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Cytotoxic Effect of *Lippia nodiflora* Leaf Extract against the Prostate Cancer Cell Line

R. Sharmila [a], P. Elumalai [b≡*], S. Raghunandhakumar [b≡], T. Lakshmi [b≡] and Anitha Roy [b≡]

[a] Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Chennai, Tamil Nadu, 600 077, India.
[b] Department of Pharmacology, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences, Chennai, Tamil Nadu, 600 077, India.

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

Prostate cancer is the second most common cause of cancer deaths for men. *Lippia. Nodiflora (L. nodiflora)* has been used as a natural remedy for various diseases, because of its antioxidant, anti-inflammatory, anti-bacterial, and anti-tumor effect. This study was to investigate the cytotoxic effect of *L. nodiflora* ethanolic leaf extract in prostate cancer cell lines (PC-3). The growth inhibitory effect of *L. nodiflora* ethanolic leaf extract was assessed by MTT assay. The cell morphological changes in *L. nodiflora* leaf extract-treated cells were observed using an inverted phase-contrast microscope. Apoptosis induction by *L. nodiflora* was determined by AO/EtBr (acridine orange and ethidium bromide) dual staining. MTT test results showed dose-dependent cell growth inhibition in PC-3 cells treated with *L. nodiflora* leaf extract (10-120 µg/mL). The IC-50 dose was observed at 40µg/ml. Morphological changes such as reduction in the number of cells, cell shrinkage, and cytoplasmic membrane blebbing were observed in the treated cells. Induction of apoptosis by *L. nodiflora* (40µg/ml) treated cells showed an increased number of early apoptotic and late apoptotic cells. The above data indicate that *L. nodiflora* inhibits cell proliferation and induces apoptosis in prostate cancer cells. Therefore, it can be concluded that *L. nodiflora* exhibits anti-cancer activity, and thus it raises new hope for its use in anti-cancer therapy.

[≡] *Dr.;*
**Corresponding author: E-mail: elumalaip.sdc@saveetha.com;*

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1. INTRODUCTION

Cancer has become one of the major causes of the death of people worldwide [1]. There are various types of cancer occurring all over the body such as Oral cancer, breast cancer, colon cancer, prostate cancer, etc. Oral cancer has also become the leading cause of morbidity and mortality [2,3]. Prostate cancer is the second most common malignancy in men that leads to death. The increase in the incidence of cancer in various countries is due to the widespread adoption of the Western diet and lifestyle [4]. In recent years cancer research has made remarkable improvements in our basic understanding and technique of cancer studies and cancer biology. Conventional anti-cancer therapies with standard chemotherapeutic agents have various adverse effects [5]. It includes nausea, vomiting, metallic taste, hair loss, etc [6]. Nanotechnology has played an important role in cancer treatment [7,8]. Current studies indicate the important role of apoptosis in the improvement of therapeutic agents and treatment of cancer. The primary aim of cancer remedy is to kill the cancer cells without causing too much harm to normal cells [1,9]. The knowledge about the mechanisms of apoptosis has made us understand the origination and progression of cancers. Various studies have been done to understand the cytotoxic and apoptotic effect of different leaf and seed extract and their use in anti-cancer therapy [10,11]. It has also made us understand that cancer remedies can work in two ways, via induction of apoptosis and direct toxicity to cancer cells [12]. Therefore in the improvement of therapeutic agents and treatment of cancer, regulators of cell death and cell cycle should be targeted [13]. Plants and plant derivatives have been shown to be powerful and flexible chemoprotective agents in many cancers. The broad usage of herbal plants in cancer prevention is depicted in the traditional background of Indian medicine. Plants produce a diverse spectrum of chemicals, each with its unique set of qualities. Suppressing cancer-promoting enzymes, mending DNA, boosting the creation of antitumor enzymes in the cell, enhancing body immunity, and producing antioxidant effects are all ways to combat cancer. Herbs are utilised to minimise toxicity and boost anticancer effects [14]. The present study was designed to broaden a novel therapeutic agent from natural resources to exploit our knowledge of apoptotic mechanisms to promote apoptosis of most cancer cells and limit the concurrent dying of normal cells. The medicinal flowers which are used in conventional medication in growing countries include an extensive range of phytochemicals that may be used to deal with cancer [14]. Jalpapli, which is also known as *Phyla nodiflora*, is a small herb that is a native of California and it also grows widely in India [15]. The plant contains triterpenoids, flavonoids, phenols, steroids, etc. It has various pharmacological values [16]. It is used for lack of bowel movement, in swollen cervical glands, and also used for pain in knee joints, in ulcers, and boils [17]. It has been used in treating various skin diseases and as a cosmetic agent [18]. It is also used in gonorrhea and hepatitis. It also has antibacterial effects against various bacteria such as *E.coli, Pseudomonas, Staphylococcus*. The sensitive stalks and leaves are bitter and are used to treat dyspepsia in youngsters as well as pregnant women after birth. A research found that extract concentrations of 90-120 µg/ml were effective at inhibiting cancer cell proliferation. Previous research found that certain Lippia nodiflora leaf extracts produced DNA laddering in breast cancer cells. Researchers discovered that bioactive natural products have potential physiological actions owing to polyphenols, which function as cytoprotective, anti-inflammatory, and hepatoprotective agents, making them a significant source of health care [19]. It has been the source of medicine for liver disorders, dandruff control, indigestion in children, an anticonvulsant effect, etc. Furthermore, studies reveal that *L. nodiflora* has antimicrobial, antifungal, antitumor, antidiuretic, anti-inflammatory, antiurolithiatic antidiabetic, and neuropharmacological activity [19]. Our team has extensive knowledge and research experience that has translated into high quality publications [20]. This study was done to investigate the cytotoxic effect of *L. nodiflora* leaf extract in the prostate cancer 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. Acridine orange (AO), ethidium bromide (EtBr), Dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), 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

Prostate cancer cell lines (PC-3) were obtained from the National Centre for Cell Science (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% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

2.3 Preparation of the Herbal Extract

L. nodiflora leaf powder obtained from The Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd (IMPCOPS) (Chennai, India) was used for the present study. About 50g of *L. 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. The fine filtrate of total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4˚C.

2.4 Cell Viability (MTT) Assay

The cell viability of *L. nodiflora* extract-treated PC-3 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. PC-3 cells were plated in 48 well plates at a concentration of 2x104 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 *L. nodiflora* at different concentrations (10-120µg/ml) for 24 hours. At the end of treatment, the medium from control and *L. 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 CO2 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 a percentage of control cells cultured in a 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 the MTT assay we selected the optimal doses (IC-50: 40µg/ml) for further studies. Analysis of cell morphology changes by a phase-contrast microscope. 3×104 cells were seeded in 6 well plates and treated with *L. nodiflora* (40µg/ml for PC-3 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.

2.6 Determination of Mode of Cell Death by Acridine Orange (AO)/Ethidium Bromide (EtBr) Dual Staining

The effects of *L. nodiflora* in PC-3 cell death were also determined by AO/EtBr dual staining as described previously (Cury-Boaventura et al., 2004). The cells were treated with *L. nodiflora* 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.

2.7 Statistical Analysis

Statistical analyses were performed using one-way ANOVA followed by Student–Newman–Keul's (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 *L. nodiflora* Extract on Cell Viability of Prostate Cancer Cell Line

MTT assay was used to assess the cytotoxic potential of *L.nodiflora* extract in the prostate cancer cell line. The cells were treated with different concentrations (10, 20, 40, 80, 100, and

120µg/ml) *of L.nodiflora* for 24 hours. With a gradual increase in concentration, the percentage of cell viability was reduced. 50% growth inhibition was observed at 40 µg/ml concentration and hence IC-50 dose was considered for further experiments.

3.2 The Effect of *L. nodiflora* on Cell Morphology

The cell morphological analysis of *L.nodiflora* extract-treated under prostate cancer was observed in a phase-contrast microscope. The PC-3 cells were treated with *L.nodiflora* extract (40 µg/ml) for 24h and on comparison with untreated cells, treated cells showed significant morphological changes. There was a decrease in the number of cells and cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

3.3 Pro-apoptotic Effect of *L.nodiflora* Extract in Prostate Cancer Cells (AO/EtBr Dual Staining)

To confirm the induction of apoptosis in *L.nodiflora* extract AO\EtBr dual staining was used. For evaluating the nuclear morphology of apoptotic cells AO\EtBr dual staining was used. The cells were treated with *L.nodiflora* extract (40 µg/ml) for 24h. The cells were stained with both AO/EtBr staining after treatment and it was observed under fluorescence microscopy. The results say that AO stained both live and dead cells and EtBr stains only the cells that have lost their membrane integrity. Cells stained uniform bright green nucleus represent viable cells, early apoptotic cells having bright orange areas of condensed or fragmented chromatin in the nucleus and late apoptotic cells having a uniform bright red nucleus. In the present study, control cells showed a uniform green color, and in *L.nodiflora* extract-treated cells showed orange and red color. From the above result, it can be confirmed that *L.nodiflora* extract induces apoptosis in prostate cancer cells.

4. DISCUSSION

Apoptosis is a programmed cell death that serves as an important mechanism for tissue homeostasis and cell eradication. The use of medicine-derived bioactive compounds as a replacement to anti-cancer and chemotherapeutic medicines is becoming increasingly popular. Plant-derived products are being tested as apoptotic inducers in anticancer research [21]. Several cancer treatments based on plant-derived bioactive compounds, such as flavonoids and limnoids have recently been proposed. By inhibiting metabolic activation, boosting detoxification, or offering alternate targets for electrophonic metabolites, these substances aid in the prevention of carcinogenesis [13,20].

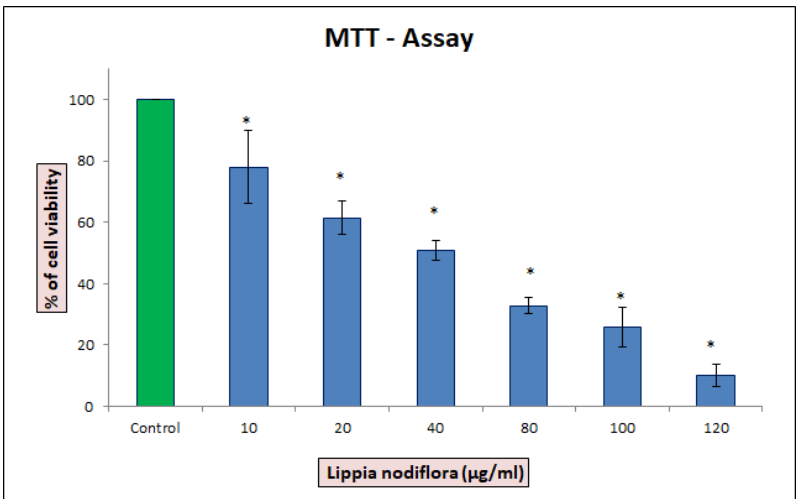
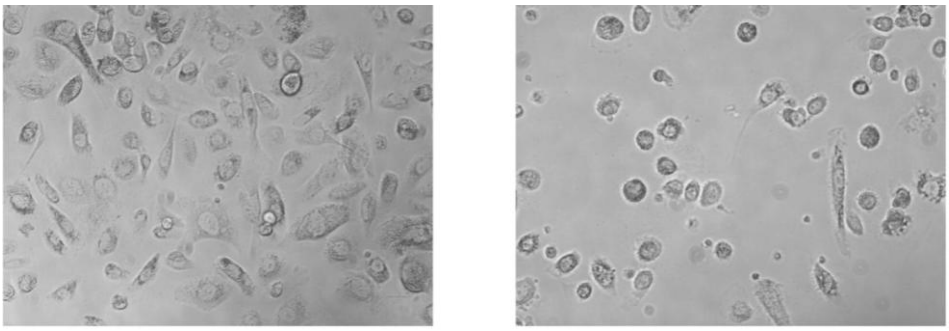
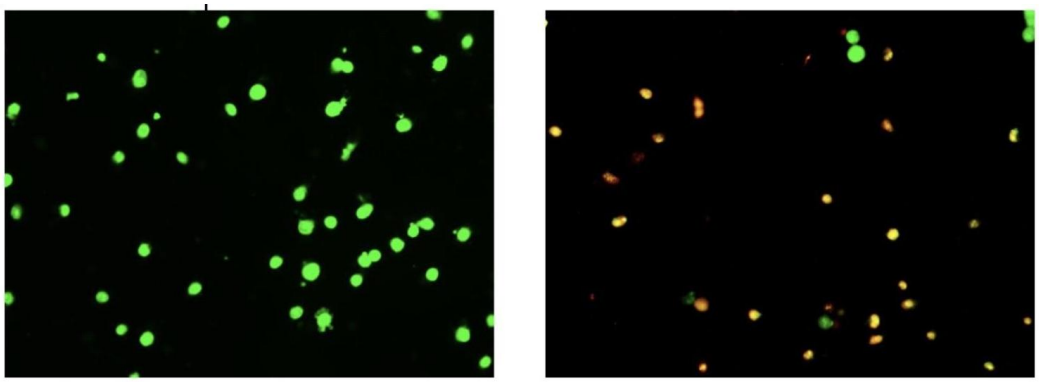


Fig. 1. Cytotoxic effect of L. nodiflora leaf extract against the prostate cancer cell line. The cells were treated with different concentrations (10, 20, 40, 80, 100, and 120µg/ml) L. nodiflora leaf extract for 24hrs. The 50% of inhibition observed at 40 µg/ml (p-value: 0.0037) concentration, which 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–Keuls test



(A) (B)

Fig. 2. Effect of L. nodiflora leaf extract on cell morphological changes in the prostate cancer cell line. After treatment with L. nodiflora leaf extract 40 µg/mL for 24hrs and observed under phase-contrast microscope at 20x magnification. (A) Control cells, (B) lippia nodiflora leaf extract (40µg/ml). Here, the number of cells was decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing



(A) (B)

Fig. 3. Induction of apoptosis in L. nodiflora leaf extract treated prostate cancer cell line. The PC-3 cells treated with L. nodiflora leaf extract at 40 µg/mL concentration for 24hrs, treatment cells were stained with and viewed under fluorescence microscope at 20x magnification. (A) Control cells, (B) lippia nodiflora leaf extract (40µg/ml). The viable cells possess a uniform bright green nucleus, early apoptotic cells having bright orange areas of condensed or fragmented chromatin in the nucleus and late apoptotic cells having a uniform bright red nucleus

The cytotoxic effect of *L.nodiflora* extract on cell lines was calculated by micro-culture tetrazolium assay (MTT). The multiple concentration of *L.nodiflora* extract was used and IC50 doses were calculated. The MTT assay results showed that dose-dependent (10-120µg/ml) cell growth inhibition was observed in *L. nodiflora* leaf extract treated against PC-3 cells. The IC-50 dose was observed at 40µg/ml. Since *L.nodiflora* has better cytotoxic activity against PC-3 cells it was used for further investigation.

Morphological investigation of apoptosis revealed that *L.nodiflora* extract at a concentration of 20µg/ml induced cell death in the PC-3 cell line by apoptosis (Fig. 2). Phenotypically apoptosis is characterized by cell shrinkage, DNA fragmentation, chromatin condensation, plasma membrane blebbing, and collapse of the cell into small membranes. The phase-contrast image shows that *L.nodiflora* extract has shown morphological changes such as reduction in the number of cells, cell shrinkage, and cytoplasmic membrane blebbing in the treated cells when compared with untreated cells. Isolated compound-like flavonoids exhibited exceptional antioxidant activity in all antioxidant tests and significantly reduced lipid peroxidation at a concentration of 50 µg/ml, according to prior research. The findings showed that extracts or

phytocompounds derived from *L. nodiflora* might be utilised as a bioactive source of natural antioxidants with health benefits.

AO\EtBr (acridine orange and ethidium bromide) dual staining was performed to determine if exposure to *L. nodiflora* leaf extract causes cell death by apoptosis in PC-3 cell lines. In the AO/EtBr staining, the viable cells will possess a uniform bright green nucleus. The early apoptotic cell will have bright orange areas of condensed or fragmented chromatin in the nucleus. Late apoptotic cells will have a uniform bright red nucleus. It was found that untreated cells were mostly green with an intact nucleus (Fig. 3). AO\EtBr analysis showed that *L. nodiflora* leaf extract was cytotoxic towards PC-3 cells through apoptosis when treated with IC50 concentration (40 µg/mL). In the AO\EtBr analysis, cells treated with IC50 value of *L. nodiflora* leaf extract showed a red color nucleus, which further confirmed the induction of late apoptosis in PC-3 cells by *L.nodiflora* extract. This finding was characterized by membrane blebbing and nuclear shrinkage. The above data indicate that *L.nodiflora* inhibits cell proliferation and induces apoptosis in prostate cancer cells.

5. CONCLUSION

In summary, the present results indicate that *L. nodiflora* leaf extract induced apoptosis which was further confirmed by characteristic morphological changes and cytotoxic effect. Therefore, *L. nodiflora* leaf extract leaf extracts exhibit anti-cancer activity and can be used for developing anti-cancer agents [11,22-34]. However, further investigations and studies are required to employ them as anticancer drugs for prostate cancer.

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’s not applicable.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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