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Antiproliferative Effect of Methanolic Extract of *Saraca asoca* bark and its Possible Targets of Action

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ABSTRACT

Background: The use of phytochemicals as anticancer drugs has gained attention in scientific and industrial approaches. In this context, the present study was undertaken to determine the antiproliferative effect of methanolic extract of *Saraca asoca* bark in the C127I cell line and its possible targets of action by *in silico* analysis. **Method:** Methanolic extract of *S. asoca* bark was assessed for its cytotoxicity in the C127I cell line by 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay at concentrations of 320, 160, 80, 40 20 and 10 µg/mL and the half maximal inhibitory concentration (IC_{50}) was calculated using Graph Pad Prism 5.0. The cells were seeded in 6 well plates at a concentration were treated for 24 hours with extract of *S. asoca* bark at IC_{50} concentration. The cells were trypsinised and subjected to Acridine orange - Ethidium bromide staining (AOEB) staining for morphological evaluation of apoptosis. Fourier transform infrared (FTIR) spectroscopic analysis was performed to identify the chemical nature of the extract. *In silico* analysis was done to assess the affinity of various phytochemicals in the extract towards Caspase and BCl_2 proteins. **Results:** Dose-dependent reduction in cell viability was noticed when the cells were subjected to different concentrations of the extract and the IC_{50} value of *S. asoca* was found to be 16.55 µg/mL. AO/EB staining detected proliferating cells with green fluorescence in the control cells whereas the cells with *S.asoca* extract showed a dose-dependent shift from orange to red fluorescence indicating apoptosis in treated cells. Ellagic acid present in the extract was found to have a maximum affinity towards Bcl_2 and Caspase proteins. **Conclusions:** From the study, it could be concluded that the methanolic extract of *Saraca asoca* was found to possess an antiproliferative effect.

Keywords: *S. asoca*, C127I, FTIR.

INTRODUCTION

India is one of the most ethnoculturally diverse nations, with residents of all creeds and civilizations who utilise herbs and natural products for treatment of various illness. The traditional medical practices like Ayurveda, Unani, and Siddha heavily rely on the usage of medicinal herbs. In recent years, several reports that established the therapeutic use of phytochemicals in the treatment of various diseases like cancer has been published. Cancer is responsible for most deaths worldwide, out of which breast cancer is the most common in women.[1] The common methods of therapy like surgery, radiation, and use of chemotherapeutic agents are associated with severe side effects such as nausea, vomiting, anaemia, loss of hair, pain in joints, lymphedema, and even the development of secondary cancers [2]. In this context, phytotherapy has become very significant as a substitute or adjuvant for conventional anticancer drugs. Preclinical anticancer medication development utilizes screening methods that employ cancer cell lines as model. Murine Breast Carcinoma cell line C127I is one such cell line that can be used to analyze a drug's anti-tumor activity.

Cancer is the uncontrolled proliferation of cells that can be trigerred by various stimuli including infection, inflammation, influence of environmental contaminants, radiation and excessive use of alcohol and smoking [3]. The proto-oncogenes are activated or the role of tumor suppressor genes are compromised either due to ineffective check points or counteraction of these restrictions by the cells. Mutations in the genes especially in p53 may augment proliferation of cells and now even the role of epigenetics [4] in cancer progression is being explored. The p53 gene keeps the cell in check points and augments apoptosis in an event of any damage to the cell function *Saraca asoca* (family Caesalpiniaceae), also known as *Saraca indica*, is one of the oldest trees found all over the Indian subcontinent. *Saraca asoca* had been used medicinally in several ways, according to Charaka Samhita (100 A.D.) [5]. Different parts of the plant exhibit several pharmacological effects like antihyperglycemic, antipyretic, antibacterial and anthelmintic activity. Extracts of the stem bark of *Saraca asoca* contain secondary metabolites such as flavonoids, terpenoids, lignin, phenolic compounds, and tannins which contributes to the therapeutic action [6].

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In this context, the present study was undertaken to establish the antiproliferative effect of methanolic extract of *Saraca asoca* bark in the C127I Cell line, to identify the possible compounds that provide the antiproliferative effect and *in silico* docking analysis to establish a causal relation on the possible targets of action.

MATERIALS AND METHODS

Plant Material

The bark of *Saraca asoca* was collected from different parts of the district of Thrissur, dried under shade, and pulverized. Then it was extracted using methanol in the Soxhlet extraction apparatus, dried using a rotary vacuum evaporator, and stored under refrigeration till further use. A qualitative phytochemical analysis was performed [7].

Fourier transform infrared spectroscopy (FTIR) analysis

A Perkin- Elmer spectrum two FTIR spectrometer with attenuated total reflectance was used for FTIR analysis. An overhead ATR attachment was installed at the sample station. A small quantity of the sample was carefully placed on the diamond crystal surface to cover the ATR diamond window to focus the laser beam. Each spectrum's absorbance under the 60 N value was recorded.[8]

In Silico screening

Lignin glycosides such as lyoniside, nudiposide,5-methoxy-9-β-xylopyranosyl, isolariciresinol and schizandriside; flavonoids such as catechin, epicatechin,epiafzelechin-(4β- 8)-epicatechin, procyanidin B2, deoxyprocyanidin B, leukocyanidins and leucopelargonidin have been reported from *S. asoca* bark [9]. Also, antioxidants such as polyphenolics, gallic acid, and ellagic acid have also been found in *S. asoca* bark [10]. A molecular docking study was conducted with different phytochemicals of *S. asoca* bark as ligands against murine caspase-11 (PDB ID: 6NS7) and Bcl2 (B- cell lymphoma 2) (PDB ID: 1G5M) receptors using Autodock V4.2.

Cell lines

C127I**,** mouse mammary tumor cells were cultured in 25 cm^2 tissue culture flasks with the supplementation of Dulbecco's Modified Eagle Medium (DMEM) with 10 percent foetal bovine serum and 1 percent ceftriaxone. The cells were maintained in a humidified incubator at 37° C with five percent carbon dioxide (CO_2).

Cytotoxicity studies: 3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

Microtiter plate (96 wells) was seeded with $5x10^4$ cells/mL and incubated for 24 hrs. Then the cells were exposed to extract which was diluted to concentrations 320, 160, 80, 40, 20, and 10 µg/mL and incubated for 24 hrs [11]. Then MTT at a concentration of 5mg/mL was added to each well at 10µL and incubated for 4 hours with serum-free media. With the addition of 100 L of DMSO, the process was stopped. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The percent cell viability was calculated from which percent cell inhibition and subsequently, IC_{50} value was calculated by Graph Pad Prism 5.0.

Acridine orange / Ethidium bromide (AO/EB) staining

The cells were seeded in 6 well plates at a concentration of $1x10^5$ cells/mL and were treated for 24 hours extract of *S. asoca* at IC_{50} concentration. Twenty-five microlitres of the treated or untreated cells were stained with five microlitres of acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) and analyzed under Trinocular Research fluorescence microscope, DM 2000 LED, Leica with blue excitation (488 nm) and emission (550 nm) filters at 10X magnification [12].

RESULTS

Phytochemical analysis

Phenolics, saponins, tannins, glycosides, and flavonoids were present in the methanolic extract of *S.asoca* bark whereas alkaloids were absent in the extract.

Table 1: Phytochemical analysis of a methanolic extract of *S. asoca* bark

Active principle- Test	**Methanolic extract of *S. asoca* bark**
Alkaloids	
Mayer's test	-
Wagner's test	-
Hager's test	-
Phenolic compounds detection test	+
Tannins	
Ferric chloride test	+
Flavonoids	
Lead Acetate test	+
Ferric chloride test	+
Glycosides	
Sodium hydroxide test	+
Saponins	
Foam test	+

Fourier transform infrared spectroscopy (FTIR) analysis:

The compounds obtained by comparing the spectra of the extract using FTIR and FLUKA library are enlisted in table 3 and the spectrum of the methanolic extract of *S. asoca* bark is given in Figure 1.

Table 2: List of structurally similar compounds of methanolic extract of *S. asoca* bark

Absorption (cm^{-1})	Appearance	Group	Compound Class
3329.5	strong	O-H stretching	Alcohol
1607.72	medium	C≡C stretching	Cyclic alkene
1518.32	strong	N-O stretching	nitro compound
1371.58	medium	O-H bending	Alcohol
1283.33	strong	C-Fstretching	fluro compound
1246.95	strong	C-Fstretching	fluro compound
1102.99	strong	C-Fstretching	fluro compound
1063.31	strong	C-Fstretching	fluro compound

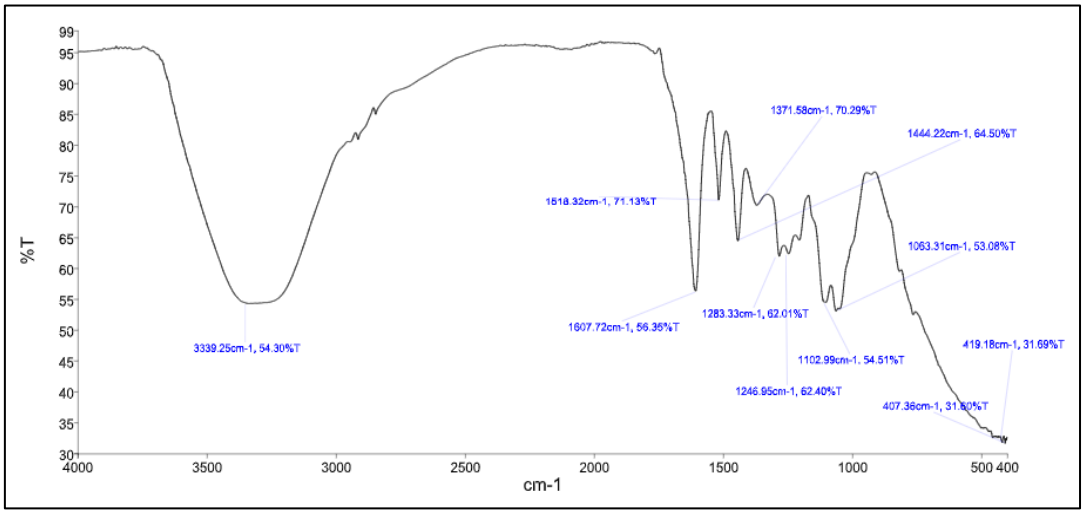

Figure 1: FTIR spectrum of methanolic extract of *S. asoca* bark.

In Silico screening

The result of molecular docking studies is given in table 3.

Table 3: (a) Minimum binding energy obtained by docking phytochemicals of *S. asoca* bark against murine caspase-11 (PDB ID: 6NS7) enzyme

Plant compound	Minimum binding energy (kCal/mol)
Catechin (Pubchem id: 9064)	-5.69
Ellagic acid (Pubchem id: 5281855)	-6.19
Isolariciresinol (Pubchem id: 160521)	-5.36

Table 4: (b) Minimum binding energy obtained by docking phytochemicals of *S. asoca* bark against Bcl2 (B- cell lymphoma 2) (PDB ID: 1G5M) receptor

Plant compound	Minimum binding energy (kCal/mol)
Isolariciresinol (Pubchem id: 160521)	-4.57
Ellagic acid (Pubchem id: 5281855)	-5.9

Cytotoxic evaluation of methanol extract of *Saraca asoca* bark in C127I cell line:

Dose-dependent reduction in cell viability was noticed when the cells were subjected to different concentrations of the extract and the IC_{50} value of *S. asoca* was found to be 16.55 µg/mL. The graph showing the analysis of IC_{50} is depicted in Fig 2.

Table 5: Per cent Inhibition of cells exposed to different concentrations of methanolic extract of *S.asoca* bark, presented as Mean±SE, with n=3 replicate

The concentration of *S. asoca* bark extract (µg/mL)	Percentage Cell Inhibition (Mean±SEM)
320	54.5±0.39
160	49.1±0.41
80	43.9±0.12
40	38.5±0.35
20	36.9±0.68
10	8.6±0.34
IC_{50} (µg/mL)	16.55

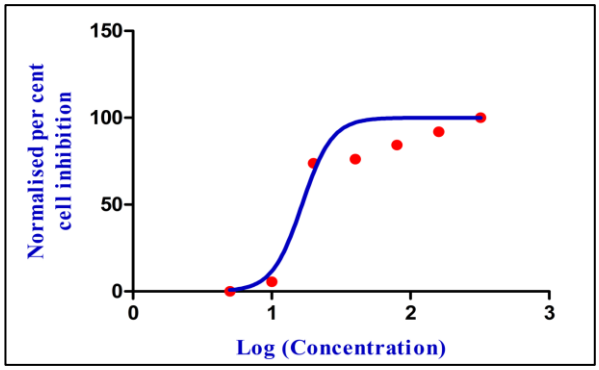

Figure 2: Calculation of IC_{50}.

Acridine Orange/ Ethidium Bromide staining:

After treatment with extract, an acridine orange/ethidium bromide (AO/EB) staining procedure was followed and the live, apoptotic, and necrotic cells were differentiated. The representative pictures of cells exposed to the treatment are depicted in figure 3. In control cells, greenish fluorescence with a circular pattern and evenly dispersed nuclei across the centre were seen. The late apoptotic stage of orange-to-red luminous cells was detected in cells treated with IC_{50} concentration of the extract. Cells that are exposed to treatment also revealed morphological changes like nuclei fragmentation, membrane blebs, and apoptotic bodies.

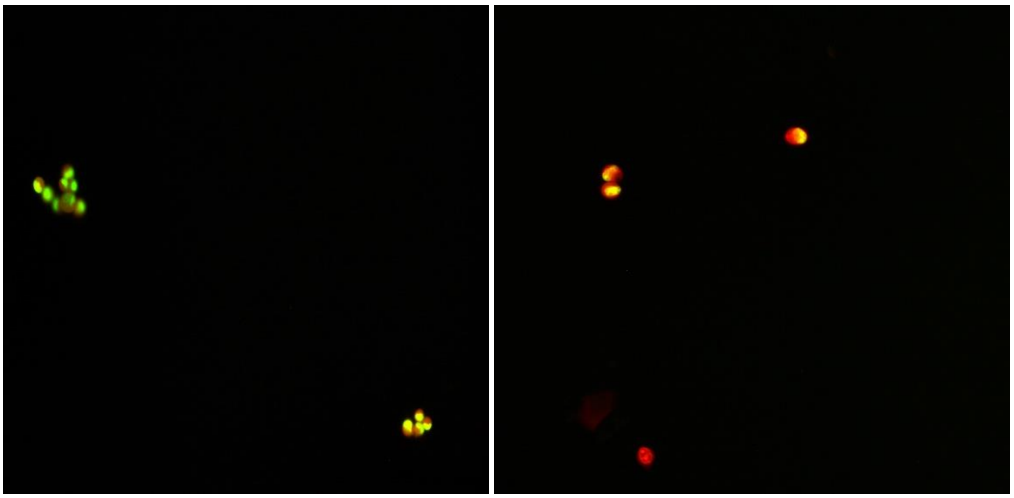

Figure 3: AO/EB staining of C127I cells after treatment with *S. asoca* bark extract at IC_{50} concentration.

DISCUSSION

The present study demonstrated the antiproliferative nature of the methanolic extract of S. asoca in C127I cell lines. Different studies have been conducted in different cell lines indicating the antiproliferative or cytotoxic nature of different compounds. Methanolic extract of *Mallotus phillippensis* was found to be cytotoxic to MCF-7 cells [13]. The antiproliferative nature of glycoalkaloids and metabolites found in *Solanum tuberosum* (potato), *Solanum melongena* (eggplant), and *Solanum lycopersicum* (tomato) were studied in liver cancer cells (HepG2) using the MTT assay [14]. Studies on the activities of solamargine demonstrated that it inhibited the growth of HepG2 cells [15].

From the present study, the phytochemical screening of the methanolic extract of *S. asoca* bark revealed the presence of glycosides, phenolic compounds, tannins, flavonoids, and saponins. FTIR analysis confirmed the presence of alcohol, cyclic alkenes, nito-group, and fluro- group compounds. *S. asoca* bark contains phytochemicals like catechin, epicatechin, procyanidin B_2, leukocyanidine, gallic acid, ellagic acid, isolariciresinol [16], and these compounds were docked against murine caspase-11 (PDB ID: 6NS7) and Bcl2 (B- cell lymphoma 2) (PDB ID: 1G5M) receptors to get the minimum binding energy. Caspases are a family of protease enzymes that provide critical links in cell regulatory networks controlling inflammation and cell death [17]. Caspases and Cytochrome C and other mitochondrial components are involved in the induction of apoptosis where in the key nuclear alterations seen including chromatin condensation, nuclear marginalization, early nuclear breakdown, and nucleosomal ladder building occur [18]. This docking study revealed that compounds like catechin, ellagic acid, and isolaricirecinol have an affinity towards the murine caspase-11 enzyme.

Bcl-2 (B-cell lymphoma 2), a protein encoded by the *BCL2* gene, is a member of the Bcl-2 family of regulator proteins that regulate cell death [19]. The release of inter-mitochondrial membrane proteins is significantly influenced by B-cell lymphoma 2 (Bcl2) family proteins. They include Bax and Bak (proapoptotic molecules) and Bcl2 and BclxL, (antiapoptotic molecules). By preventing the release of cytochrome c from mitochondria and preventing the subsequent activation of caspases linked to apoptotic cell death, Bcl2 inhibits apoptosis [20]. Compounds like isolaricirecinol and ellagic acid have an affinity towards Bcl-2 receptor. An inhibition of the activity of BCl2 proteins can initiate the progression of cancer cells to apoptosis. So, from the molecular docking study, it is evident that these compounds contributed to the antiproliferative effect of the methanolic extract of *S. asoca* bark.

The direct reduction of MTT into purple formazan product by cells in active metabolism illustrates a direct relationship between colour change and cell viability [21]. *In vitro* cytotoxic studies of *Saraca asoca* bark extracts on HT-29 cancer cell line showed a significant inhibition percentage on HT-29 cancer cell line and in MTT assay withe IC_{50} values were 174.1 and 163.2µg/mL on alcohol and aqueous bark extracts, respectively [22]. In the present study, the *in-vitro* cytotoxic potential of the methanolic extract of *S. asoca* bark was assessed in the C127I cell line using MTT reduction assay. Dose-dependent reduction in cell viability was noticed when the cells were subjected to different concentrations of the extract and the IC_{50} value of *S. asoca* was found to be 16.55 µg/mL. As per NCI guidelines, the IC_{50} limit for selecting the plant extracts for anticancer studies is less than 30 µg/mL after 72 h of exposure [23]. Since the IC_{50} value obtained is within the range, the extract can be further studied for its anticancer property.

Apoptotic cells can be distinguished on the basis of morphological traits, such as blebbing of the plasma membrane with irregular outlines, chromatin condensation, and shrinkage of cytoplasm with the presence of apoptotic bodies, [24]. Acridine orange/ethidium bromide dual staining is used to detect apoptosis based on the differential uptake of the two fluorescent DNA binding dyes to determine the live,

early and late apoptotic cells. The nuclei of normal cells stained by AO penetration which emits green fluorescence by attaching to DNA, EB, on the other hand, dyes the nuclei of late apoptotic and necrotic cells with red colour and the result shows that *S. asoca* induced apoptosis.

From the results of the study, it can be evidenced that the alcoholic extracts *of S. asoca* contains potent phytochemicals that can be developed into an anticancer drug. The isolation of the potent biomolecule(s) and their characterisation should be done and tested in animals for their cytotoxicity.

CONCLUSION

From the study, it could be concluded that the methanolic extract of *S. asoca* bark induces apoptosis in cancer cells in a dose-dependent manner and it could be developed as a lead molecule for cancer management after conducting clinical trials in vivo and human subjects.

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Competing of interest

The authors declare that they have no competing interest.

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