

Evaluation of anti-cancer activity of *Saraca asoca* flower extract against lung cancer cell line

Running title - Anticancer activity of *Saraca Asoca* flower extract against lung cancer cell line

ABSTRACT

Lung cancer is the second most common type of cancer, affecting one out of every five men and one out of every nine women. Lung cancer treatment is dictated by the cell type of the cancer, the amount to which it has spread, and the patient's overall condition. It is well known that cancer cells, in part due to apoptotic pathway malfunction, confer resistance to chemotherapy drugs or radiation. *Saraca Asoca* has been used as medicinal remedies for various ailments due to its antioxidant, anti-inflammatory, anti-bacterial, anti-tumor activity. The objective of the study was to evaluate the anti-cancer activity of *S. asoca* flower extract against lung cancer cell line. The cytotoxic effect of *S. asoca* flower extract was carried out by MTT assay against the lung cell line (A-549). Different concentrations of *S. asoca* flower ethanolic extract (20-120 μ g) were treated for 24h. Furthermore, the morphological changes were analysed using phase contrast microscopy. The nuclear morphology of *S. asoca* flower ethanolic extract treated lung cancer was analyzed by DAPI staining. The MTT assay clearly showed that the extract of *S. asoca* flower treatment has significantly reduced the lung cancer cell viability in a dose dependent manner. We observed the IC-50 dose at 60 μ g/ml concentration. The DAPI staining results showed increased apoptotic cells with condensed chromatin and nuclear fragmentation in ethanolic extract of *S. asoca* flower treatment in lung cancer cells. The present study shows ethanolic extract of *S. asoca* flower treatment inhibits cell growth and induces the apoptosis in lung cancer cells. More research is needed to understand the cytotoxicity mechanisms of this plant extract.

KEYWORDS: *S. asoca* flower extract, Lung cancer, Cytotoxicity, Apoptosis

INTRODUCTION

Cancer is one among the foremost dreaded diseases from the 20th century spreading further with continuance and elevating its incidence in the 21st century. In the United States, as the leading cause of death, presently it accounts for 25% of all the deaths in humans (1) and the chemoprevention of cancer has led to a new frontier in research ever, human lung cancer continues to be the leading prime cause of death from cancer among males rather than females in the developing countries (2), (3). Till now, the five-year survival rate is less than 15% for human lung cancer (4). Hence, new tactics to enhance the therapy of lung cancer have received much attention.

Since ancient times, medicinal plants were used as key therapeutic agents all over the globe, especially among the rural areas of the developing countries due to easy accessibility and affordable primary health care system (5),(6). According to the World Health Organization (WHO), 80% of people across the globe use medicinal plants to treat various diseases (7,8). A wide range of biological and pharmacological properties of medicinal plants manifest their therapeutic potential, for the treatment of various diseases (9), (10). Plants have a wide history in the treatment of cancers and the interest in nature as a source of potential chemotherapeutic agents continues (11). Uses of herbal medicine in the treatment of lung cancer and other types of cancers are well proven in the literature (12), (13). Hartwell, (1982) described more than 3000 medicinal plants, possessing anticancer properties and subsequently used as potent anticancer drugs (14). Medicinal plants have the ability to provide accessible, cost effective, and also a relatively safe treatment when compared to standard methods (15), (16). Although medicinal plants are considered nontoxic, numerous safety studies reported that they can cause various side effects; hence safety evaluation of medicinal plants is also required (17).

S. asoca also referred to as *S. indica* is an indigenous herbal drug belonging to the family Caesalpiniaceae and one among the foremost ancient sacred plants widely distributed throughout the Indian subcontinent (18), (19). *S. asoca* is reported to contain glycoside, flavonoids, tannins and saponins. The *asoca* tree has many health benefits and has long been utilised in traditional Indian medicine as a main key ingredient in various therapies and cures(20) (21) (22) (23). It is used as a protective drug for spasmogenic, oxytocic, uterotonic, anti-bacterial, anti-implantation, anti-tumour, anti progestational, antiestrogenic activity against menorrhagia and anti-cancer (24) (25). Useful parts of the plant are barks, leaves, flowers and seeds. Flowers are fragrant. Flowers are Polygamous apetalous, yellowish orange turning to scarlet, in brief laterally placed corymbose, auxillary panicles, bract small, deciduous and calyx petaloid (26), (27). However, the presence of gallic acid, an antioxidant molecule, has already been reported in *S. asoca* flower (28). During ‘ashoka-sasthi’ the flower buds are taken orally by women. Aqueous suspension of *S. asoca* flower has antiulcer activity in albino rats (29), (30) and chemopreventive activity of the flavonoid fraction of *S. asoca* is reported in skin carcinogenesis (31), (32). Dried flower buds are reported to possess antibacterial activity. *S. asoca* bark and flowers exhibit antitumor activity against DLA, S-180 and Ehrlich ascites carcinoma tumour cell lines, Larvicidal activity has also been recorded (33), (34). The present study attempts to evaluate the anti-cancer activity of *Saraca asoca* flower extract against lung cancer cell line.

MATERIALS AND METHODS

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.

Cell line maintenance

Lung cancer cell lines (A549) 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.

Preparation of the Herbal Extract

S. asoca obtained from Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd (Chennai, India) was used for the present study. About 50g of *S. asoca* flower 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 whatmann 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.

Cell viability (MTT) assay

The cell viability of *S. asoca* flower 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 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 37°C. After starvation, cells were treated with *S. asoca* flower extract in different concentrations (20 to 120µg/ml) for 24 hours. At the end of treatment, the medium from control and *S. asoca* flower extract 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 37°C 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 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 = [A_{570 nm} of treated cells/A_{570 nm} of control cells]×100.

Morphology study

Based on MTT assay we selected the optimal doses (IC-50: 60µg/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 *S. asoca* flower extract (60µg) 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 nuclear morphological changes of cells (DAPI staining)

For the nuclear morphological analysis, after the treatment, 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.

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.

RESULTS

The cytotoxic potential of *S.asoca* flower extract in lung cancer cells was assessed by MTT assay.

The cells were treated with *Saraca asoca* flower extract for 24h in different concentrations (20, - 120 µg/ml). The treatment significantly decreased the viability of A-549 cancer cells compared to control at 24 h time point (Figure-1). With increase in the concentration, the percentage of cell viability reduced gradually. We observed the 50% growth inhibition at (60 µg/ml) concentration. Hence, IC-50 dose (60 µg/ml) was considered for the further experiments.

The cell morphological analysis of *S.asoca* flower extract treated lung cancer cells was observed in an inverted phase contrast microscope.

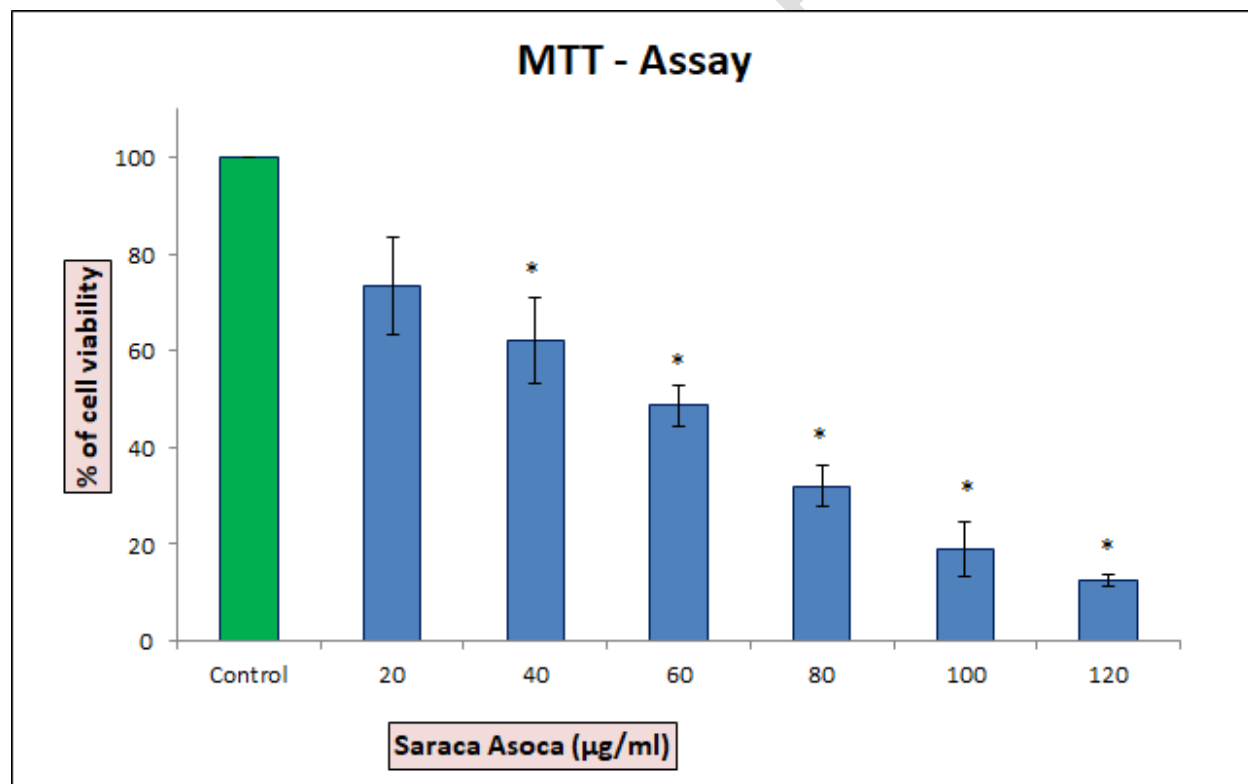
The A-549 cells were treated with *S.asoca* flower extract (60 µg/ml) for 24 h, compared with the untreated cells, treated cells showed significant morphological changes, which are characteristic

of apoptotic cells, such as cell shrinkage and reduction in cell density (Figure-2). Other types of morphological changes such as rounded up cells that shrink and lose contact with neighboring cells were also observed in cells undergoing apoptosis. Some sensitive cells were even detached from the surface of the plates.

The induction of apoptosis in *Saraca asoca* flower extract (60 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 (Figure-3).

FIG:1-Effect of *Saraca Asoca* flower extract on the viability of lung cancer cells



This graph represents the anticancer activity of *S. asoca* flower extract against lung cancer cell line was determined by MTT assay. The 50% of growth inhibition was observed in concentration of 60µg/ml (*p* value: 0.0015), which has been taken as IC50 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-Effect of ethanolic extract of Saraca Asoca flower on cell morphological changes in lung cancer cell line

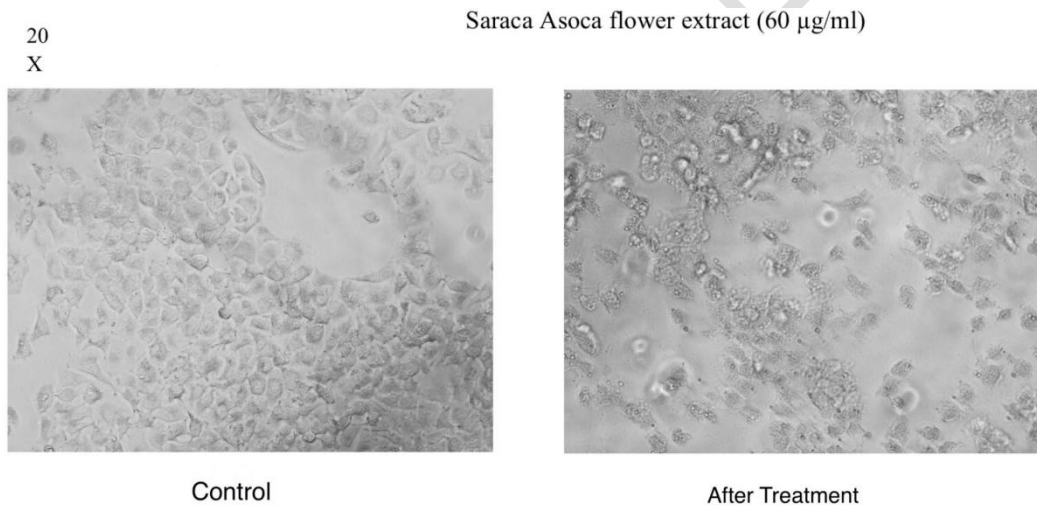


Figure 2: Represents the morphological changes in A-549 lung cancer cell line upon and without and with the treatment of Saraca asoca at 60 $\mu\text{g/mL}$ for 24hrs by phase-contrast microscope at 20x magnification. The figure represents that, the number of cells decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

FIG:3- Induction of apoptosis in Saraca Asoca flower extract treated lung cancer cell line

20
X

Saraca Asoca flower extract (60
 $\mu\text{g/ml}$)

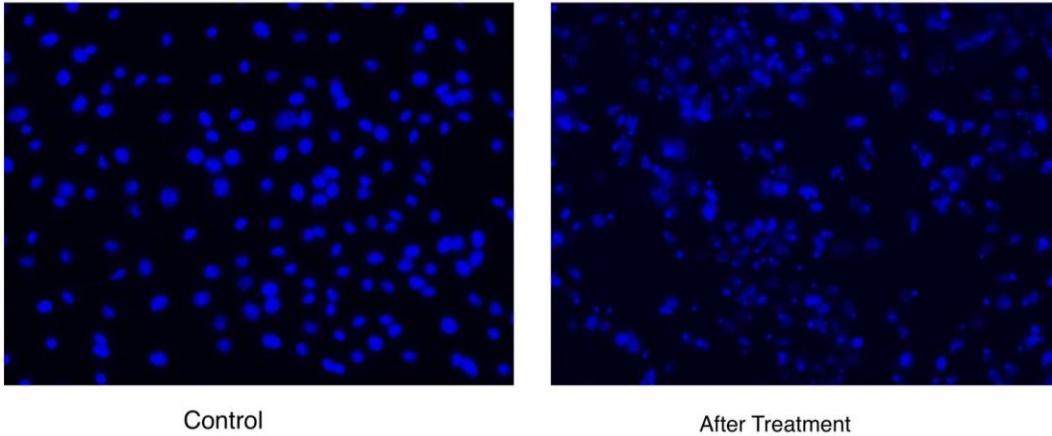


Figure 3: Induction of apoptosis in Saraca asoca at 60 $\mu\text{g/mL}$ treated lung cancer cell line visualised under a fluorescent microscope at 20x magnification. The nuclei were stained with DAPI staining and observed under a fluorescence Microscope. The figure represents that the treated cells clearly showed condensed chromatin and nuclear fragmentation.

DISCUSSION

Medicinal plants are contested as an alternative drug for cancer prevention and/curation in many nations around the world. A greater therapeutic effect of the above plant-derived active compounds will be found to be an effective anti-cancer drug against cancer cells (35), (36). Plants also have the opportunity to supply newer therapies, making them a potentially rich source of novel anticancer substances (37), (38). Recently, numerous treatments have been proposed for cancer, which uses plant-derived materials. Any herbs and plants include chemicals that influence human physiology, and these bioactive compounds include tannins, sugars, hormones, and flavonoids (39). The compounds that inhibit cancer initiation are traditionally termed blocking agents, Extracts of medicinal plants are multi composed mixtures of active components; they show their synergistic effect by acting at the same or different nodes of a cancer signaling network increasing therapeutic potential many folds, in comparison to a single drug-target therapy, and also compensate the toxicity and increased bioavailability of active compounds

(40), (41). The ability to target the multiple nodes of the cancer signaling network may restrict the cancerous cells to develop resistance against medicinal plant extracts (42).

Lung cancer is a type of cancer that originates in the lungs and is a prevalent form of cancer worldwide (39,43). Increased incidence of smoking and rising levels of air pollution have brought many people into the high-risk category of lung cancer. Primarily, two types of lung cancers have been identified. The small cell lung cancer, which is evidenced mostly in those who have the habit of smoking; and the non-small cell lung cancer which is further classified as - squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (44,45). Natural products have supplied a number of the most effective anticancer leads in the beyond, several already being into a successful business product (46), (47). Ethnobotanical studies of *S. asoca* also have found out its flavonoid fraction (from flora) to save you -degree pores and skin carcinogenesis and preferentially act towards Dalton's lymphoma ascites and Sarcoma-180 tumors cells, even as being non-poisonous to ordinary lymphocytes. The ethanolic extract of Ashoka was shown to inhibit breast cancer. The lectin 'saracin' remoted from the seed integument has been suggested to set off apoptosis in human T- lymphocytes in an in vitro assay (48).

In this present study, anticancer activity of *S. asoca* flower extract against lung cancer cell line cells was determined by MTT assay. The anti cancer activity of *S. asoca* flower extract was evaluated in lung cancer cell line (A549). *S. asoca* flower extract inhibited proliferation of A549 cells and IC50 dose was observed with 60µg/mL concentration. It inhibits the proliferation of cells in dose as well as time dependently at 24hr (figure-1). Effect of ethanolic extract of *S. asoca* flower on cell morphological changes in lung cancer cell line was observed as the number of cells decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing (figure-2). We also confirmed, induction of apoptosis which represents that the treated cells clearly showed condensed chromatin and nuclear fragmentation (figure-3). Further research studies are still required to find out the mechanism of action of specific bioactive compounds responsible for anti lung cancer activity of *S. asoca* flower extract(49) (50) (51) (52) (53) (54) (55) (56) (57) (58) (59) (60) (61) (62) (63).

CONCLUSION

Within the study limitations, the present study shows the ethanolic extract of *S. asoca* flower treatment significantly inhibits cell growth in a dose dependent manner. We found 50 % inhibitory concentration at 60µg/mL, and it is concluded that the ethanolic extract of *S. asoca* flower had a strong effect on the cell viability on A549 lung cancer cell line. And also it disturbs the cell morphology and induces apoptosis in lung cancer cell lines. However more research is needed to understand the mechanisms of anti-cancer activity of *S. asoca* flower extract.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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.

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