Anti-cancer effects of Saraca Asoca flower extract on prostate cancer cell line

Running Title: Anti-cancer potential of *Saraca asoca* flower extract on prostate cancer cell line

ABSTRACT

Prostate cancer is the second most common cause of cancer deaths for men. The role of plants in the prevention and treatment of disease has been known from the dawn of civilization. Plants maintain the health and vitality of individuals and also cure diseases, including cancer without causing toxicity. More than 50% of all modern drugs in clinical use are of natural products, many of which have the ability to control cancer cells. Saraca asoca (Roxb.), De. Wild or Saraca indica is one of the most ancient trees of India, frequently known as a "Ashok briksh", or "Ashoka" belonging to the family Caesalpiniaceae means "without sorrow" or which gives no grief. Saraca Asoca plant has several pharmacological activities such as anti inflammatory, antimicrobial, Antimenorrhagic, antidiabetic and anti cancer activities. Aim of this study is to study the Anti-cancer effects of Saraca Asoca flower extract on prostate cancer cell line. The anti-cancer effects of *saraca asoca* extract on prostate cancer cell lines was assessed by cell viability assay, cell and nuclear morphological studies. The multiple concentration of *saraca asoca* extract (0, 20, 40, 60, 80, 100 and 120 µg/ml) was used and IC-50 doses were calculated. The MTT assay results showed the percentage of cell viability significantly decreased in treated cells compared to control groups without any treatment was represented as 100% and we observed inhibitory concentration was 60µg/ml. Morphologic changes observed showed cell shrinkage, cytoplasmic membrane blebbing, and collapse of cells into small membranes when viewed in a phase-contrast microscope. The apoptotic nuclei (intensely stained, fragmented nuclei, and condensed chromatin) were viewed under a fluorescent microscope. Overall, the present study results demonstrated the cytotoxic and proapoptotic effects of Saraca Asoca flower extract on prostate cancer cell line. However, more research is needed to understand the mechanisms of anti cancer effects.

KEYWORDS: *Saraca asoca*, prostate cancer, cell line, anti -cancer.

INTRODUCTION

The role of plants in the prevention and treatment of disease has been known from the dawn of civilization. Plants maintain the health and vitality of individuals and also cure diseases, including cancer without causing toxicity. More than 50% of all modern drugs in clinical use are of natural products, many of which have the ability to control cancer cells (Prasad and Nicol, 2006). Saraca asoca (Roxb.), De. Wild or Saraca indica is one of the most ancient trees of India, frequently known as a “Ashok briksh”, or “Ashoka" belonging to the family Caesalpiniaceae means “without sorrow” or which gives no grief. The Ashoka tree has been mentioned in some of the oldest Indian texts apart from Ayurveda. Across India, Ashoka tree is believed to be sacred and apart from Ramayana, Ashoka tree is mentioned in Buddhism and Jainism as well. Charaka Samhita which is believed to have been composed in 1000 BC describes the Ashoka tree and its medicinal benefits (Harborne, 2011); (Kumar *et al.*, 2019). Ashoka is one of the most significant Ayurvedic drugs for the treatment of several feminine disorders especially in menorrhagia. Its bark for keeping a woman healthy and youthful. It is useful in internal bleeding, hemorrhoids, ulcers, uterine affections, menorrhagia especially due to uterine fibroids, meno-metrorrhagia, leucorrhoea and pimples. The plant possesses several medicinal values and is widely used in Ayurvedic formulation for treating a number of diseases like to treat painful conditions, improves complexion of the body, improves digestion and assimilation, alleviates excessive thirst, to kill all infectious agents, in blood disease, inflammation (Yadav, State Forest Research Institute and Pradesh., 2019).

Plant has several pharmacological activities such as anti inflammatory, antimicrobial, Antimenorrhagic, antidiabetic and anti cancer activities. One study shows the principle from Saraca asoca flowers indicated 50 percent cytotoxicity (in vitro) in Dalton's lymphoma ascites and Sarcoma-180 tumour cells at a concentration of 38 mug and 54 mug respectively, with no activity against normal lymphocytes but preferential activity for lymphocytes derived from leukemia patients (U. and S., 2017). Prostate cancer is the second most frequent malignancy (after oral cancer) in men worldwide, counting 1,276,106 new cases and causing 358,989 deaths (3.8% of all deaths caused by cancer in men) in 2018 (Bray *et al.*, 2018); (Parkin *et al.*, 2018).

The incidence and mortality of prostate cancer worldwide correlate with increasing age with the average age at the time of diagnosis being 66 years. Also Colon cancer incidence is increasing worldwide. In this study, AgNP was synthesized using β-sitosterol and its cytotoxic potential was evaluated in human colon cancer (HT-29) cells (Shathviha *et al.*, 2021); (M, Sohaib and Ezhilarasan, 2020).

Human cancer cell lines have been the most commonly used experimental models because they retain characteristic features of cancer cells, purity, are easily propagated and can be genetically manipulated to provide reproducible results; results obtained with cell lines are often extrapolated to human tumors *in vivo('An experimental analysis on the influence of fuel borne additives on the single cylinder diesel engine powered by Cymbopogon flexuosus biofuel', 2017)* (Campeau *et al.*, 2014) (Sathish and Karthick, 2020) (Krishnaswamy *et al.*, 2020). In past years, a number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). *Saraca asoca* Roxb. de Wilde, syn. *S. indica* auct non L. (Ashoka) is an evergreen tree belonging to the Caesalpiniaceae subfamily of the legume family. Several studies have reported that the plant has uterotonic, antibacterial,antitumor and anti-oestrogenic activity (A. C. Gomathi *et al.*, 2020). Other studies have reported that Histone deacetylase (HDAC) enzyme inhibitors possess potential anti-cancer effects (Anirudh and Ezhilarasan, 2020) and cytotoxic potentials of *S. cumini* methanolic seed kernel extract against human hepatoma HepG2 cells (Solai Prakash and Devaraj, 2019). One study reported that the syringic acid has anti cancer effects against OSCC (Abijeth and Ezhilarasan, 2020) and the cytotoxic potentials of ethanolic banaba leaves extract (EBLE) against human hepatocellular carcinoma (HepG2) cell line (Rohit Singh and Ezhilarasan, 2020). Other study showed possible mechanism on eco friendly synthesized nanoparticles on hazardous dyes degeneration (Nandhini, Rajeshkumar and Mythili, 2019),Collective influence of 1-decanol addition, injection pressure and EGR on diesel engine characteristics fueled with diesel/LDPE oil blends (Rajasekaran *et al.*, 2020), hepatic stellate cell-specific therapeutic targets(Ezhilarasan, Sokal and Najimi, 2018), oxidative stress is bane in chronic liver diseases an experimental perspective(Ezhilarasan, 2018), The potential of siRNA based drug delivery in respiratory disorders: recent advances and progress (Dua *et al.*, 2019), Comparative estimation of sulfiredoxin levels between chronic periodontitis and healthy patients - A case-control study (Ramesh *et al.*, 2018), Aberrations of m6A regulators are associated with tumorigenesis and metastasis in head and neck squamous cell carcinoma

(Arumugam, George and Jayaseelan, 2021),Is photodynamic therapy a viable antiviral weapon against COVID-19 in dentistry?(Joseph and Prasanth, 2021),Syzygium cumini extract induced reactive oxygen species-mediated apoptosis in human oral squamous carcinoma cells(Ezhilarasan, Apoorva and Ashok Vardhan, 2019), Compatibility of Nonoriginal Abutments With Implants: Evaluation of Microgap at the Implant-Abutment Interface, With Original and Nonoriginal Abutments (Duraisamy *et al.*, 2019),Aquaculture: An overview of chemical ecology of seaweeds (food species) in natural products (Gnanavel, Roopan and Rajeshkumar, 2019), Mesenchymal stem/stromal cells as a valuable source for the treatment of immune-mediated disorders (Markov *et al.*, 2021).

One more study showed the biosynthesis of zinc oxide nanoparticles using Mangifera indica leaves and evaluation of their antioxidant and cytotoxic properties in lung cancer cells (Rajeshkumar *et al.*, 2018) and synthesis of nanoparticles from phenerochaete chrysosporium and their antibacterial activity against human pathogenic bacteria (Saravanan *et al.*, 2018). Aim of this study is to study the Anti- cancer effects of Saraca Asoca flower extract on prostate 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. 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

Androgen dependent (PC3) Prostate 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% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Preparation of the Herbal Extract

Saraca Asoca leaf powder obtained from IMPCOPS (Chennai, India) was used for the present study. About 50g of Saraca Asoca 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 (Santhoshkumar *et al.*, 2019). Fine filtrate was subjected to **rota** evaporation after that 3g of the material was obtained. The total ethanol extract was concentrated in a vacuum and immediately stored at 4˚C. **One study has shown evaluation of antibacterial activity using Gymnema sylvestre extract (M. Gomathi *et al.*, 2020).**

Cell viability (MTT) assay

The cell viability of Saraca Asoca extract treated PC3 cells was assessed by MTT assay. One study revealed cytotoxicity evaluated by MTT assay in human colorectal cancer cells (Vairavel, Devaraj and Shanmugam, 2020). The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. PC3 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 Saraca Asoca in different concentrations for 24 hours. At the end of treatment, the medium from control and Saraca Asoca 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 pippeting 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.

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 Saraca Asoca (concentration PC3 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. One study has shown Morphology-related apoptotic changes were analyzed by annexin V staining (Raj R, D and S, 2020)

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.

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 $p<0.05$.

RESULTS

Effect of Saraca asoca flower extract on cell viability of prostate cancer cell line

The cytotoxic potential of Saraca asoca flower extract in prostate cancer cells was assessed by MTT assay. The cells were treated with different concentrations (20 -120 µg/ml) of Saraca asoca flower extract for 24h. Saraca asoca flower extract treatment significantly decreased the viability

of PC-3 cancer cells compared to control at 24 h time point (Figure-1). The percentage of cell viability reduced gradually with increase in the concentration. 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 effect of Saraca asoca on cell morphology

The cell morphological analysis of Saraca asoca flower extract treated prostate cancer cells were observed in inverted phase contrast microscope. The PC-3 cells were treated with Saraca 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 reduced cell density were observed in the Saraca asoca flower 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.

Pro-apoptotic effect of Saraca asoca flower extract in prostate cancer cells (DAPI staining).

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).

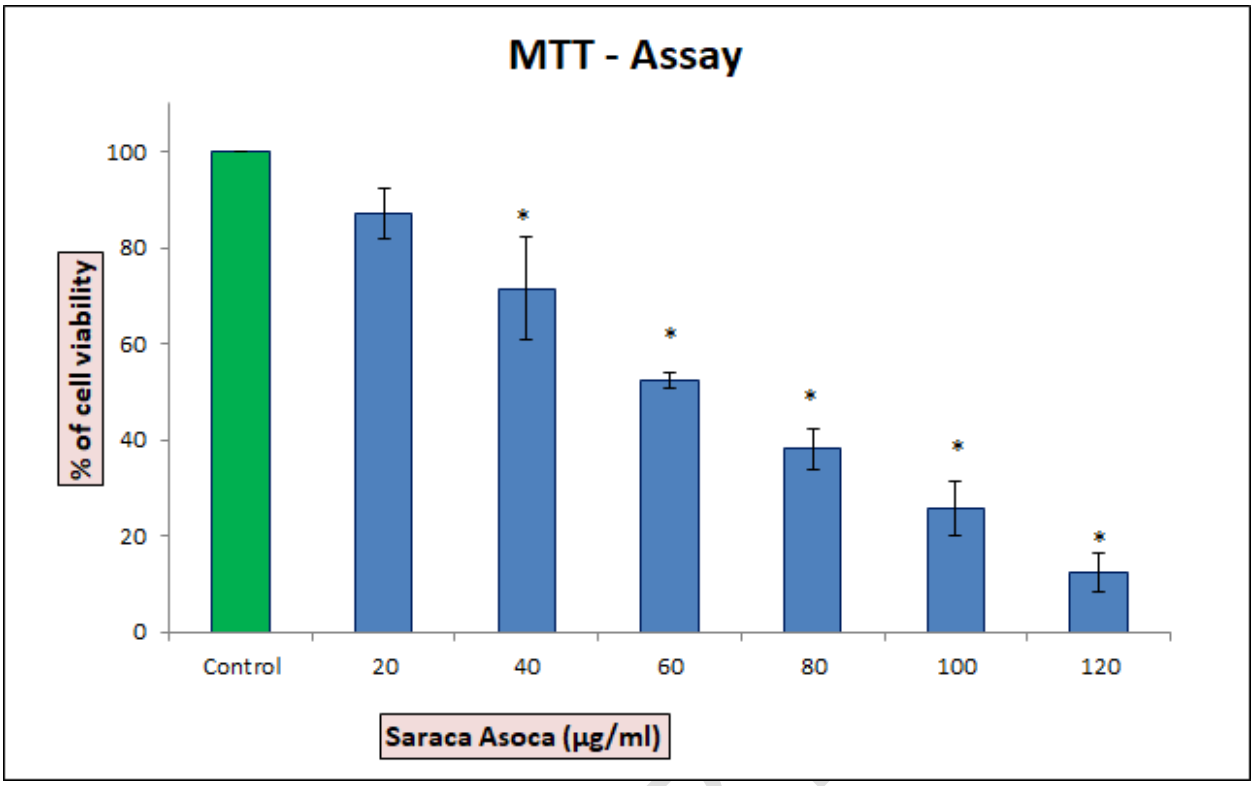
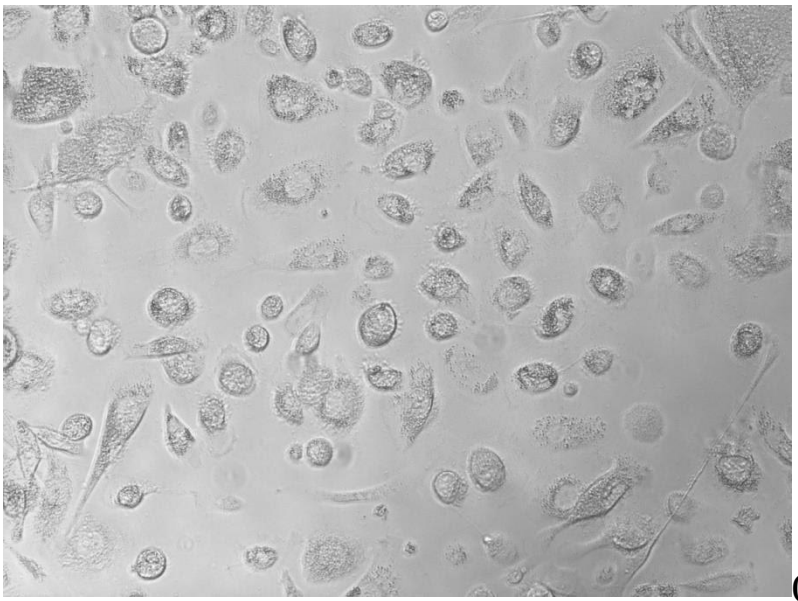
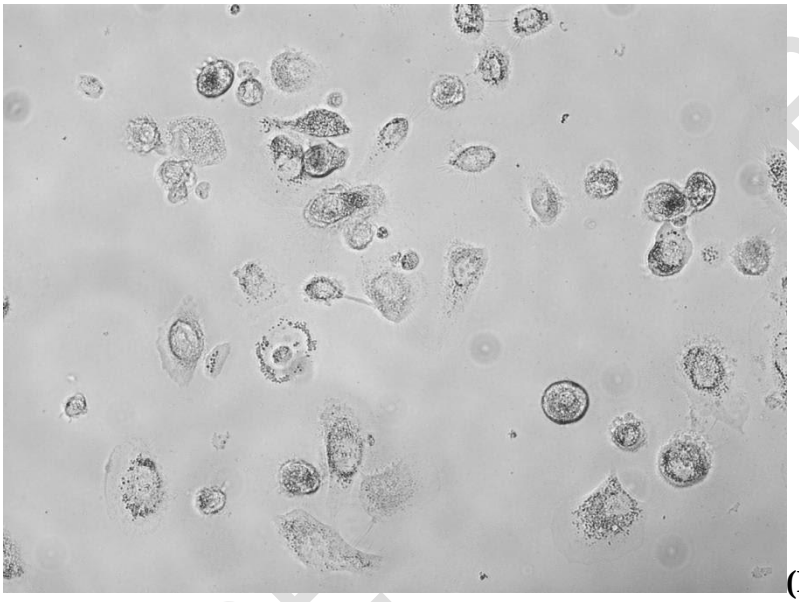


*Figure 1. Effect of Saraca Asoca flower extract on the viability of prostate cancer cells PC-3. The cells were treated with different concentrations (0,20,40,60,80,100&120 µg/ml) for 24 hrs. Inhibitory concentration (IC-50) dose: 60µg/ml (p-value: 0.0046). *' represents statistical significance between control versus treatment groups at p < 0.05 level using Student's–Newman–Keul's test.*

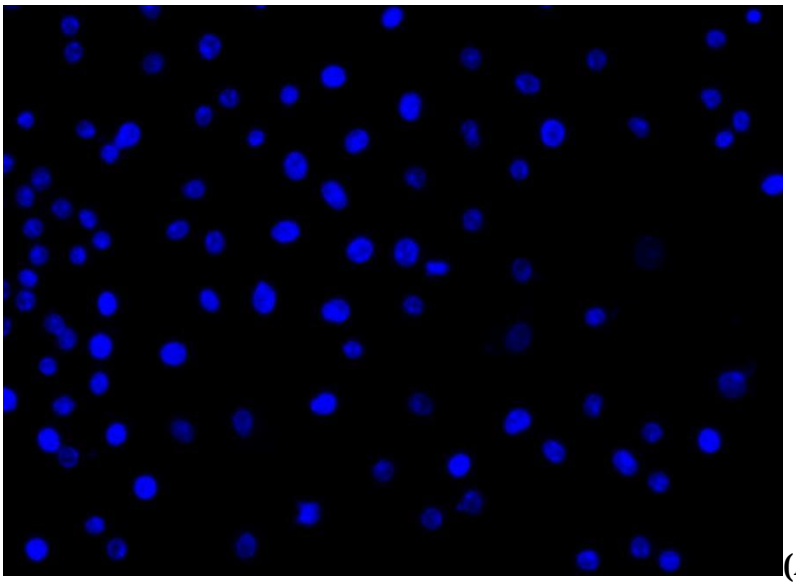


(A)

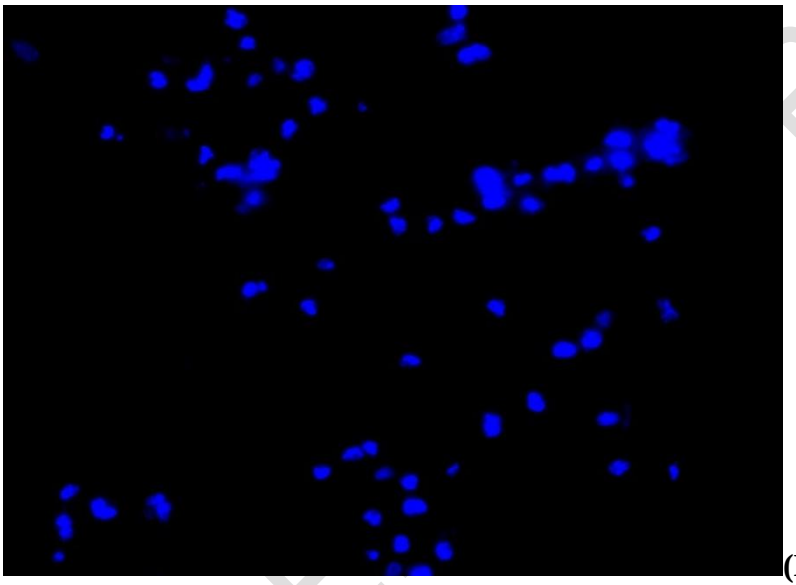


(B)

Figure 2: The effect of ethanolic extract of Saraca Asoca flower on cell morphological changes in prostate cancer cell line is visualised in a phase-contrast microscope at 20x magnification. (A) Control cells; (B) saraca asoca leaf extract (60µg/ml). The number of cells decreased after the treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.



(A)



(B)

Figure 3: Induction of apoptosis in saraca asoca leaf extract treated prostate cancer cell line (PC-3) visualised under a fluorescent microscope at 20x magnification. (A) Control cells ; (B) saraca acosa leaf extract (60µg/ml).The nuclei were stained with DAPI and observed under a fluorescent microscope. The treated cells clearly showed condensed chromatin and nuclear fragmentation.

DISCUSSION

Prostate cancer is the second most frequent malignancy (after oral cancer) in men worldwide, counting 1,276,106 new cases and causing 358,989 deaths (3.8% of all deaths caused by cancer in men) in 2018 (Bray *et al.*, 2018); (Parkin *et al.*, 2018). The incidence and mortality of prostate cancer worldwide correlate with increasing age with the average age at the time of diagnosis being 66 years. Of note, for African-American men, the incidence rates are higher when compared to the White men, with 158.3 new cases diagnosed per 100,000 men and their mortality is approximately twice as White men (Panigrahi *et al.*, 2019). Reasons for this disparity have been hypothesized to be differences in social, environmental and genetic factors. Although 2,293,818 new cases are estimated until 2040, a small variation in mortality will be observed (an increase of 1.05%). Prostate cancer may be asymptomatic at the early stage and often has an indolent course, and may require minimal or even no treatment. However, the most frequent complaint is difficulty with urination, increased frequency, and nocturia, all symptoms that may also arise from prostatic hypertrophy. More advanced stages of the disease may present with urinary retention and back pain, as axis skeleton is the most common site of bony metastatic disease. Before prostate cancer, Oral cancer was one of the most leading cancers responsible for significant morbidity and mortality (Ezhilarasan *et al.*, 2021).

The cell viability of Saraca Asoca extract treated PC3 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. PC3 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 Saraca Asoca in different concentrations for 24 hours. At the end of treatment, the medium from control and Saraca Asoca 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% (**Figure 1).** Morphologic changes observed showed cell shrinkage, cytoplasmic membrane blebbing, and collapse of cells into small membranes when viewed in a phase-contrast microscope (**Figure 2)**. 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. One study showed that ethanolic neem leaf extract significantly inhibited the growth of PC-3 cells in vitro. This predominantly involved apoptosis-induced cell loss and a lowering in the proliferation rate of PC-3 cells. These results suggest that ethanolic neem leaf extract may be an attractive option for the "drug hunters" as a potential agent for the management of human prostate cancer (Kumar *et al.*, 2006). The nuclei were stained with DAPI and observed under a fluorescent microscope. The apoptotic nuclei were intensely stained, fragmented, and had condensed chromatin (**Figure 3).** One study showed that Morphology-related apoptosis was analyzed by dual staining with acridine orange/ethidium bromide (Rithanya and Ezhilarasan, 2021)(Ezhilarasan, Apoorva and Ashok Vardhan, 2019) (Danda, Krishna, *et al.*, 2010) (Ramadurai *et al.*, 2019) (Sathivel *et al.*, 2008) (Panda *et al.*, 2016) (Neelakantan *et al.*, 2012) (Govindaraju, Neelakantan and Gutmann, 2017) (Sekhar, Narayanan and Baig, 2001) (DeSouza *et al.*, 2014) (Nasim *et al.*, 2010) (Danda, Muthusekhar, *et al.*, 2010) ('Molecular structure and vibrational spectra of 2,6-bis(benzylidene)cyclohexanone: A density functional theoretical study', 2011) (Putchala *et al.*, 2013) (Neelakantan, Grotra and Sharma, 2013) (Suresh *et al.*, 2014)

. The effects of Saraca Asoca in PC3 cell death were also determined by AO/EtBr dual staining as described previously (Cury-Boaventura, Pompéia and Curi, 2004). The cells were treated with Saraca Asoca 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.

CONCLUSION

Overall, the present study results demonstrated the Anti- cancer effects of Saraca Asoca flower extract on prostate cancer cell line at 20µg/ml concentration and 24h incubation period. However, more research is needed to understand the mechanisms of anti cancer effects.

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