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Cytotoxic Evaluation and HPLC Profiling of *Saraca asoca* Ethanolic Extract for the Treatment of Endometriosis

R J Reshmi Nair[1], S Lakshmi[2], K P Suja[2] and K K Sabu[1]
[1]Jawaharlal Nehru Tropical Botanic Garden & Research Institute (JNTBGRI), Palode, Thiruvananthapuram 695 562, India. (Research Centre of University of Kerala)
[2]CRDC, HLL Lifecare Limited, Akkulam, Thiruvananthapuram 695 017, India.

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***Corresponding Author Email:** sabu@jntbgri.res.in

Abstract
This study investigated antioxidant activity of *Saraca asoca* extract using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and phytochemical analysis to evaluate the presence of secondary metabolites. *In-vitro* cytotoxic analysis was investigated in MDA-MB-231 (non-estrogenic) and MCF-7(estrogenic) cell lines along with endometrial cancer cell line Ishikawa. Viability of the extract was tested in L929 normal cell line with MTT assay. Column chromatography was performed and its HPLC profile were evaluated for the presence of bioactive compound, catechin. The extract exhibited significant concentration-dependent antioxidant and cell line activities. The safety profile of the extract with 85.86% cell viability in 500µg shows its efficacy. Its effect in MCF7 with IC_{50} value (416µg/ml) and MDA-MB-231 (518µg/ml) and prominent activity in Ishikawa cell lines (122µg/ml) indicate its significant use for endometriosis treatment. In the present study, DPPH activity HPLC profile and cytotoxic effects of the extract significantly substantiated the utility of the plant for endometriosis treatment.

Keywords
DPPH, cell lines, HPLC, *S.asoca*, Endometriosis

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INTRODUCTION:
Endometriosis is a chronic inflammatory disease that predominantly affects women of reproductive age. It is defined by the presence of uterine endometrial tissue seen mainly in ovaries, pelvic cavity etc. These tissues containing glands and stroma that are functionally capable of responding to endogenous, exogenous or to local hormonal stimuli leading to endometriosis disease condition. It is a highly estrogen-dependent disease associated with the biological effect of estrogen in the target tissue [1]. Current treatment method includes hormonal medications or surgeries [2] that carries risk of complications with their side-effect profiles. The secondary plant metabolites with structural similarity to estradiol (E2) has the ability to modulate estrogen receptors and their pathways [3]. *Saraca asoca* bark is well mentioned in Indian treatise *Charaka Samhita* and *Bhavprakash Nighantu* as main source for treating uterine related issues and for regularizing menstrual disorders. The bark part of this plant is reported with significant amounts of biologically active phenolic compounds that have stimulating effects on endometrium [4] and a wide range of evident biological activities such as anti-mutagenic, genoprotective properties [5], antioxidant and anti-breast cancer, [6], anti-bacterial, anti-menorrhagia [7], anthelmintic effects [8] etc.
Saraca asoca of the family Fabaceae has been used in Indian traditional medicine as an excellent source

to cure gynecological problems. The plant parts are being used in preparation of various herbal drug formulations as it is a rich source of catechin, epicatechin, catechin polymers and glycosides [9]. This plant is having selective estrogen receptor modulator (SERM) activity required for endometriosis treatment [10] and the secondary metabolites found in this plant can exert its beneficial effects through synergistic action with several chemical compounds acting in single or multiple target sites with low or no toxicity [5].

As endometriosis is occasionally accompanied by tumours that can lead to adenocarcinomas, the present study was to evaluate antioxidant activity of the extract with DPPH assay and its *in-vitro* assessment in estrogenic (MCF7), non-estrogenic (MDA-MB-231) and endometrial carcinoma cells (Ishikawa) with MTT assay along with safety assessment in normal cell line (L929). *S. asoca* extract with active marker compound detected through HPLC profile and its antiproliferative effect in ishikawa cells thereby hypothesizing significant effect of the extract for endometriosis treatment.

MATERIALS AND METHODS:

All solvents used in this study were of analytical grades and were purchased from Merck.

Collection of plant material:

Bark of *Saraca asoca* was collected from a home garden Thiruvananthapuram, Kerala and the species was confirmed by a taxonomist at JNTBGRI.

Processing of plant material and Preparation of crude powder:

The collected plant material was cleaned and air-dried in hot air oven to remove moisture content at 40°C in the laboratory. The dried bark was crushed and powdered in electric mixer-grinder to fine powder and stored in airtight container or poly bags [11].

Preparation of ethanolic extracts:

24 gm powdered plant material was subjected to pressurized sequential extraction using accelerated solvent extractor (ASE 150, Dionex Inc., USA). The powder with diatomaceous earth was mixed at 4:1 ratio and were placed in a 100 ml sample cell and loaded onto the system. Extraction was performed under 1500 psi pressure at 60°C with a flush volume of 90% using three static cycles using HPLC grade ethanol. The process was repeated several times to get the required amount of extract [12]. The solvents were then evaporated in a rotary evaporator (Buchi, Switzerland). One portion of the dried extracts was weighed and used for isolation of marker compound and the other dissolved in DMSO to the desired concentrations and were assayed for cell line studies.

Preservation of extracts:

The extract was labelled and kept in airtight glass bottles at 4°C in refrigerator for further use.

Phytochemical analysis of extracts:

Ethanolic extract of *S. asoca* obtained was subjected to qualitative phytochemical tests for the determination of active secondary metabolites such as alkaloids, tannins, flavonoids, saponins, glycosides, triterpenes, reducing sugars and proteins by standard procedures [13].

Determination of DPPH radical scavenging assay:

Evaluation of antioxidant activity of *S. asoca* bark extract was determined using stable 1, 1 –diphenyl-2-picrylhydrazyl (DPPH) method. It has been largely used as a reliable and reproducible method to determine the antioxidant property of a plant [14].

Typically, 3.96 mg DPPH (Alfa Aesar) was prepared in 100 ml MeOH. 2ml of DPPH in methanol was added to 1 ml of varying concentrations (2 to 50 µg/ml) of ethanolic extract, vortexed and allowed to react at room temperature in a dark chamber for 30 min with respective control. The experiment was analysed at 517 nm using UV-visible spectrophotometer (Shimadzu model UV-1700). Methanol (Merk) and ascorbic acid (Sigma Aldrich) were used as blank and standard respectively. The radical scavenging activity or percentage inhibition was measured with decrease in the absorbance of DPPH and calculated using the following equation:

Inhibition (%) = [Absorbance control - Absorbance test sample] / Absorbance control x 100

Higher the percentage inhibition, the colour of the solution faded from violet to yellow. IC_{50} value was calculated by plotting % inhibition against respective concentrations. The experiments were performed in triplicates.

Compound isolation and thin layer chromatography:

The compounds from the plant extract were separated through repeated column chromatography on silica gel (60-120 mesh) loaded glass column. The dried ethanolic extract was scrapped and loaded onto top of the packed column and eluted with chloroform: methanol (95:5 to 60:40). Fractions of 50 ml were collected and concentrated. These fractions were then monitored on silica gel pre-coated TLC plates (Merck TLC Silica gel $60F_{254}$ plates, 0.2 mm thickness, 20×20 cm) using toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2) as the mobile phase. The spots were visualized by anisaldehyde reagent. All the fractions containing similar compounds (appearing as single spots) were combined, concentrated and vacuum dried. Purity and authenticity of the isolated compounds were ascertained by running on TLC

plates along with reference standard and R_f values were calculated using the formula:

Retention factor = Distance travelled by the solute. / Distance travelled by the solvent.

Preparation and sample profiling of *Saraca asoca* for RP-HPLC analysis:

Ethanolic extract of *S. asoca* and standard catechin were dissolved in methanol, then sonicated for 5 min to obtain 5 mg/ml and 1 mg/ml concentrated solutions respectively. Filtered the solution through 0.2 mm NY-membrane (Sartorius, Germany) before injecting into HPLC. The reference standard catechin (Sigma Aldrich) was subjected to HPLC analysis (HPLC; LC Agilent, Agilent Technologies, Boblingen, Germany) using a Reverse phase sun fire column (4.6 ×150 mm, C18 5µm; Waters Corporation, Milford, USA). HPLC solvents were purchased from Merck, Mumbai, India. The isocratic solvent system was acetonitrile: 0.1% orthophosphoric acid in water (20:80). The flow rate was 0.7 ml/min and the detection wavelength were set at 280 nm with 33-min run time for both standard and sample.

Cell proliferation inhibition assay and treatment procedure:

Antiproliferative activity of the extract was determined with standard MTT assay. The cell lines for the study, MCF-7 (ER positive cells) and MDA-MB-231 (ER negative) and L929 (normal cells) were obtained from NCCS, Pune. All cells were grown in Dulbecco's Eagles Minimal. Essential Medium (DMEM, Invitrogen) and Ishikawa in (DMEM F12) containing 10% fetal bovine serum (FBS) (GIBCO BRL Laboratories, New York, USA) and 1% penicillin-streptomycin solution (Sigma Aldrich). Trypsin-ethylene diamine tetra acetic acid (EDTA) was used for detaching monolayer cells to single cell suspensions. 100 µl per well were seeded into 96-well plates with 10,000 cells/well and incubated for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 hr, these cells were treated with different concentrations of the extract for 24 and 48 hrs. The extracts were initially dissolved in dimethyl sulfoxide (DMSO) and further diluted in cell culture medium to produce four concentrations. Hundred microliters per well of each concentration was added to plates to obtain final concentrations of 500, 250, 125 and 62.5 µg/ml. Culture medium without samples were served as control. After incubation 100µl of MTT (7mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4 hr. The medium with MTT was then aspirated and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using ELISA plate reader. Triplicate was maintained for all concentrations. The percentage cell toxicity was determined using the following formula.

% Cell cytotoxicity = 100 - Absorbance (sample)/Absorbance (control) x100.

% Cell viability =100 - % cell cytotoxicity

In terms of cytotoxicity, lower the IC_{50} higher the cytotoxicity [15]

RESULTS:

Plants contain phytochemicals that are produced as primary and secondary metabolites which have diverse pharmacological activities and play vital roles in treatment of various diseases. *Saraca asoca* is a traditional by popular plant with many medicinal uses. Qualitative phytochemical screening of the extract showed alkaloids, flavonoids, tannins, terpenoids, glycosides, Phenols and flavanols (Table 1).

DPPH radical scavenging assay

The antioxidant activity was expressed based on the scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the solution. The degree of colour change can be expressed as the amount of antioxidant present in the extract, and it is proportional to the concentration and potency of the antioxidant [16]. The IC_{50} is the concentration of antioxidant required to reduce the original amount of radical by 50 % and in the present study scavenging activity was observed as 88.84% in 50 µg concentration with an IC_{50} value of 15.94 compared to that of standard ascorbic acid.

Values are expressed as percentage mean of 3 replicates.

TLC and HPLC profiling of *S. asoca* extract

S. asoca is a traditionally important medicinal plant which is widely used commercially in modern Ayurvedic medicine for various gynaecological disorders. TLC and HPLC profiling of the extract has been carried out in the present study and identified the active marker compound catechin. Optimization of mobile phase for TLC was carried out with toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2) as the mobile phases and isocratic elution based on 0.1% orthophosphoric acid in water as solvent A and ACN as solvent B for HPLC. The results showed best resolution and sharp peaks. The RP C 18 column has shown better peak resolution in the above-mentioned analytical conditions. Optimal detection wavelength was 280 nm. The standard catechin showed an RT 6.5 which was detected in sample as well.

Activity of *S. asoca* extract in different cell lines

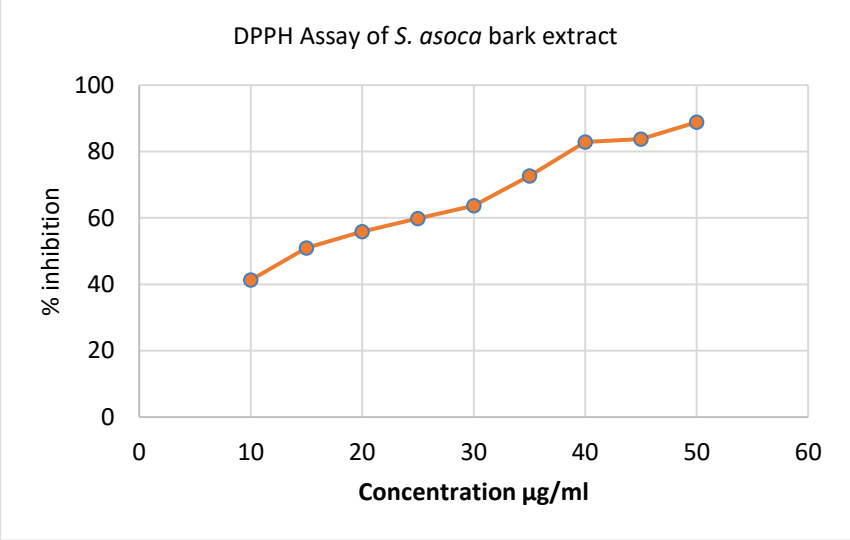
All the cells were seeded and treated with *S. asoca* ethanol extracts at 500, 250, 125 and 62.5 µg/ml of concentration and cytotoxicity was evaluated. The bark extract inhibits cell growth in dose-dependent manners (Fig5). The extracts exposure demonstrated cytotoxicity of 57.98% in MCF7 and 49.24% in MDA-MB-231 at 500 µg/ml concentration in 48hr of incubation. Meanwhile in Ishikawa cells a higher toxicity of 74.1%, 68.8%, 52.8% and 40.9% cell death were shown in 48 hr of incubation for the above-mentioned respective concentrations. Prominent cytotoxicity with IC_{50} values (416µg/ml, 518 µg/ml and 122 µg/ml) were observed in MCF7, MDA-MB-231 and ishikawa cell lines respectively. The ethanolic extract that elicited cytotoxicity in MCF-7 (ER positive cells) and MDA-MB-231 (ER negative) along with prominent activity in Ishikawa cells encourages the investigation in normal cell line.

In-vitro evaluation done in normal L929 cell line (Fig 6) did not induce significant cytotoxicity up to the concentration of 500 µg/ml. The result showed 85.86% viability of these cells even in 500 µg/ml concentration. All the experimental data were represented as a mean of triplicates.

Table 1. Qualitative phytochemical analysis of ethanolic extract

Tests	*Saraca asoca* EtOH extracts
Tannins	+
Saponins	-
Flavonoids	+
Quinine	+
Terpenoids	+
Glycosides	+
Cardiac Glycoside	-
Triterpenoids	-
Phenols	+
Steroids	+
Carbohydrates	+
Proteins	+
Alkaloids	+

Fig 1: *In-vitro* antioxidant activity of *Saraca asoca* bark



Values are expressed as percentage mean of 3 replicates.

Fig 2: Antioxidant activity of standard ascorbic acid

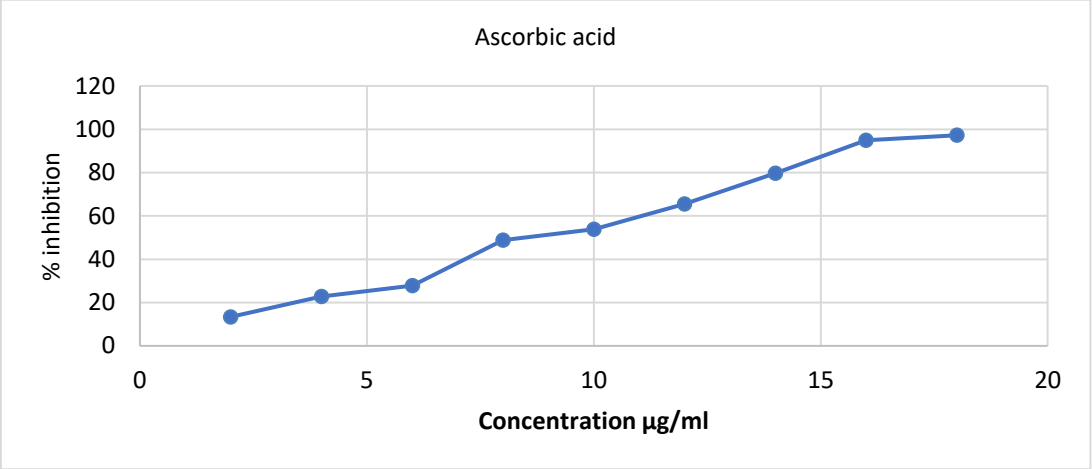


Fig 3: HPLC profile of *S. asoca* ethanolic extract

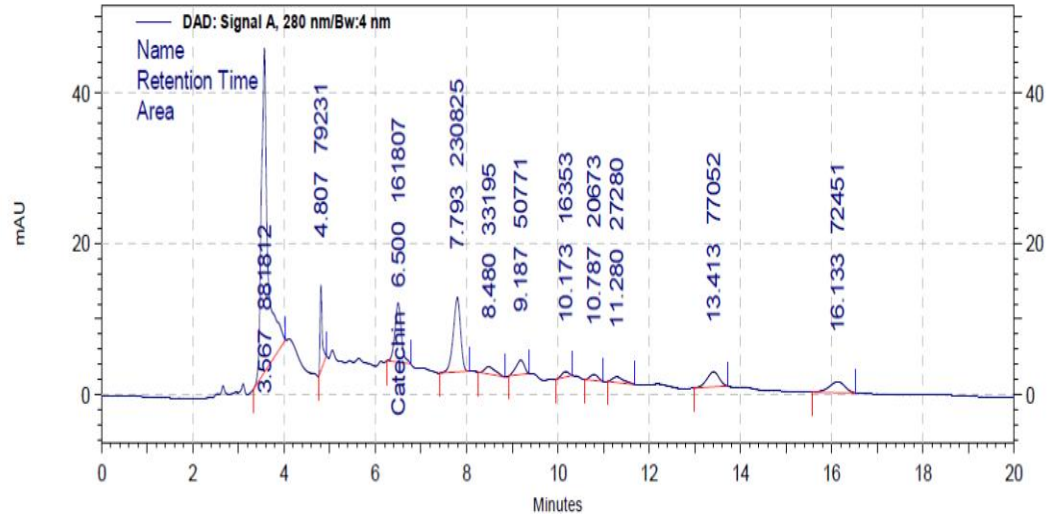


Fig 4: HPLC profile of standard catechin

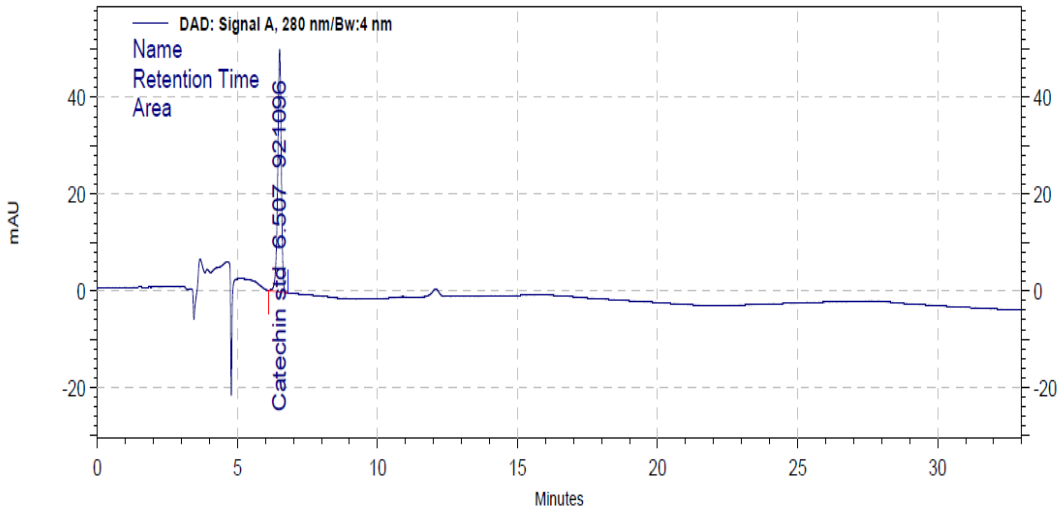


Fig 5: Cytotoxicity in MCF-7, MDA-MB-231 and Ishikawa cell lines with 48hr of treatment.

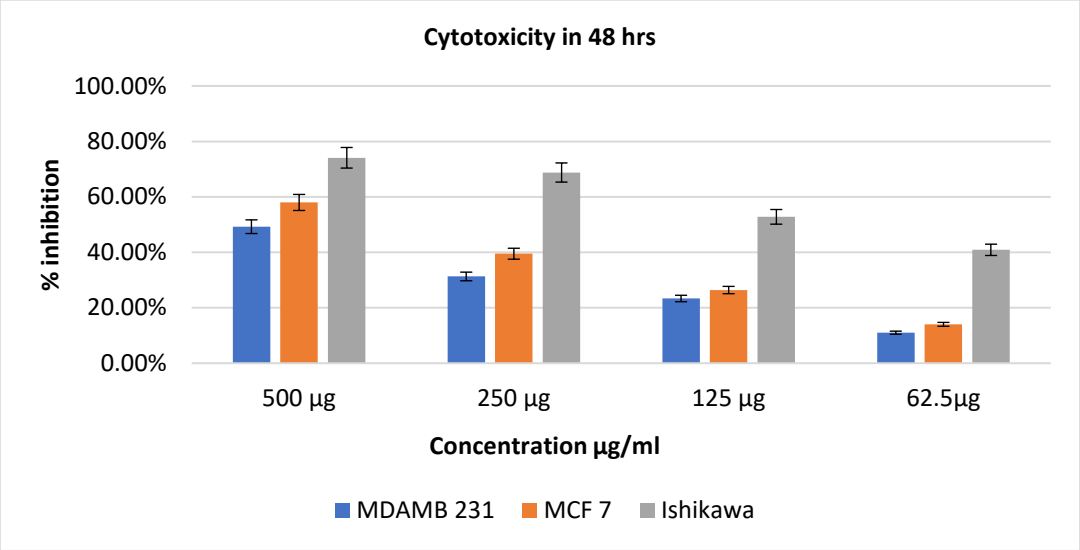
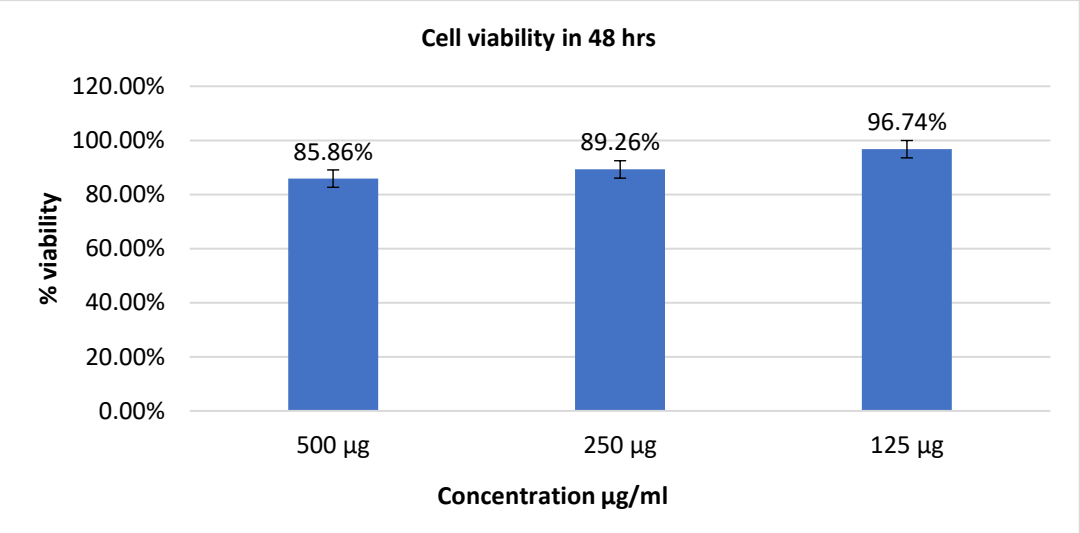


Fig 6: Viability of L929 cell lines with *Saraca asoca* extract



DISCUSSION:

Unfavourable side effects and lack of appropriate medicines have led to a serious problem in endometriosis treatment. These lead to a growing interest in the use of plant-based compounds to develop safe and more effective therapeutic agents for the disease. *Saraca asoca* is a drug of choice for its stimulating effect on the ovarian tissue, endometrium and for treating leucorrhoea and internal bleeding [17,18]. Bark extract of the plant was reported with a number of compounds including catechin, epicatechin and epigallocatechin [19,20,21,] with other secondary metabolite compound from flavonoids, terpenoids and lignin may found responsible for these therapeutic action.

It was reported that active compounds from plants can lead to increase in antioxidant activity [22] and higher phenol and flavonoid contents were observed to have a positive correlation with it [23]. In DPPH assay significant activity with an IC_{50} value of 15.94 µg/ml was obtained in the present study. As anti-oxidants can play a protective role in normal healthy cells, many studies suggested that they prevent the spreading of ROS (reactive oxygen species) and were able to protect adjacent cells from oxidative DNA damage[24,25]. Although the pathogenesis of endometriosis is not fully elucidated, it is suggested that abnormal increase in ROS and oxidative stress can aggravate the disease condition [26]. Several clinical trials have also shown evident that intake of antioxidants can protect the healthy cells from oxidative stress as well as reduce toxic side effects of cancer therapy without affecting therapeutic efficacy [27,28].

Medicinal plant extracts have been used for treating various diseases all over the globe as they can be

easily prepared and cost effective. Chromatographic fingerprints and active compound identification of marker compounds may further be useful for laboratory authentication of plant-based drug samples. We have identified the marker compound catechin in the extract with HPLC at RT 6.5. However, their synergistic effect in biological pathways should be evaluated. DPPH assay and phytochemical investigation of major compounds in the extract lead to the evaluation of its effect on ER positive and ER negative cell lines. In the present work growth inhibition of (triple positive), which is known to express estrogen, progesterone and human epidermal factor receptor 2 (HER2) to that of MDA-MB-231 (triple negative) cell lines were revealed using MTT assay. We got significant activity which can be related to some recent studies of SERM drugs in which both these cell lines demonstrate toxicity level due to different modes of actions [29]. It can induce cytotoxic effect either in an estrogen-dependent pathway (for estrogen-positive cells such as MCF7) or estrogen independent pathway (for estrogen-negative cells such as MDA-MB-231).

Similarly, in this study it has been observed clearly that the crude extract has significant activity against endometrial cancer cell line Ishikawa which are highly responsive cell lines that maintain functional steroid receptors to estrogen, progesterone and androgen, making them particularly useful for *in-vitro* investigations in endometrial biology.

Remarkable antiproliferative effect of the extract was observed in a dose dependent manner. Although these plants are being used from ancient time, to determine whether the extract is having toxicity to normal cells, safety evaluation was done in L929.

S. asoca are having phytoestrogenic compounds like flavonoids that are non-steroidal estrogen probably responsible for its antioxidant and cytotoxic effects. The present studies on different cell lines suggests that *S. asoca* are safer to be used as a therapeutic agent with selective estrogen receptor modulator activity (SERM) beneficial for endometriosis treatment.

CONCLUSION:

The bark part of the plant was reported to have a stimulating effect on ovarian tissue and endometrium and were also used in the treatment of menorrhagia and uterine fibroid. It is a versatile plant with reliable source of medicinal properties due to the presence of various types of phyto compounds. *In-vitro* investigations demonstrated the activity of the extract in MCF-7, MDA-MB-231 and Ishikawa cell lines through MTT assay. Moreover, the extract did not show cytotoxic effect on L929 cell line. As it has shown inhibitory effect in estrogenic and nonestrogenic cell lines along with significant effect on endometrial cancer cells, it can be used as a promising candidate for endometriosis treatment. Finding herbal drug that are efficient and are able in reducing the cost of treatments for patient suffering from the disease should be taken seriously. As this plant is very popular as nutrient supplements, they are potentially practical for preclinical and clinical studies. Detailed *in-vivo* research and further investigation in molecular level are in need to explore and establish it as a standard drug for endometriosis treatment.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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