Journal of Pharmaceutical Research International

33(61B): 436-446, 2021; Article no.JPRI.77871
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Evaluation of Proapoptotic Potential of *Saraca asoca* Flower Extract on Skin Cancer Cell Line

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Authors' contributions

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

Article Information

DOI: 10.9734/JPRI/2021/v33i61B35708

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/77871

Original Research Article

Received 17 October 2021
Accepted 27 December 2021
Published 28 December 2021

ABSTRACT

Background: Cancer persists as a major health issue globally due to its high rate of morbidity and mortality. Skin cancer is the most common cancer and accounts for at least 40% of cancer cases worldwide. Search for tumour-selective and novel anticancer compounds with lesser side effects remains a major focus of cancer research. *Saraca asoca* is a traditional Indian medicinal plant, known to have anti-cancer, anti-menorrhagic, anti-oxidant, anti-oxytocic and anti-microbial activities. Though phytoconstituents of the *Saraca asoca* leaves, bark, and flowers have been reported in few studies, the cytotoxic potential of *Saraca asoca* flowers has not been evaluated.
Aim: To evaluate the proapoptotic potential of *Saraca asoca* flower extract on skin cancer cell line.
Materials and Methods: In this present study, the cytotoxic potential of *Saraca asoca* flower extract (10 to 60µg/ml) was evaluated by MTT assays in B16-F10 skin cancer cells. According to the MTT assay, we determined the optimal doses (IC-50: 30µg/ml) which were used for further analyses. Analysis of changes in cell morphology is examined by a phase-contrast microscope. The impacts of Saraca asoca in B16-F10 cell death were also determined by AO/EtBr dual staining under a fluorescence microscope.
Results: In our study, the cell viability assay results showed that 50% of growth inhibition was observed at 30 µg/ml concentration of *Saraca asoca* flower extract treated B16-F10 cells, which

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has been taken as an inhibitory concentration (IC-50) dose value and fixed for further experiments. The morphological changes in B16-F10 skin cancer cell line with the treatment of *Saraca asoca* at 30 µg/mL for 24hrs has significantly altered the morphology of B16-F10 cell lines. AO/EtBr dual staining results showed the early apoptotic cells having bright orange areas of condensed or fragmented chromatin in the nucleus after *Saraca asoca* flower extract treated skin cancer cells.
Conclusion: The results of this present study showed that the flower extracts of *Saraca asoca* were cytotoxic and induced apoptosis to the cancer cells at a concentration of 30µg/ml at the 24th-time point.

Keywords: Saraca asoca; flower extract; skin cancer; cytotoxicity; apoptosis.

1. INTRODUCTION

Cancer persists as a major health issue globally due to its high rate of morbidity and mortality [1]. Skin cancer is the most common cancer and accounts for at least 40% of cancer cases worldwide [2]. Skin cancers are cancers that emerge from the skin due to the development of abnormal or mutated cells which can invade or spread to other parts of the body. The main types of skin cancers include basal-cell skin cancer (BCC), squamous-cell skin cancer (SCC) and melanoma [3]. The basal-cell skin cancer (BCC), squamous-cell skin cancer (SCC) along with a number of less common skin cancers, are recognized as nonmelanoma skin cancer (NMSC) [4]. Non-melanoma skin cancer, which occurs in at least 2–3 million people per year, is the most common type of skin cancer [5]. An alarming rate of incidence of skin cancer in India has been seen in recent times [6]. The most important reason for the occurrence of skin cancer in India, as well as other countries, is attributed to increased urbanization, increased pollution due to the vehicles, smoke emitted from different types of industries causing the depletion of the ozone layer. These changes result in the passage of ultraviolet rays from the sun into the earth and cause genetic mutations in humans, leading to skin cancer [7].

Developing novel strategies for the management of skin cancer represents a desirable goal due to the increasing rise in the incidence of skin cancer patients throughout the world. Search for tumour-selective and novel anticancer compounds with lesser side effects remains a major focus of cancer research [8]. Moreover, the side effects linked with synthetic drugs severely influence the quality of life of patients [9]. Also, drugs obtained from natural sources have attained considerable attention worldwide. *Saraca asoca* belongs to the family Caesalpiniaceae is one of the most ancient trees seen in India, and is frequently known as an “Ashoka " [10]. *Saraca asoca* is a traditional Indian medicinal plant, known to have anti-cancer, anti- menorrhagic, anti-oxidant, anti-oxytocic and anti-microbial activities [11]. Researchers have reported that almost every part of the plant is known to have a medicinal property and has a role in ayurvedic medicine for centuries [12]. Flowers of *Saraca asoca* have been widely used in the Ayurvedic system of medicine for year's extremely owing to its wound healing property. Studies documented the phytochemical constituents of *Saraca asoca* flowers containing oleic, linoleic, palmitic, and stearic acids, quercetin-3-0-P-D-glucoside, P-sitosterol, gallic acid, kaempferol-3-0-P-D-glucoside, p and y sitosterols [13] quercetin, apigenin-7-0-p-D-glucoside, pelargonidin-3,5-diglucoside, cyanidin-3,5-diglucoside, and leucocyanidin [14]. The cytotoxic activity of *Saraca asoca* bark and its possible application in cancer prevention has been recently reported. Though phytoconstituents of the *Saraca asoca* leaves, bark, flowers have been reported in few studies the cytotoxic potential of *Saraca asoca* flowers has not been evaluated.

Apoptosis means the ability of a cell to cause self-destruction by the activation of intrinsic cellular suicidal programs when the cells are no longer needed, genetically mutated or when they are extremely damaged [15]. Induction of apoptosis in tumour cells is the most established anticancer mechanism and employed in many cancer therapies [16]. Human cancer cell lines have been the most commonly used as experimental models in research as they can retain the characteristic features of cancer cells, purity, easily assessed and can be manipulated genetically to contribute reproducible findings [17]. Outcomes obtained with cell lines are often used to understand human tumours in vivo. Our team has extensive knowledge and research experience that has translated into high quality publications [18-38]. With this background, our study aims in evaluating the proapoptotic potential of Saraca asoca flower extract on skin cancer cell lines [39-42].

2. MATERIALS AND METHODS

2.1 Reagents Used

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), AO/EtBr were purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were extra pure of molecular grade and were obtained from SRL, India.

2.2 Cell Line Maintenance

Skin cancer cell lines B16-F10 were acquired from the NCCS, Pune. After that, the cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics.

2.3 Preparation of the Herbal Extract

Saraca asoca flower powder acquired from IMPCOPS (Chennai, India) was used for this current study. 50g of the *Saraca asoca* flower powder was soaked in 500ml of 95% ethanol and was maintained at room temperature for 3 days under static condition.

2.4 Cell Viability (MTT) Assay

The cytotoxic potential of *Saraca asoca* flower extract treated with B16-F10 skin cancer cells was evaluated by using MTT assays. The principle of this assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. The concentration of $2x10^4$ cells/well was plated in 48 well plates and incubated for 24 hours and further starved by incubating it in a serum-free medium for 3 hours at a temperature of 37ºC. Later then the cells were treated with *Saraca asoca* flower extract at different concentrations (10 to 60µg/ml) for 24 hours. 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 appropriately by pipetting up and down. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (200µl) and incubated in dark for an hour. The intensity of the colour developed was assayed with the help of a Micro ELISA plate reader at 570nm. The number of viable cells was expressed as the percentage of control cells cultured in a serum-free medium. The cell viability is calculated using the following formula: % cell viability = [A570nm of treated cells/A570 nm of control cells]×100.

2.5 Morphology Study

According to the MTT assay, we determined the optimal doses (IC-50: 30µg/ml) which were used for further analyses. Analysis of changes in cell morphology is examined by a phase-contrast microscope. $3×10^4$ cells were seeded in 6 well plates and treated with Saraca asoca (30µg/ml for B16-F10 cells) for an interval of 24h. After the incubation period, the medium was removed and the cells were washed once by phosphate buffer saline (PBS pH 7.4). The obtained 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 impacts of Saraca asoca in B16-F10 cell death were also determined by AO/EtBr dual staining. The cells were treated with *Saraca asoca* flower extract for a duration of 24 h and further, the cells were harvested. The pellets were resuspended in acridine orange and ethidium bromide dual staining and the cell death was examined under a fluorescence microscope.

2.7 Statistical Analysis

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

3. RESULTS

3.1 The Cytotoxic Potential of *Saraca asoca* Flower Extract on the B16 - F10 Skin Cancer Cell Line was Determined by Using an MTT Assay

The cells were treated with different concentrations (10, 20, 30, 40, 50 and 60µg/ml) over a time duration of 24hrs. Our study observed, *Saraca asoca* flower extract treatment significantly decreased the viability of B16 - F10 skin cancer cells in comparison to the control at

24 hrs time point (Fig. 1). The percentage of cell viability decreased gradually with an increase in the concentration. At a concentration of 30 µg/ml, we observed a 50% of growth inhibition which has been taken as inhibitory concentration (IC-50) dose value and was considered for further experiments.

3.2 The Cell Morphological Analysis of *Saraca asoca* Flower Extract Treated Skin Cancer Cells were Observed through a Phase-Contrast Microscope

The B16 - F10 skin cancer cell line was treated with Saraca asoca flower extract (30 µg/ml) for a duration of 24 hrs and compared with the untreated cells, the treated cells demonstrated significant morphological changes, such as cell shrinkage and reduced cell density which are characteristic of apoptotic cells were observed in the *Saraca asoca* flower extract-treated cells (Fig. 2). In addition to it, cells undergoing apoptosis also exhibited other types of morphological changes such as rounded up cells that shrink and lose contact with neighbouring cells. Few sensitive cells were also detached from the surface of the plates.

3.3 Acridine Orange/Ethidium Bromide (AO/EtBr) Dual Staining Were Used to Confirm the Induction of Apoptosis in *Saraca asoca* Flower Extract-treated Skin Cancer Cells

AO/EtBr dual staining is used in assessing the nuclear morphology of apoptotic cells for that the cells were treated with *Saraca asoca* flower extract (30 µg/ml) for 24h. After treatment, the cells were stained with both AO/EtBr stain for 20 mins and examined under fluorescence microscopy. The obtained result showed that AO stained both live as well as dead cells, whereas EtBr stains only that have lost their membrane integrity. Cells stained green represent viable cells, whereas yellow staining represents early apoptotic cells, and orange staining represents late apoptotic cells. In the present study, control cells expressed a uniform green colour and in *Saraca asoca* flower extract-treated cells showed a yellow, orange and red signal (Fig. 3). From these results obtained, it is confirmed that *Saraca asoca* flower extract induces apoptosis in skin cancer cells.

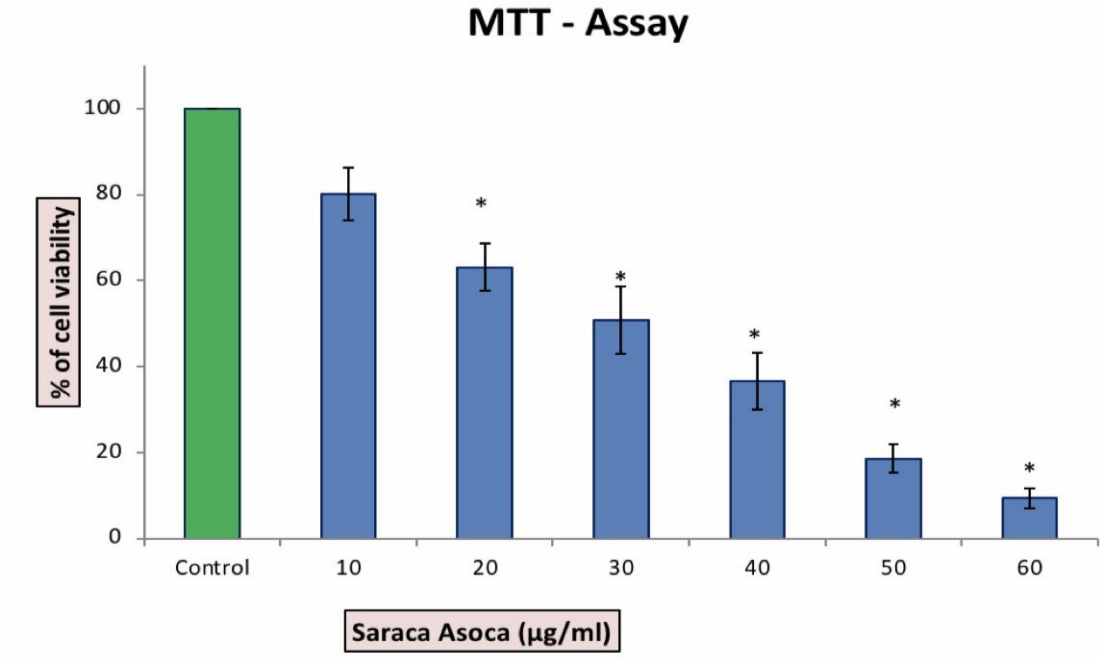

Fig. 1. The cytotoxic potential of Saraca asoca on the B16 - F10 cell line was determined by MTT assay. The cells were treated with different concentrations (10, 20, 30, 40, 50 and 60µg/ml) for 24hrs. The 50% of inhibition observed in a concentration of 30 µg/ml, (p value: 0.0086) which has been taken 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*

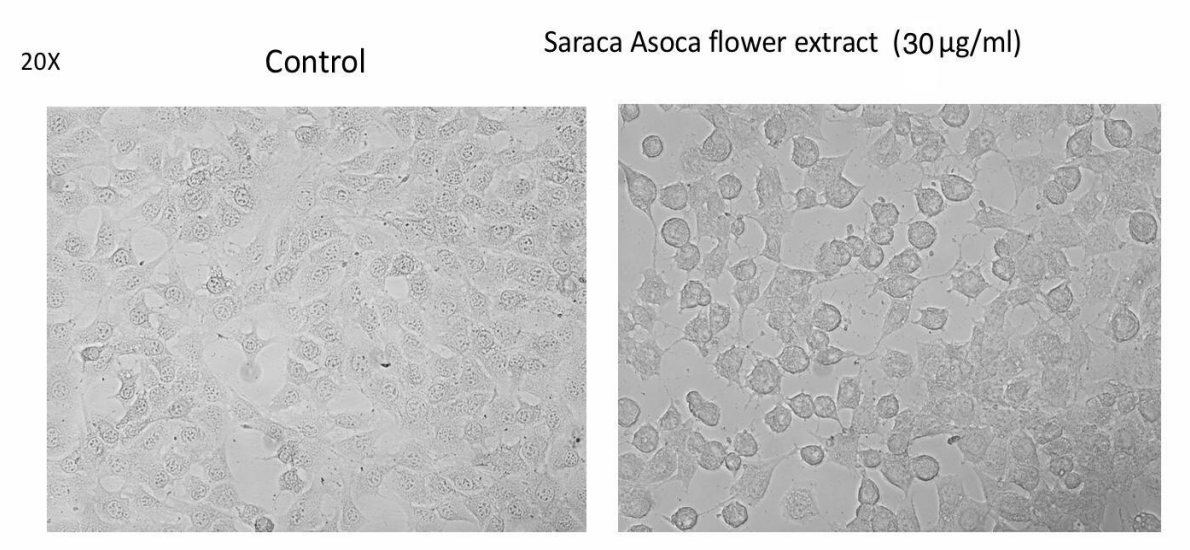

Fig. 2. Represents the morphological changes in B16 - F10 skin cancer cell line upon and without and with the treatment of Saraca asoca at 30 µg/mL for 24hrs by phase-contrast microscope at 20x magnification

The number of cells decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing

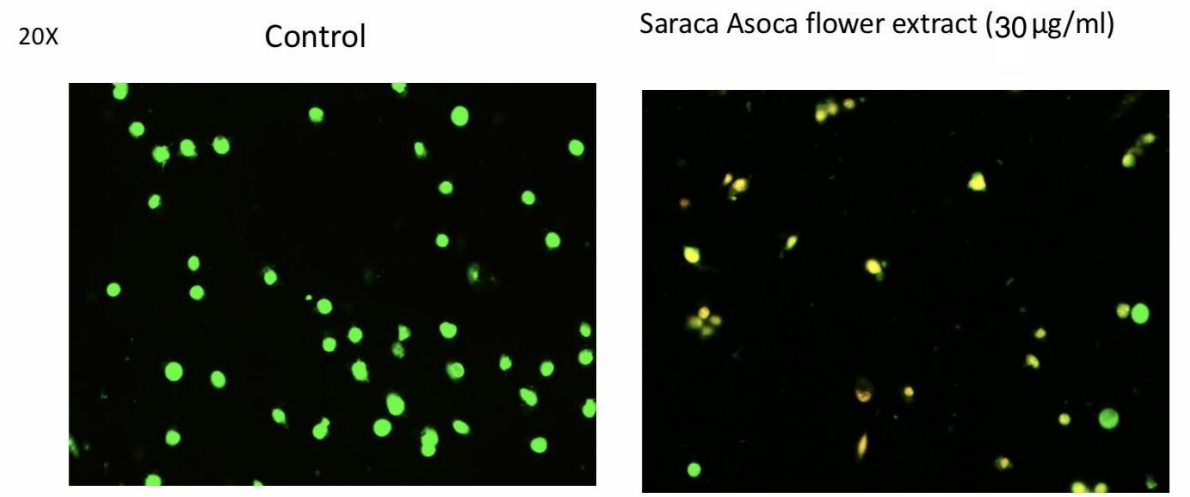

Fig. 3. Represents the acridine orange and ethidium bromide dual staining of treated B16 - F10 skin cancer cell line upon without and with the treatment of *Saraca asoca* at 30 µg/mL for 24hrs, viewed under fluorescence microscope at 20x magnification

The viable cells are possessing 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

4. DISCUSSION

Plants are notable for their therapeutic value and are considered to be a common source of medicine globally [43]. Plants have been used for treatments in several countries over the years. *Saraca asoca* is an important ancient medicinal plant seen in India, Sri Lanka, Burma and Malaysia. Studies have reported *S.asoca* extracts to have antitumor and cytotoxic effects. As the skin is the most widely exposed tissue to environmental carcinogens, skin cancer accounts for the most common type of cancer [14]. With an increasing prevalence of skin cancers, there is an urgent requirement for the development of various treatment options. Apoptosis or programmed cell death is represented by cell shrinkage, condensation of chromatin, DNA

fragmentation and the activation of specific enzymes known as caspases [44]. The process of apoptosis is being arrested during cancer progression. Induction of apoptosis in tumour cells is the most established anticancer mechanism and employed in many cancer therapies [45].

In our present study, the cytotoxicity potential of flower extracts of *Saraca asoca* is observed at a concentration of 30 µg/mL for 24hrs. Previous studies evaluated the flavonoid fraction from *Saraca asoca* as a potent chemopreventive property against DMBA-induced skin carcinogenesis and observed that the apoptosis-inducing biochemical markers were significantly restored to near-normal levels [46]. The inhibitory concentration (IC-50) dose value found in our study from the flower extracts of *Saraca asoca* was 30 µg/ml. Similar study evaluated the cytotoxicity of crude methanolic extract of Saraca indica bark extract on the cervical cancer cell lines and the IC50 value was determined to be 14.63 µg/ml [47]. In our current study, the morphological changes in B16 - F10 skin cancer cell line upon treatment of *Saraca asoca* at 30 µg/mL for 24hrs has been observed, and the number of cells decreased after treatment, also the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

Induction of apoptosis in cancer cells is observed in our study after the acridine orange and ethidium bromide dual staining of treated B16 - F10 skin cancer cell line with the treatment of *Saraca asoca* at 30 µg/mL for 24hrs, when viewed under a fluorescence microscope. The viable cells were possessing 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. Studies reported that the presence of compounds such as quercetin and gallic acid in *S.asoca* flowers shows chemopreventive properties. The same study also documented a significant reduction in the expression of ornithine decarboxylase, which is a key enzyme in the promotion stage of 2-stage skin cancer, was observed in the S.*asoca* treated group [48]. Another study assessed the anticancer activity of endophytic fungi associated with the medicinal plant *Saraca asoca* and provided promising lead molecules for the development of novel anti-cancer agents [49].

From this study, it can be concluded that the flower extracts of *Saraca asoca* possess potent cytotoxic properties against skin carcinogenesis. Also, further studies are required to understand and ascertain the component that plays a role in proapoptotic potential and their various mechanisms regulating the cytotoxic action of the *S.asoca* flower. The use of the flowers of the *Saraca asoca* can be suggested due to its edible nature, easy availability, and cost effectiveness [13,50-63]. This work determines significance since the current trend worldwide is to identify therapeutics from natural sources particularly because most of the plants and their products are extensively free from adverse effects and also to interpret traditional knowledge to a scientific platform.

5. CONCLUSION

Overall, the results of the current study revealed that the flower extracts of *Saraca asoca* were cytotoxic and induced apoptosis to the skin cancer cells at a concentration of 30µg/ml at the 24th-time point. However, further research is required to understand the mechanisms of anti-cancer effect of this *Saraca asoca* flower extract.

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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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/77871