RESEARCH ARTICLE

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Caspase-Dependent Apoptosis Induced by Simarouba Glauca on Human Non-Small-Cell Lung Cancer, A549 Cells

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Abstract

Background: The leaves of *Simarouba glauca* (*S. glauca*) have been used as a potential source of anticancer agents in traditional medicine. Attempts have been made to isolate anticancer agents from the leaves of *S. glauca*. The objective of the present study was to demonstrate the anticancer and apoptotic effect of the leaf extract of petroleum ether (LPE) on human non-small-cell lung cancer A549 cells. **Methods:** MTT assay was used to investigate the effect of LPE on the viability of A-549 cells. The apoptotic effect of human lung cancer cells was evaluated using fluorescence staining, acridine orange/ ethidium bromide staining, Hoechst staining, flow cytometry analysis, annexin V staining, and caspase assay. **Results:** The results showed a direct correlation between the dose and the rate of cytotoxicity. Fluorescence staining revealed apoptotic features, such as blebbing and chromatin condensation. Flow cytometry analysis and annexin V staining revealed phosphatidyl serine externalization. Caspase assay confirmed that the extract inhibited cell death. Caspase 3 expressions indicated that the cell death occurred either through the mitochondrial pathway or the death receptor. **Conclusions:** The study revealed that the LPE induced the apoptosis of human non-small-cell lung cancer, A549 cells, either through mitochondrial or death receptor pathway.

Keywords: *Simarouba glauca*- apoptosis- Non-small cell lung cancer- cytotoxicity- caspase 3

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Introduction

Medicinal plants are studied for pharmacological and biological properties in recent years. To understand their mechanism of action, the researchers have done molecular and phytochemical analysis. Medicinal plants have been found to be very effective against experimental as well as clinical cases of tumors. Undesired side effects sometimes occur during chemotherapy. Plant-derived products may reduce adverse side effects associated with cancer treatments (Jose et al., 2018; Jose et al., 2020). This type of experimental research has led to the commercialization of a number of important plant-based drugs used in chemotherapy. Currently, a lot of plant products are being used to treat cancer. Scientists all over the world are concentrating on medicinal plants to boost the immune cells of the body against cancers. They are also a significant source of synthetic and herbal drugs. So far, pharmaceutical companies have screened more than 25,000 plants to discover anticancer drugs. The herbal system of medicine has been practiced for thousands of years (Sakarkar and Deshmukh, 2011). Drug discovery and development from plant-based compounds are more cost-effective than conventional synthetic compounds (Rayan et al., 2017).

Medicinal plant extracts have a large diversity of chemical structures, which might prove to be suitable for specific medicinal applications (Vikas et al., 2019). There are only limited suggestions about how plants tightly regulate their cell cycle machineries endogenously even after enormous exposure to hazardous components. Plant-derived compounds, such as vinblastine, topotecan, and other compounds have been used as anticancer drugs (Brahmer and Ettinger, 1998; Qu et al., 2015; Carrato et al., 2020).

Simarouba glauca (*S. glauca*) has been used for medicinal purposes in several counties. *S. glauca* is an evergreen edible oil tree, which is commonly known as 'Lakshmi taru' or 'paradise tree' belonging to the family Simaroubaceae. *S. glauca* is one of the important herbal drugs used against various diseases (Jibi et al., 2016; Yeo et al., 2016; Osagie-Eweka et al., 2021). The leaf extract of Simarouba is known for its pharmacological properties, such as haemostatic, antihelmenthic, antiparasitic, antidysentric, antipyretic, and anticancerous (Rivero-Cruz et al., 2005; Mathew et al., 2016; Balu et al., 2020). Several

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quassinoids from *S. glauca* have exhibited cytotoxic effects against human oral epidermoid carcinoma (KB) cells, including glaucarubin, glaucarubol, glaucarubinone, and glaucarubolone (Kupchan et al., 1976; Polonsky et al., 1978). In Brazil, Simaroubaceae is represented by the genera *Picrolemma* and *Quassia* within the Amazon, *Picrasma*, and *Castela* to the South, and *Simaba, Simarouba*, and *Picrolema*, which can be seen throughout the country (Almeida et al., 2007; Alves et al., 2014).T wo hundred quassinoids have been isolated and identified (Vieira and Braz-Filho, 2006). The chloroform extract of *S. glauca* exhibited significant cytotoxicity against several human cancer cell lines (Likhitwitayawuid et al., 1993; Seo et al., 2001; Jose et al., 2018; Prajapati et al., 2018). The objective of this study was to demonstrate the anticancer and apoptotic effect of the leaf extract of petroleum ether (LPE) on human non-small-cell lung cancer A549 cells.

Materials and Methods

Plant

The leaves of *S. glauca* were collected from the Trivandrum district and authenticated with voucher specimen 95212 and deposited at JNTBGRI herbarium. The plant leaves were then shade dried and powdered. This powder was used for sequential solvent extraction using petroleum ether (LPE).

Cell lines and culture conditions

A-549 cells of human non-small-cell lung cancer cells were obtained from National Centre for Cell Sciences (NCCS) Pune, grown in DMEM media and supplemented with 10 % FBS, HEPES buffer, penicillin (100 units/ml), and streptomycin (100 µg/ ml). Cells were maintained at 370 C containing 5% CO2.

Cytotoxicity assay using MTT assay

MTT (3-(4,5 Dimethylthiazol-2yl)-2,5- Diphenyl Tetrazolium Bromide) assay relies on the ability of a mitochondrial dehydrogenase enzyme from healthy cells to cleave the tetrazolium rings of the pale yellow MTT and form impermeable, dark blue formazan crystals, which accumulate within healthy cells (Mossman et al.,1983). A detergent was added causing solubilization of the cells, and consequently the liberation of the crystals, which were solubilized. The color was quantified using a multi-well plate reader. The in vitro response was studied using an MTT assay. The lung cancer cell line was maintained in a DMEM medium with 10% FCS. Briefly, cells were harvested, counted, and seeded (5×10^3 cells/well in 100 µl) in 96 well titre plates (Axygen), and PBS was added to the outer wells (200µl/well). After 24 hours of incubation at 37°c and 5% CO2 to achieve cell attachment, media were removed and cultures were treated with varying concentrations of drugs (12.5-800 µg/ ml) diluted with the medium. The negative controls were also kept (i.e. cells and media). The plates were further incubated for 24 and 48 hours. Following the completion of the incubation, media were removed without disturbing the cells. Then, 100µl of 5mg/ml stock solution of MTT was added to each well, and plates were further incubated for 2 hours in dark at 37ºC and in a CO_2 incubator. Next, 100 µl of lysis buffer was added to each well. The plates were then incubated for 4 hours in dark and in a CO_2 incubator. The absorbance was recorded using multiplate reader. Three replicates were set up for each concentration. The concentration required to reduce absorbance by 50% lC_{50} in comparison to control cells was determined. The IC_{50} values were derived by substituting the percentage inhibition values of drugs for their respective curves.

$$\% \text{ of Growth Inhibition} = \frac{100 - \text{absorbance of the drug-treated cells X } 100}{\text{The absorbance of untreated control cells}}$$

Morphological determination of the apoptotic cells

Analysis of cell death (apoptosis) using dual acridine orange/ethidium bromide fluorescent staining, visualized under a fluorescent microscope: For the assessment of apoptosis after treatment of A-549 cells for 48 hours, dual acridine orange/ethidium bromide fluorescent staining of unfixed A-549 cells was used. The mixture of fluorescent dyes consisted of acridine orange at 5µg/ml in PBS. This stain makes visible the DNA-condensed chromatin of apoptotic cells. Slides were observed under a fluorescent microscope. Acridine orange was observed using standard narrow band FITC excitation (excitation wavelength 450-490nm and barrier filter 520-560nm). Ethidium bromide only stains cells in the late stages of apoptosis and secondary necrosis when membrane integrity has been lost. Early apoptotic cells are impermeable to the dye. The early stages of apoptosis are readily detectable using acridine orange (Haque et al., 1997). Cells were cultured in test tubes 1x 10^6. A-549 cells were incubated in DMEM medium with 10% FCS, and incubated with LPE in a CO2 incubator at 37°C for 48 hours (Kirsch-Volders et al., 1997). For the assessment of apoptosis 48 hours after exposure to different concentrations of leaf extracts, dual acridine orange/ethidium bromide staining of unfixed A-549 was used. These dyes stained the DNA, allowing the chromatin of apoptotic cells to be observed. The medium was removed, and the gel was gently pelleted. Then, 1µl of acridine orange and 100g of ethidium bromide in 1ml waswere added to cells and immediately washed once with buffer saline (PBS) and re-suspended in 10ml of 10% glycerol in PBS. The slides were analyzed by fluorescent microscopy. The number of cells manifesting morphologic features of apoptosis, such as chromatin condensation, and the loss of nuclear envelope (Kerr et al., 1994) were counted as the function of total number of cells presenting in the field.

Nuclear counter stain for fluorescence microscopy

Hoechst 33342 nucleic acid stain is cell-permeant nuclear counterstain that emits blue fluorescence. One advantage of Hoechst 33342 is that it is membrane-permeant and stains live cells. Hoechst 33342 binds to adenine-thymine-rich regions of DNA in the minor groove. On binding to DNA, the fluorescence greatly increases. Cells were cultured in test tubes. 1x106 cells were incubated in DMEM medium with 10% FCS containing LPE in a CO_2 incubator at 37ºC for 48 hours. For the assessment of apoptosis, 48 hours

after exposure to different concentrations of LPE, cells were stained with 5 µM Hoechst 33342. These dyes stain the DNA and allowed the visualization of condensed chromatin of apoptotic cells.

Detection of apoptotic cells by annexin V-FITC and PI staining

The cells were centrifuged with cold PBS at 1500-2000 rpm for 10 minutes. Then, 100µl binding buffer (1X) was added to the pellet. Next, 5µl of annexin V FITC and 5µL of propidium iodide was added. The cells were gently vortexed and kept for incubation at room temperature in the dark for 15 minutes. About 400µl of 1X binding buffer was added to each tube. Then, the tubes were analyzed by flow cytometry.

Caspase 3 expression

Caspase-3 is a key protease that is activated during apoptosis. The cells were treated with 10µg/ml LPE. After 48 hours of incubation, the cells were washed twice with cold PBS and prepared for acquisition using FITC conjugated monoclonal active Caspase-3 antibody apoptosis detection kit. The cells were fixed in cytofix solution at a concentration of $1x10^6$ cells/0.5 ml. The cells were then fixed in ice for 30 minutes, resuspended in perm wash buffer containing the antibody, and incubated for 30 minutes at room temperature. Cells were then analyzed by flow cytometry. Finally, 10,000 cells were acquired, and the results were interpreted using Diva software analysis.

Results

MTT assay on A-549 cells

Different concentrations of LPE (12.5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml), 800µg/ml)) were treated on A-549 cell lines and were assayed to check the cytotoxicity at 24 and 48 hours. It was seen that when treated with LPE, the cytotoxicity varied depending on the concentration and time. In A-549 cell line at lower concentration of 12.5µg/ml, the percentage of cytotoxicity at 24 and 48 hours were 19% and 26%. At the concentration of 800µg/ml, the extracts showed maximum activity of 65% and 81%, respectively (Fig.1). The IC50 value of LPE at 24 hours on A-549 cells was 159µg/ml. LPE-induced maximum cytotoxicity in A-549 was 81% at the concentration of 800µg/ml and following 48 hours of incubation

Acridine orange/ ethidium bromide dual staining

Acridine orange/Ethidium bromide dual staining was used for detecting apoptosis of the 10µg/ml and 15 µg/ml LPE treated A549 cells for 48hours. While examining under inverted fluorescent microscope, viable cells were seen as fluorescent green; whereas, the apoptotic cells were seen as fluorescent orange. The findings showed nuclear fragmentation, membrane blebbing, and membrane invagination (Figure 3).

Hoechst staining

In Hoechst staining the control cells, A-549 cells, appeared light blue in color and the apoptotic cells appeared fluorescent blue in color, showing chromatin condensation and DNA fragmentation after 48 hours of incubation of LPE at the concentrations of 10 µg/ml and 15 µg/ml (Figure 4).

Annexin V status

A number of annexin-positive cells were observed after 48 hours of treatment with LPE at concentrations of 10 µg/ml, indicating an apoptosis induction of 67% in A-549 cells (Figure 5).

Caspase 3 expression

After treating with LPE at the concentration of 10 µg/ml, the A-549 cells showed 63% caspase expression at 48 hours, compared to untreated control cells (Figure 6).

Statistical analysis

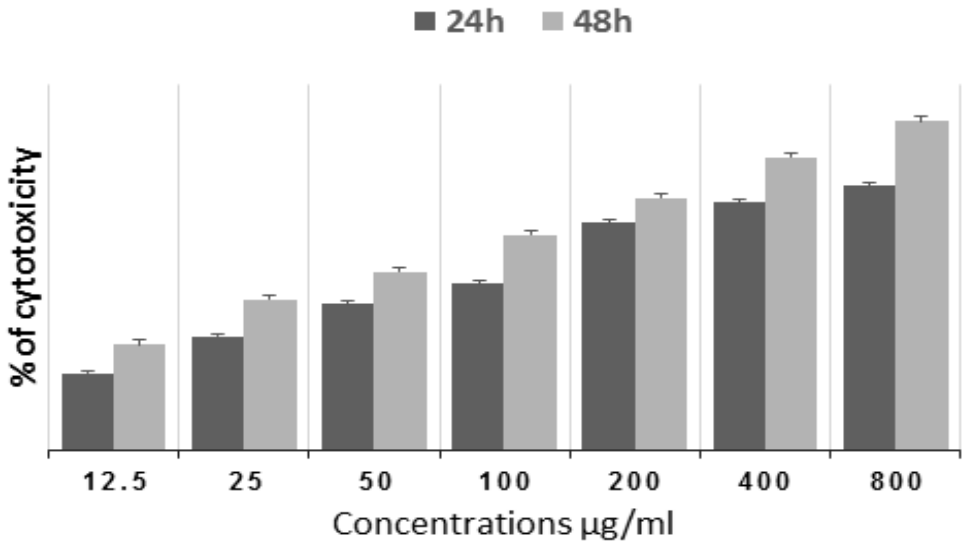

Figure 1. MTT Assay LPE on A-549 Cells. Leaves of Simarouba gluaca petroleum ether extract induced cell death in lung adenocarcinoma cells.

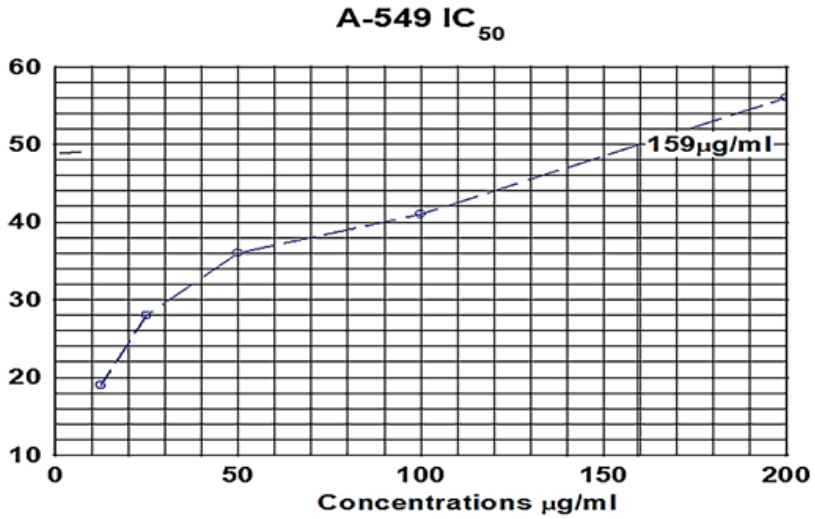

Figure 2. IC_{50} Value of LPE Induced in Lung Cancer Cells after 24 Hours

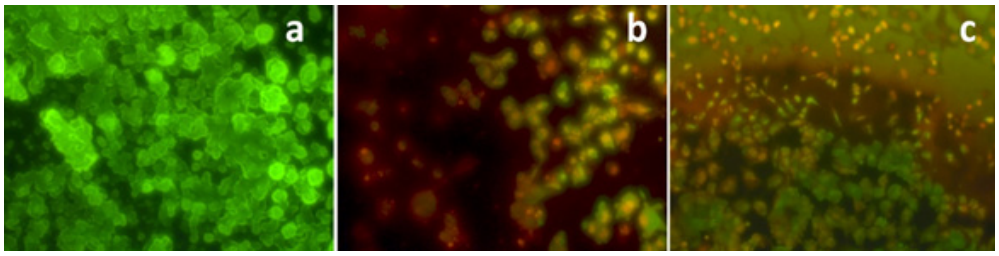

Figure 3. Acridine Orange/Ethidium Bromide Staining of LPE Treated A-549 Cells (a) Untreated A-549 control cells (b) A-549 cells treated with LPE (c) A-549 cells treated with LPE 15µg/ml

The results are represented as the mean + SD. The data were analyzed by using excel and Easy plot.

Discussion

From ancient times, medicinal plants posse pain-relieving and healing capabilities and today we still rely on the curative properties of plants. Plant-based- medicines have a vital role in the prevention and treatment of cancer. In addition, medicinal plants are economical and readily available. A great deal of pharmaceutical research done in technologically advanced countries has considerably improved the quality of the herbal medicines used in the treatment of cancer. All over the world, scientists are concentrating on medicinal plants to boost the immune cells of the body against cancer. They are also an important source of synthetic and herbal drugs. So far, the pharmaceutical companies have screened more than 25,000 plants for their therapeutic properties (Deshmukh and Sakarkar, 2011).

In this study, the anticancer and apoptotic properties of the leaf extract of S. glauca was assessed against human non-small-cell lung cancer, A549 cells. Many compounds isolated from different members of this family have been previously reported to have anti-cancer properties. For example, early cancer screening in *S. glauca* indicated that an alcohol extract had cytotoxic activity against cancer cells at a low dosage. Following that, scientists discovered that several of the quassinoids in S.glauca had anti-leukemic action against lymphocytic leukemia in vitro (Sakarkar and Deshmukh, 2011; Jose et al., 2018). Researchers found that yet another quassinoid present in *S. glauca*, holocanthone, also possesses antileukemic and anti-tumorous properties (Narayanan, 2011).

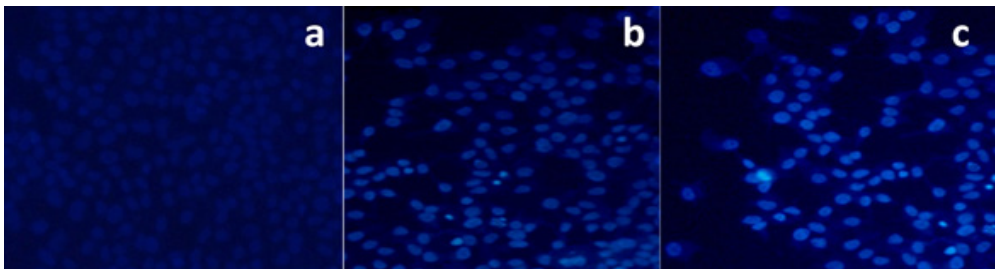

Figure 4. Hoechst Staining of LPE Treated A-549 Cells (a) Untreated A-549 control cells (b) A-549 cells treated with LPE 10µg/ml, (c) A-549 cells treated with LPE 15µg/ml

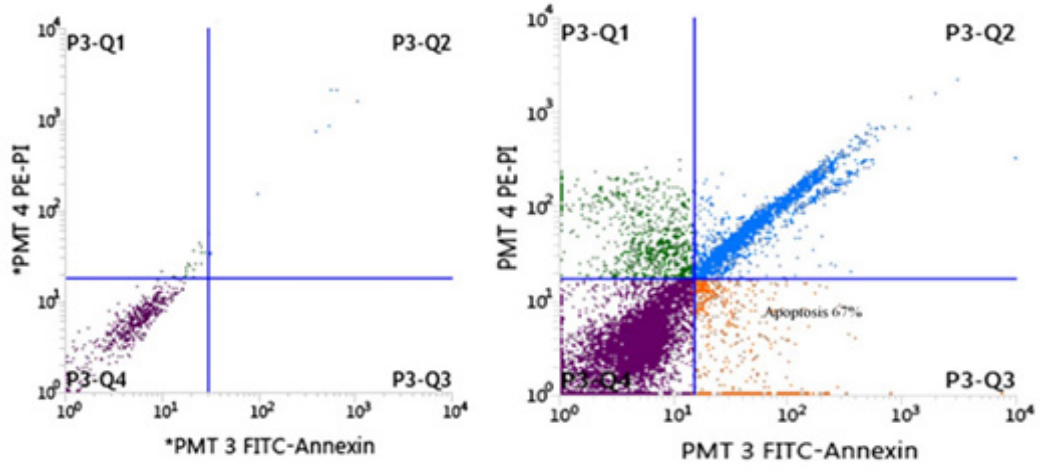

Figure 5. Annexin V Staining by Flow Cytometry of LPE Treated A-549 cells. a, A-549 control cells; b, A-549 LPE 10µg/ml

In the present study, petroleum ether extract (LPE) obtained from *S. glauca* was screened for cytotoxicity using an MTT assay and the findings indicated that the viability of the MTT assay conveniently provided drug sensitivity information. Appropriate cytotoxicity was proved for cancer cell lines treated with *S. glauca* petroleum ether leaf extract. We evaluated the apoptotic and anti-proliferative properties of *S. glauca* as a member of the Simaroubaceae family. Various compounds isolated from different members of this family have been previously reported to have anti-cancer properties. For example, the most potent quassinoids from *S. glauca* with such antitumor properties, such as bruceantin, bruceantinol, glacarubinone, and simalikalctone D, deserve special attention (Guo et al., 2005).

Using human non-small-cell lung cancer, A549 cells, the extract of the plant demonstrated remarkable cytotoxicity in the present study. Different fluorescence staining techniques were applied to A-549 cells treated 10 µg/ml and 15 µg/ml LPE in order to determine apoptotic cell death and apoptotic characteristics. In the acridine/ethidium bromide staining, acridine orange enters the living cell and intercalates the G-C base pairs of the DNA. At the A-T base pairs, ethidium bromide enters the dead cell and intercalates the DNA. The viable cells fluoresce green, while the dead cells fluoresce orange. The apoptotic feature like membrane blebbing, chromatin condensation and DNA fragmentation were observed in this study. Analyzing the data of Annexin using flow cytometry revealed early and end stages of apoptosis. Caspase 3 using flow cytometry determined that cell death occurred through either mitochondrial or death receptor pathway. In a nutshell, it was discovered that *S. glauca* possessed the potential to induce apoptosis in cancer cells.

In conclusion, this study yielded that LPE had remarkable anticancer activity via apoptotic induction through mitochondrial and death receptor pathways. This substantiated that *S. glauca* posed potential anticancer properties. Further research is recommended to develop a drug for treating malignancies.

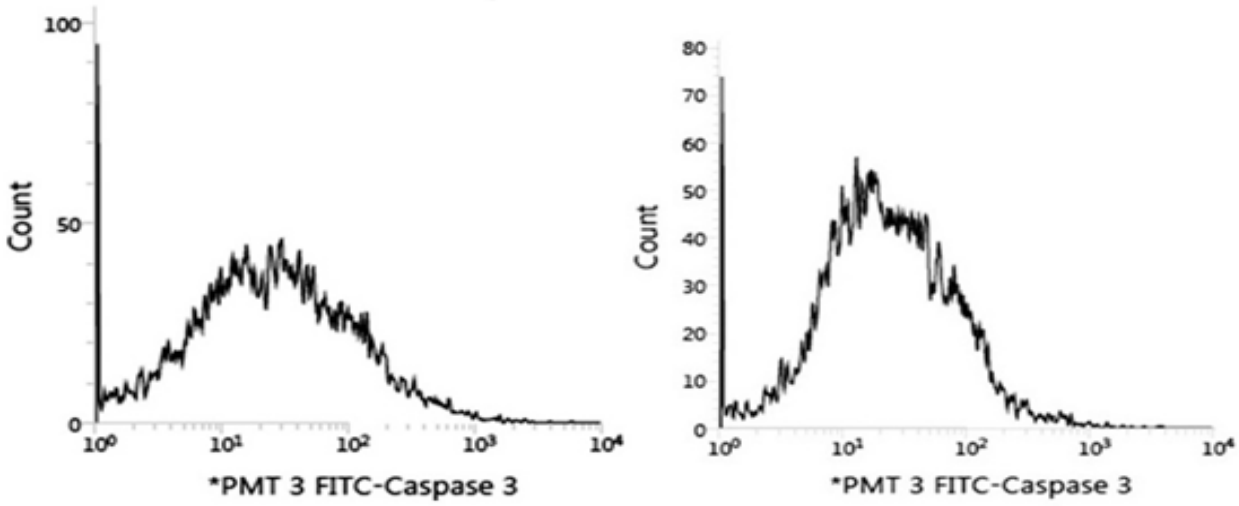

Figure 6. Caspase 3 Assay by Flow Cytometry of LPE Treated A-549 cells. a, A-549 control cells; b, A-549 LPE 10µg/ml

Author Contribution Statement

BV and SA conceived and designed the analysis; BV collected the data; BV, SR, KS contributed to the analysis and interpretation of data; BV and SA performed the analysis; BV wrote the paper, SA and KS edited the paper. All authors read and approved the manuscript.

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Any conflict of interest

None.

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