL of 95% ethanol and kept at room temperature for 3 days in a static condition. Then the solution was filtered with crude filter paper followed by whatman paper. Fine filtrate was subjected to rota evaporation after that 3g of the material was obtained. The total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4˚C [14].

2.4 Cell viability (MTT) Assay

The cell viability of *Lippia nodiflora* extract treated A549 cells was assessed by MTT assay [15]. 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 48 well plates at a concentration of $2x10^4$ cells/well 24 hours after plating, cells were washed twice with 500µl 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 *Lippia nodiflora* of different concentrations (10-120 µg) for 24 hours. At the end of treatment, the medium from control and *Lippia nodiflora* treated cells were discarded and 200µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37ºC in the CO_2 incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS. The crystals were then dissolved by adding 200µl of solubilization solution and this was mixed properly by pipetting up and down. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (200µl) 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 the percentage of control cells cultured in serum-free medium. Cell viability in the 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.

2.5 Morphology Study

Based on MTT assay we selected the optimal doses (IC-50: 20µg/ml) for further studies. Analysis of cell morphology changes by a phase contrast microscope. $3×10^4$ cells were seeded in 6 well plates and treated with *Lippia nodiflora* extract (20µg/ml) 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.

2.6 Determination of Nuclear Morphological Changes of Cells (DAPI Staining)

For the nuclear morphological analysis, the monolayer of cells was washed with PBS and fixed with 3% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and incubated with 0.5µg/ml of DAPI for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei, and

condensed chromatin) were viewed under a fluorescent microscope [16].

2.7 Statistical Analysis

Statistical analyses were performed using one-way ANOVA followed by Student–Newman–Keuls (SNK) tests for comparison between treatment values and control values. Data were expressed as mean ± SEM. The level of statistical significance was set at $p<0.05$.

3. RESULTS

3.1 Effect of *Lippia nodiflora* Leaf Extract on Cell Viability of Lung Cancer Cell Line

The cytotoxic potential of *Lippia nodiflora* leaf extract in lung cancer cells was assessed by MTT assay. The cells were treated with different concentrations (10-120 µg/ml) of *Lippia nodiflora* leaf extract for 24h. *Lippia nodiflora* leaf extract treatment significantly decreased the viability of A-549 cancer cells compared to control at 24 h time point (Fig. 1). The percentage of cell viability reduced gradually with increase in the concentration. We observed the 50% growth inhibition at (20 µg/ml) concentration. Hence, IC-50 dose (20 µg/ml) was considered for the further experiments.

3.2 The Effect of *Lippia nodiflora* on Cell Morphology

The cell morphological analysis of *Lippia nodiflora* leaf extract treated lung cancer cells was observed in an inverted phase contrast microscope. The A-549 cells were treated with *Lippia nodiflora* leaf extract (20 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 *Lippia nodiflora* leaf extract treated cells (Figs. 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.

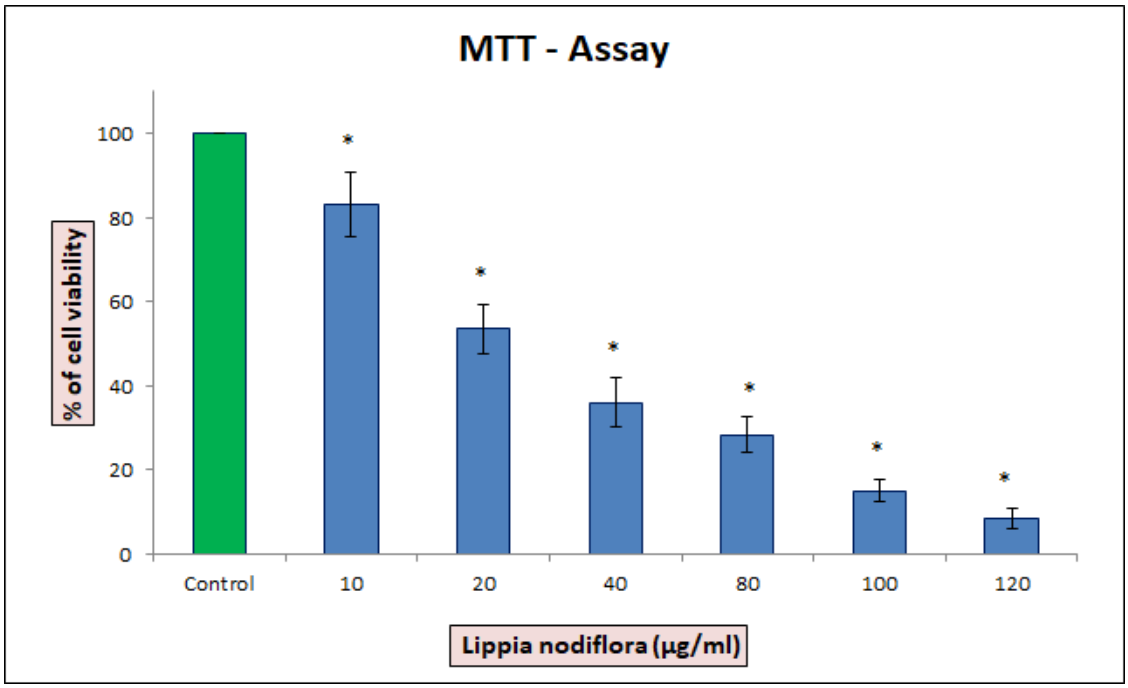

Fig. 1. The cytotoxic potential of Lippia nodiflora leaf extract on A549 cell line was determined by MTT assay. The cells were treated with different concentrations (10, 20, 40, 80, 100 and 120 µg/ml) of Lippia nodiflora leaf extract for 24 hrs. The 50% of inhibition observed in a concentration of 20 µg/ml, (p-value: 0.0108) which has been taken as Inhibitory concentration (IC-50) dose value and fixed for further experiments. * represents statistical significance between control versus treatment groups at $p<0.05$ level using Student's-Newman-Keul's test

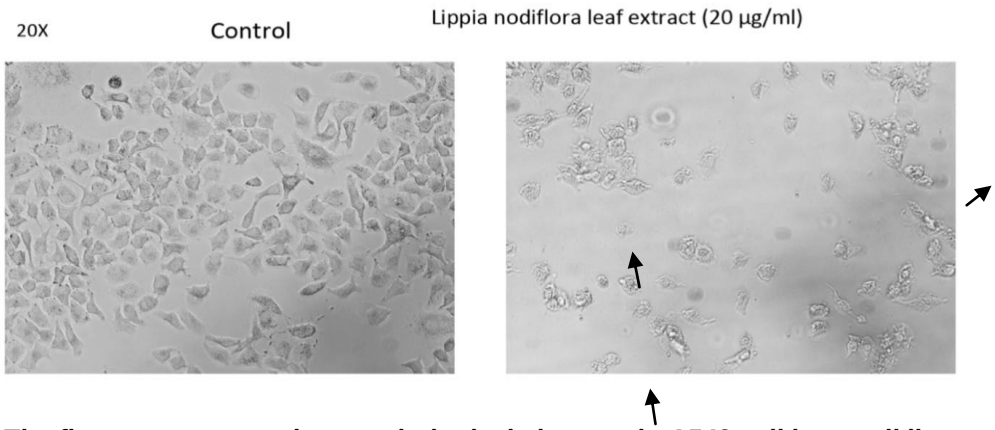

Fig. 2. The figure represents the morphological changes in A549 cell lung cell line upon, without and with the treatment of Lippia nodiflora leaf extract at 20 µg/ml for 24 hrs by phase contrast microscope at 20x magnification. The number of cells decreased after the treatment and the arrow indicates the cells exhibited shrinkage and cytoplasmic membrane blebbing

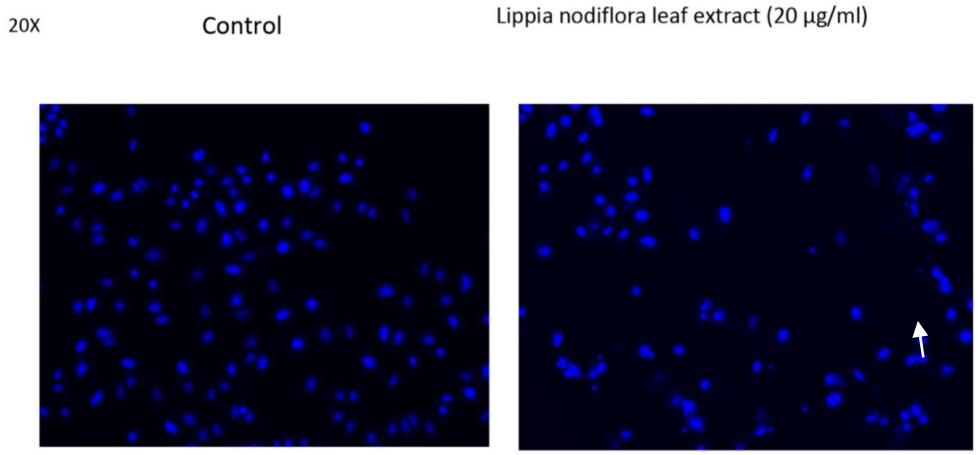

Fig. 3. This figure represents the DAPI staining of A549 lung cancer cell line upon, without and with treatment of Lippia nodiflora leaf extract at 20 µg/ml for 24 hrs, viewed under a fluorescence microscope at 20x magnification. The viable cells possess a uniform blue colour nucleus, and arrow indicates the treated cells possessing condensed chromatin and nuclear fragmentation

3.3 Pro-apoptotic Effect of *Lippia nodiflora* Leaf Extract in Lung Cancer Cells (DAPI Staining)

The induction of apoptosis in *Lippia nodiflora* leaf extract (20 µg/ml) treated cells was analyzed by DAPI staining. After a 24h treatment period, the cells were stained with nuclear staining (DAPI) 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).

4. DISCUSSION

Medicinal plants are found to be the source of healing around thousands of years. It does not provide any toxic effects when compared to the allopathy medicines. Patients can access herbal medicines easily and there is an increase in the demand for these medicines [17]. *Lippia nodiflora* is an important member of the family, verbenaceae that could be used to treat a wide range of diseases. It is considered to be a herbal product as well as traditional medicine. In the present study, we have elucidated the anticancer effect of *Lippia nodiflora* leaf extract. The results

show that *Lippia nodiflora* leaf extract inhibits the growth of the A549 lung cell line by inducing apoptosis and perturbing cell survival. IC-50 dose was obtained in 20 µg/ml concentration. It has the potential to exhibit chemoprevention and chemotherapeutic purposes. Apoptosis is a physiological process that functions as an essential mechanism of tissue hemostasis and is regarded as a way to eliminate the unwanted cells [18]. Deregulation expression of MYC genes could be noticed in lung cancer. MYC is said to be a powerful inducer of apoptosis, especially under immense stress or depleted factors [19]. It could be seen that sensitizing cells triggers apoptosis including DNA damage, nutrient deprivation, interferon, hypoxia etc. it plays an important role in eliminating the mutated cells. Apoptotic cell death is characterised by the activation of caspases, which belongs to the family of cysteine proteases with specificity for aspartic acid residues. In the case of SCLC cell lines had a homogenous depletion at 2q33 encompassing the chromosomal location of CASP8 gene [20]. All the lung cancer cell lines expressed CASP10, CASP3. The MYC family is found to be amplified often as it is a potent inducer. Several mitochondrial proteins were found to activate apoptosis directly [21]. A similar study with the breast cell line of this plant revealed that there is an alteration of genes like AIFMI, BIRC3, CFLAR, CASP9 and CYCS in which AIFMI, a pro apoptotic factor causes nuclear disassembly in apoptotic cells [22]. Another study revealed that *Lippia nodiflora* produced apoptosis in NCI-H460 cells with the IC-50 seen in less than 30 µg/ml in the preliminary assay. Many herbal products and phytochemicals have been reported for their cytoprotective effect which was seen through the MTT assay. Based on the results obtained on the morphological changes (Fig. 2), cell death was initiated by the mitochondria as the loss of mitochondrial membrane could be seen in these cells when treated with the leaf extract of *Lippia nodiflora* [23]. The main morphological changes including the chromatin condensation, nuclear remodelling and membrane blebbing are mainly due to the role of caspase substrate cleavage during apoptosis. Morphological changes were seen 24 hrs with the extract being subjected to the A549 cell line. The most important feature of these morphological changes is the apoptotic changes in A549 lung cell line. Production of apoptosis is a sign for anticancer development [24]. To know more about the anticancer effect of the plant, DAPI staining, fluorescent DNA binding agent was done to observe cell death and cellular morphological changes involved in apoptosis. It shows fragmented apoptotic bodies, shrunken and marginated nuclei in contrast to the normal cells and a large nucleus in the untreated cells, showing the apoptotic potential. The present study shows the decrease in the mitochondrial membrane strength from the fluorescent activity when A549 cells with IC-50 were subjected. DNA laddering is one of the hallmark indications that helps in giving a clear difference between apoptosis and necrosis [25]. This DNA laddering is initiated by the caspases and DNAases. Another study revealed the anti tumour activity using Ehrlich's ascites carcinoma bearing swiss albino mice and hence could be seen that it contained a considerable amount of anti tumour activity which is due to the increase in the antioxidant activity. Similar study but with the different plant extract of L.acutangula depicted good antiproliferative activity on human lung cell line A549. The reason could be due to the presence of the methanolic extract fraction of the fruit of that plant which shows high action of cytotoxicity, antiproliferative and apoptotic effects in the HL-60. Flavonoids are found to play the most important role in the *Lippia nodiflora* leaf extract of the plant and that causes the apoptosis [26-31]. Hence, further research should be carried out in order to confirm this compound's effect. Thus, as a result, *Lippia nodiflora* leaf extract will be a new hope for developing anti-cancer drug for treating lung cancer as it does not produce any side effects.

5. CONCLUSION

Overall, the present study results demonstrated that the plant extracts of *Lippia nodiflora* were cytotoxic and induced apoptosis to the lung cancer cells at 20µg/ml concentration and 24h incubation period. However more research is needed to understand the mechanisms of anti-cancer potential of this *Lippia nodiflora*.

NOTE

The study highlights the efficacy of "traditional medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely

no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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