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Antiproliferative effect of *Saraca asoca* methanol bark extract on triple negative breast cancer (TNBC)

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Abstract

Background *Saraca asoca* (Asoka) is reported to possess phytoestrogenic components with anticancer properties. The phytoestrogens are recognized as natural agonists for ER*β*, which acts as an antagonist to ER*α*. Despite the absence of ER*α*, studies have identified ER*β* in 50–80% of triple negative breast cancers (TNBC). Thus, the present study is intended to reveal the role of phytoestrogens of Asoka on TNBC. The cytotoxic effect of Asoka methanol bark extract was analyzed on different breast cancer cell lines by MTT assay. Estrogen-screen assay was employed to determine the proliferative/antiproliferative effect. Identification of phytoestrogens in Asoka was accomplished using LC-MS analysis and in silico docking studies were performed to investigate possible interactions of phytoestrogens with ER*α* and *β*.

Results The extract of Asoka was found to be cytotoxic against TNBC cell line, MDAMB-231 with IC_{50} of 70.22 ± 1.89 µg/mL and towards HER^+ breast cancer cell line, SKBR3 with IC_{50} of 98.41 ± 2.31 µg/mL, respectively. Whereas the extract did not show any cytotoxicity towards ER*α* cell line, MCF-7 even up to the concentration 300 µg/mL. Estrogen-screen assay emphasized an estrogenic effect of the extract on MCF-7 and an anti-estrogenic/anti-proliferative effect on MDAMB-231 cells. LC–MS analysis identified phytoestrogens such as *β*-sitosterol, quercetin, kaempferol and others. The docking results revealed good binding efficacy of phytoestrogens with ER*β* than ER*α* and quercetin shows more affinity with the highest docking score of − 9.220. Strikingly, it was found that the *S. asoca* methanol extract was preferentially cytotoxic to TNBC cells.

Conclusion The study demonstrates selective anticancer properties of *S. asoca* methanol extract on TNBC, which indicates a selective impact on ER subtypes. The identification of phytoestrogens, such as *β*-sitosterol, quercetin and kaempferol, in the Asoka methanol bark extract provides a molecular basis for its observed effects. In silico studies further support the view that these phytoestrogens may preferentially interact with ER*β* rather than ER*α*. Quercetin, in particular, demonstrated the highest binding efficacy with ER*β*, suggesting its potential role in mediating the anticancer effects observed in TNBC cells. Further research is warranted to explore the full therapeutic potential of phytoestrogens in breast cancer treatment.

Keywords Asoka, Anticancer, Phytoestrogen, Estrogen receptor, Molecular docking, TNBC

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Background

Saraca asoca (Roxb.) De Wilde, commonly known as Asoka, belonging to the family Fabaceae is considered one of the most ancient and holistic trees in India. Various ethnopharmacological uses of Asoka in different treatment aspects are well documented in Indian old classical Ayurvedic treatises, *Charaka Samhita* (1000 BC), *Susruta* (500 BC) *Vaghbatta* (sixth century) *Dhanvantari Nighantu* (ninth century) and *Chakradatta*, (eleventh century) etc. In Ayurveda, the stem bark of Asoka is used to make *Asokarishta*, a polyherbal decoction used to manage various gynecological complications, especially menorrhagia [1]. This traditional practice reflects the significance of Asoka especially in women's health, as emphasized in the traditional healing systems of India. Inspired by these treatises, several studies have validated the ethnobotanical claims and unveiled novel pharmacological properties [2] like antibacterial [3], antioxidant [4], antipyretic [5] antihyperglycemic [6], anthelmintic [7] and anticancer [8] activities. The cytotoxic activity of *S. asoca* on the breast (MDAMB-231, MCF-7), cervical (HeLa), colon (HT-29), and lung (A549) cancer cell lines were reported [9–11]. *Saraca asoca* exhibits chemopreventive activity against acute myeloid leukemia (AML) and DMBA/croton oil-induced skin papilloma formation in mice [12, 13]. The phytochemical analysis of the stem bark revealed the presence of alkaloids, flavonoids, phenols, phytosterols, saponins, tannins, steroids and terpenoids [14]. The phytoestrogens in this plant β-sitosterol, quercetin, kaempferol and catechin are reported to show anticancer properties including breast cancers.

Breast cancer is closely dependent on estrogen in its initiation and progression. Thus, estrogen receptors ERα/β plays a pivotal role in maintaining the homeostasis of the normal mammary gland [15]. ERα, the primary receptor of estrogen activates the cell cycle and stimulates proliferation, but ERβ functions as a counterbalance to ERα, actively inhibiting cellular proliferation and providing a regulatory mechanism to the proliferative effect of ERα [16]. This intrinsic counteraction creates a dynamic interplay between ERα and ERβ, influencing the delicate equilibrium of mammary gland homeostasis. Although ERα serves as the primary receptor for estrogen and is vital for the homeostasis of the normal mammary gland, its activation promotes cell cycle and stimulates proliferation, potentially leading to the initiation and development of cancer. Conversely, ERβ functions as a counterbalance to ERα, actively inhibiting cellular proliferation and consequently, offering potential therapeutic avenues for breast cancer [17, 18]. Various reports indicate that ERβ is expressed in triple-negative breast cancer (TNBC), accounting 50–80 % [19]. By leveraging the inhibitory properties of ERβ, researchers and clinicians can explore targeted interventions to modulate hormonal signaling and disrupt the uncontrolled cell growth characteristic of breast cancer [20]. Several consistent findings have shown that ERβ expression decreases in precancerous and cancerous breast lesions [21].

Currently, the predominant focus lies in the identification of novel selective ERβ agonists, with numerous synthetic and natural molecules demonstrating high efficacy in breast cancer prevention and treatment. Notably, recent studies indicate that phytoestrogens exhibit a heightened affinity for ERβ compared to ERα [22]. The potential for phytoestrogens to accumulate in breast tissue suggests significant clinical implications [23]. Among ERβ agonists, phytoestrogens offer a distinctive therapeutic avenue for targeting ERβ [24]. Liquiritigenin [25] and genistein [26] are notable examples, forming stable complexes with ERβ, recruiting selective co-activators, and interacting with chromatin regulatory elements in estrogen-responsive genes [27]. Both liquiritigenin and genistein have been reported as protective factors against breast cancer, demonstrating the capacity to reduce invasiveness and growth of triple-negative breast cancer (TNBC) through pathway modulation [28, 29]. Another phytoestrogen kaempferol specifically inhibits the migration and invasion of TNBC cells by blocking RhoA and Rac1 signaling pathways. Given these findings, the present study aims to investigate the role of phytoconstituents in Asoka in inhibiting breast cancer cell growth, employing MCF-7, MDAMB-231, and SKBR3 cell lines through estrogen-screen and MTT assays. Additionally, the binding efficacy of phytoestrogens on estrogen receptors will be explored through in silico molecular docking.

Methods

Collection and preparation of extract

The stem bark of *S. asoca* was collected from the Thrissur district of Kerala, India, and its authenticity was confirmed by Dr. N. Sasidharan, Taxonomist, Kerala Forest Research Institute (KFRI), Thrissur, Kerala, India. The collected specimens have been deposited in the Herbarium of KFRI, assigned the voucher specimen number KFRI 4725. The plant sample underwent thorough washing with distilled water, followed by cutting into small pieces and subsequent drying at 45–50 °C for one week. The dried bark was then powdered using a grinder and stored in light-resistant, airtight containers. About 20 g of the powdered sample was subjected to extraction with 200 mL of methanol at room temperature through overnight stirring. The resulting Asoka crude methanol extract was filtered using Whatman No.1 filter paper. The extraction process was repeated 2–3 times, and the residue was evaporated to dryness utilizing a vacuum concentrator. The weight of the dried extract was measured

to determine the percentage yield of the soluble constituents [30].

Cell lines and animals

The triple-negative breast cancer cell line, MDAMB-231, the HER-2 expressed breast cancer cell line, SKBR3, and the hormone-positive breast cancer cell line, MCF-7, were procured from National Center for Cell Science (NCCS), Pune, India. These cell lines were cultured in DMEM medium supplemented with fetal bovine serum (FBS) at a concentration of 10% v/v. For the estrogen-screen assay, phenol red-free DMEM supplemented with charcoal–dextran-treated FBS was employed. Both media were also supplemented with streptomycin (100 µg/mL) and penicillin (100 U/mL). The cell lines were incubated at 37 °C in an incubator with 5% CO_2. Murine tumor cells, including Daltons Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cell lines, were grown in the intraperitoneal cavity of Swiss albino mice and were maintained in the animal house facility at Amala Cancer Research Center. Prior approval was obtained from the Institutional Animal Ethics Committee for the use of experimental animals with Approval No: ACRC/IAEC/17(1)/P-05 dt: 22-12-2017.

Phytochemical analysis

The crude methanol extract obtained from *S. asoca* was dissolved in methanol and underwent qualitative and quantitative analysis to identify the presence and concentrations of various phytochemicals. Different standard tests were employed for qualitative assessment to identify the presence of various compounds. Flavonoids were detected using Shinoda's test, phenols with the ferric chloride test, saponins with the Froth formation test, sterols with Salkowski and Liebermann-Burchard tests, tannins with the lead acetate test, and terpenoids with Salkowski tests [31, 32]. Additionally, the *S. asoca* crude extract was quantitatively analyzed using the Folin–Ciocalteau method to determine the total phenolic content using gallic acid as standard and expressed as mg of gallic acid equivalent (GAE)/g of dry extract [33]. For the quantification of flavonoids, the aluminium chloride colorimetric method was employed utilizing standard quercetin and expressed as milligrams of quercetin equivalent (QE) per gram of dry extract [34]. The experiments were meticulously conducted in triplicates to ensure accuracy and reliability, and the results are reported as the mean ± standard deviation (SD).

Cytotoxicity assay

The short-term cytotoxic activity of the Asoka crude extract was assessed by determining the percentage viability of murine tumor cells such as DLA and EAC, employing the trypan blue exclusion method [35]. The murine tumor cells were cultivated in the peritoneal cavity of female Swiss albino mice (25–30 g, 2 months old) through intraperitoneal injection of 1×10^6 cells/mL. Cells were aspirated aseptically from the cavity of mice after 15 days of inoculation; washed with PBS and centrifuged at 1000 rpm for 5 min. Pellets were resuspended in PBS and the cell count was adjusted to 1×10^6 cells/mL. Cells were pipetted out and added into each tube having PBS with different concentrations of the extract. It was then incubated for 3 h at 37 °C. After incubation, trypan blue dye was added and observed under the light microscope using a haemocytometer. The experiments were replicated in triplicates, and the percentage of cytotoxicity was assessed by enumerating the number of dead cells relative to that of live cells and substituting in the equation:

$$\%\ \text{of cytotoxicity} = \frac{\text{No. of dead cells}}{\text{Total no of cells}} \times 100$$

The dose–response curve was fitted with the Hill equation using data analysis and graphing software, OriginPro 9 software [36].

Breast cancer cell lines such as MDAMB-231, SKBR3 and MCF-7, were employed to assess the antiproliferative activity of the extract using the MTT assay [37]. Approximately, 1×10^5 cells were seeded in 12 well plates containing medium and incubated at 37 °C for 24 h. Cells were then incubated with different concentrations of extract at 37 °C for 24 h. The test also included a blank containing a complete culture without cells. After incubation, 100 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h. The dark blue formazan crystals were dissolved in 1 mL solubilization solution containing isopropanol, concentrated HCl and Triton X 100 by continuous aspiration and re-suspension. The absorbance of the colored product was measured at 570 nm. The cytotoxicity was determined by comparing the percentage death of the treated cell population with the untreated control, indicated by their respective absorbance assessed with the MTT assay. The dose–response curve was fitted with the Hill equation [36].

$$\frac{E}{E_{\max}} = \frac{1}{1+\left(\frac{EC_{50}}{|A|}\right)^n}$$

where the maximum percentage of inhibition is E_{max}, the half-maximal effective concentration is EC_{50}, the Hill coefficient is '*n*' and the extract concentration is '*A*'. The Hill equation was computed using OriginPro 9. The assays were performed in triplicates, and statistical analysis was carried out using one-way ANOVA followed by

Tukey's multiple comparison test in GraphPad Prism 8 software.

Estrogen-screen assay

Breast cancer cell lines expressing ERβ, including MDAMB-231 and cell lines expressing ERα, such as MCF-7, were used for the study. The cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with FBS and phenol red as pH indicators with culture conditions of 5% CO_2 and 95% humidity at 37 °C. The cells were plated in well culture plates and allowed to attach. After 24 h, the seeding medium was removed and replaced with phenol red-free DMEM containing charcoal dextran treated FBS [38]. The cells were treated with different concentrations of 17 β-estradiol (0.1–1000 pM) and incubated at 37 °C for 3 days. After incubation, 100 µL of MTT was added to each well and incubated for 4 h. The dark blue formazan crystals were dissolved in 1 mL solubilization solution by continuous aspiration and re-suspension. The absorbance of the colored product was measured at 570 nm and the cytotoxicity was determined by comparing the percentage death of the treated cell population with the untreated control, indicated by their respective absorbance assessed with the MTT assay [37].

UV–visible spectroscopy

The *S. asoca* crude extract underwent centrifugation at 3000 rpm for 10 min and were subsequently filtered through the Whatman No.1 filter paper. The resulting samples were diluted with the same solvent employed for extraction, achieving a final concentration of 1 mg/mL. Standard solutions of quercetin, kaempferol, and β-sitosterol were also prepared at a concentration of 1 mg/mL of ethanol. Utilizing a UV–Vis spectrophotometer (PG Instruments, UK), the extract and standards were scanned across a wavelength range of 200 to 900 nm, and characteristic peaks were identified.

Fourier transform infrared spectroscopy (FTIR)

To characterize the functional group present in the sample, FTIR spectroscopy was conducted. A translucent sample disk was created by encapsulating 10 mg of *S. asoca* crude powder in 100 mg of potassium bromide (KBr) pellets. The FTIR spectroscopy analysis was performed using a Shimadzu IR Affinity 1 (Kyoto, Japan) within the range of 500 to 4000 cm^{-1} [39]. The obtained raw data was employed to generate FTIR spectra using OriginPro 9 software. The 'spectroscopic tools' were utilized for the analysis of the FTIR spectra (https://www.science-and-fun.de/tools/).

LC–MS analysis

The chemical profiling of the Asoka crude extract was done using High Resolution-Liquid Chromatography/Mass Spectrometry. The analysis was performed on an Agilent 6550 iFunnel Q-TOF LC/MS system (G6550A) equipped with an Agilent 1290 Infinity Autosampler (G4226A), an Agilent 1290 Infinity Binary Pump VL (G4220B), and an Agilent 1200 series thermostatted column compartment. A reverse-phase analytical column (Zorbax SB-C18, 100×2.1 mm i.d., 1.8 µm particle size) was used for separation at a flow rate of 0.3 mL/min for a total of 30 min. Sample injection involved 5 µL of the sample. The mobile phases comprised aqueous 0.1% formic acid (A) and 90% acetonitrile in 0.1% aqueous formic acid (B). Mass spectroscopy utilized a dual ion source system with full scan mode, covering a mass range of 50 to 500 m/z. Mass Hunter Qualitative Analysis software was employed for data analysis.

Molecular docking

Molecular docking was done using Schrodinger Maestro software to investigate the possible interactions between β-sitosterol, kaempferol and quercetin which are specific compounds identified in *S. asoca* extract with the targeted receptors, ERα and ERβ. Also, inbuilt ligand estradiol and the classical chemotherapy drug, tamoxifen was used to find out the binding affinity of estrogen receptors towards them. The structures of proteins with PDB IDs 3ERT and 3OLL were downloaded from the protein data bank for ERα and ERβ, respectively. The protein structures were processed before being used as a receptor for docking. Hydrogen atoms were added, atomic charges were assigned and water molecules that were not involved in ligand binding were removed during the operation. Chains and loops that were missing were also inserted. Protein preparation was done by using the protein preparation wizard. The pre-processing was done with the use of the import and process tab, while the review and modify tab was used for the generation of tautomeric states. The structures were optimized and minimized using the refine tab and protein was prepared for further studies. The optimized structures of the standards β-sitosterol, kaempferol and quercetin were used after converting them to structures with.sdf extension. The imported structures were edited using the 2D sketcher option in the Schrodinger Maestro. Then the ligprep wizard was employed to prepare each ligand using the OPLS3 force field. All tautomers of the structures were generated. Then the molecules were subjected to conformational change to form a stable conformer with the lowest energy. The glide receptor grid generating wizard was used to create a three-dimensional grid with

0.5 Å spacing and a maximum size of 20 Å × 20 Å × 20 Å. Any type of constraint, such as accuracy constraints, H-bond constraints, and so on, can be applied using the receptor grid generation wizard. The XP (extra precision) method was used for docking as it is the most powerful and discriminating procedure. After setting the location of the grid and ligands, docking was done with flexible molecules, and the proteins were used as rigid molecules [40].

Statistical analysis

The data from in vitro studies were presented as mean ± standard deviation (SD), derived from three distinct experiments. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test in GraphPad Prism 8 software. Statistical significance was assigned to p values < 0.05*, < 0.01**, and < 0.001***, while $p > 0.05$ was considered non-significant.

Results

Phytochemical analysis

The qualitative analysis of the *S. asoca* crude methanol extract indicated positive results for the presence of flavonoids, alkaloids, phytosterols, phenols, saponins, tannins and terpenoids. The quantification of polyphenolic content in the *S. asoca* crude extract, determined from the calibration curve ($R2 = 0.998$), revealed a concentration of 120 ± 6.82 mg of gallic acid equivalent (GAE) per gram of dry extract. Additionally, the total flavonoid content in the crude extract, estimated from the calibration curve ($R2 = 0.999$), was found to be 61.54 ± 4.51 mg of quercetin equivalent (QE) per gram of dry extract. These results provide a comprehensive insight into the chemical composition of *S. asoca* crude methanol extract, highlighting its rich polyphenolic and flavonoid content.

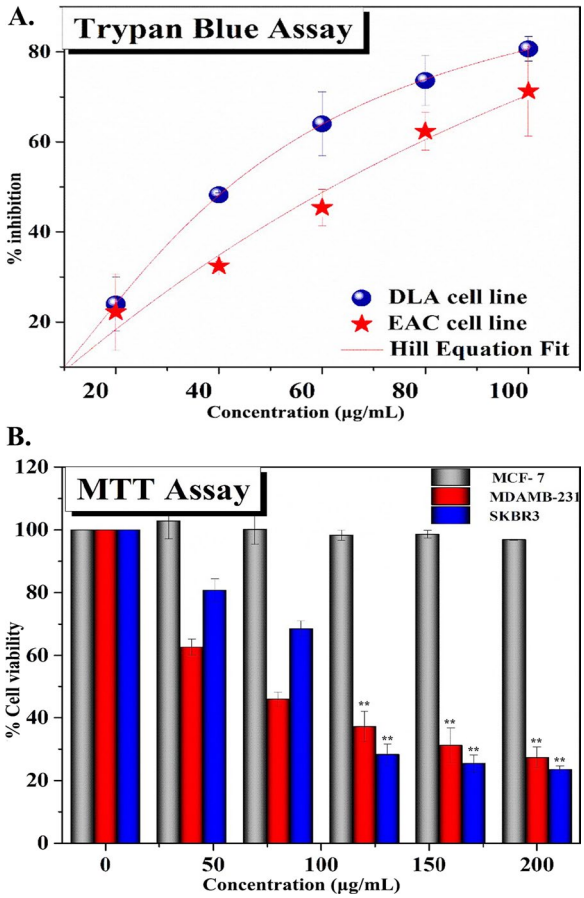


Fig. 1 **A** Cytotoxic effect of *S. asoca* crude methanol extract on murine tumor cells by trypan blue assay, **B** Antiproliferative effect of *S. asoca* on different breast cancer cells by MTT assay. The results are expressed as mean ± SD, with $n = 3$. Statistical comparisons were conducted using one-way ANOVA, followed by Tukey's multiple comparison test. Statistically significant probabilities are denoted as *$p < 0.05$ and **$p < 0.01$

Anticancer properties

The cytotoxic effect of *S. asoca* crude methanol extract was assessed using the trypan blue assay and demonstrated considerable cytotoxic effects on DLA and EAC cells. The concentrations required to achieve 50% cytotoxicity were 42.24 ± 3.65 and 65.44 ± 2.89 µg/mL for DLA and EAC cells, respectively (Fig. 1). In MTT assay, the efficacy of *S. asoca* extract was evident against triple-negative breast cancer cell lines, MDAMB-231, with an IC_{50} of 70.22 ± 1.89 µg/mL, and HER-2 positive breast cancer cell line, SKBR3, with an IC_{50} of 98.41 ± 2.31 µg/mL (Fig. 1). A statistically significant ($p < 0.05$) decrease in cell numbers was observed in both MDAMB-231 and SKBR3 following extract treatment. Treated cells exhibited a noticeable difference in morphology compared to control cells, characterized by cell shrinkage and shift in morphology from epithelial-like to round in both MDAMB-231 and SKBR3 (Fig. 2). However, the extract did not exhibit any cytotoxicity towards MCF-7, even at a concentration of 300 µg/mL.

Estrogen-screen assay

In this study, the ERα expressing MCF-7 cells exhibited a proliferative response to 17 β-estradiol (1000 pM), showing a 30% increase in cell count and a 7% increase in response to crude extract within a 72-h timeframe. Along with 17 β-estradiol, the crude extract exhibited a mild estrogenic effect. Conversely, ERβ expressing MDAMB-231 cells demonstrated a 10% decrease in proliferation with 17 β-estradiol treatment, and the cell population was halved when treated with the crude extract at a concentration of 100 µg/mL (Fig. 3). Notably, the crude extract did not induce cytotoxicity in MCF-7 cell lines even at higher concentrations, while they decreased the

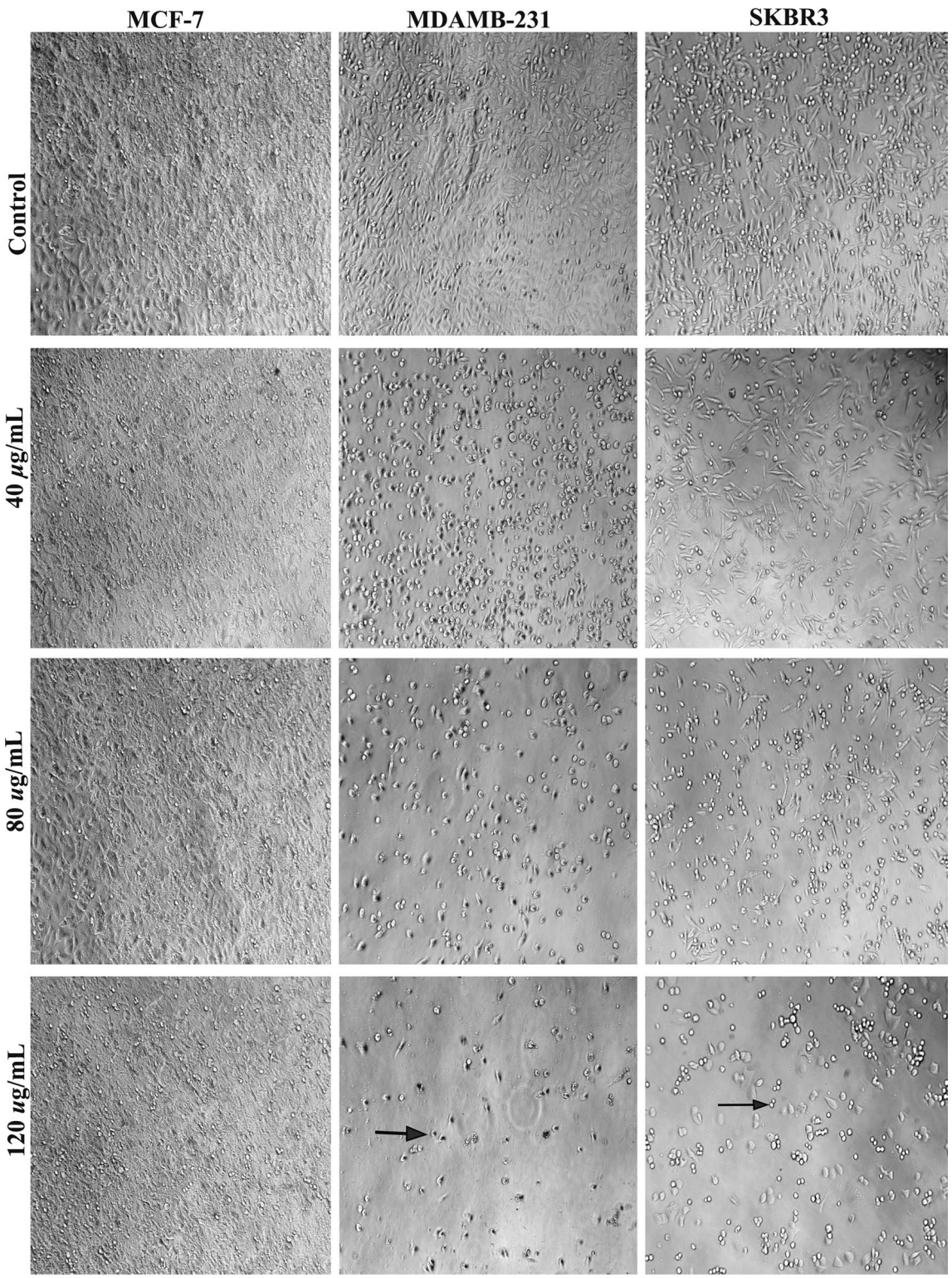


Fig. 2 Morphology of different breast cancer cells after exposure to varying concentrations of *S. asoca* crude extract (20× magnification). The black arrow indicates altered morphology from epithelial-like to round

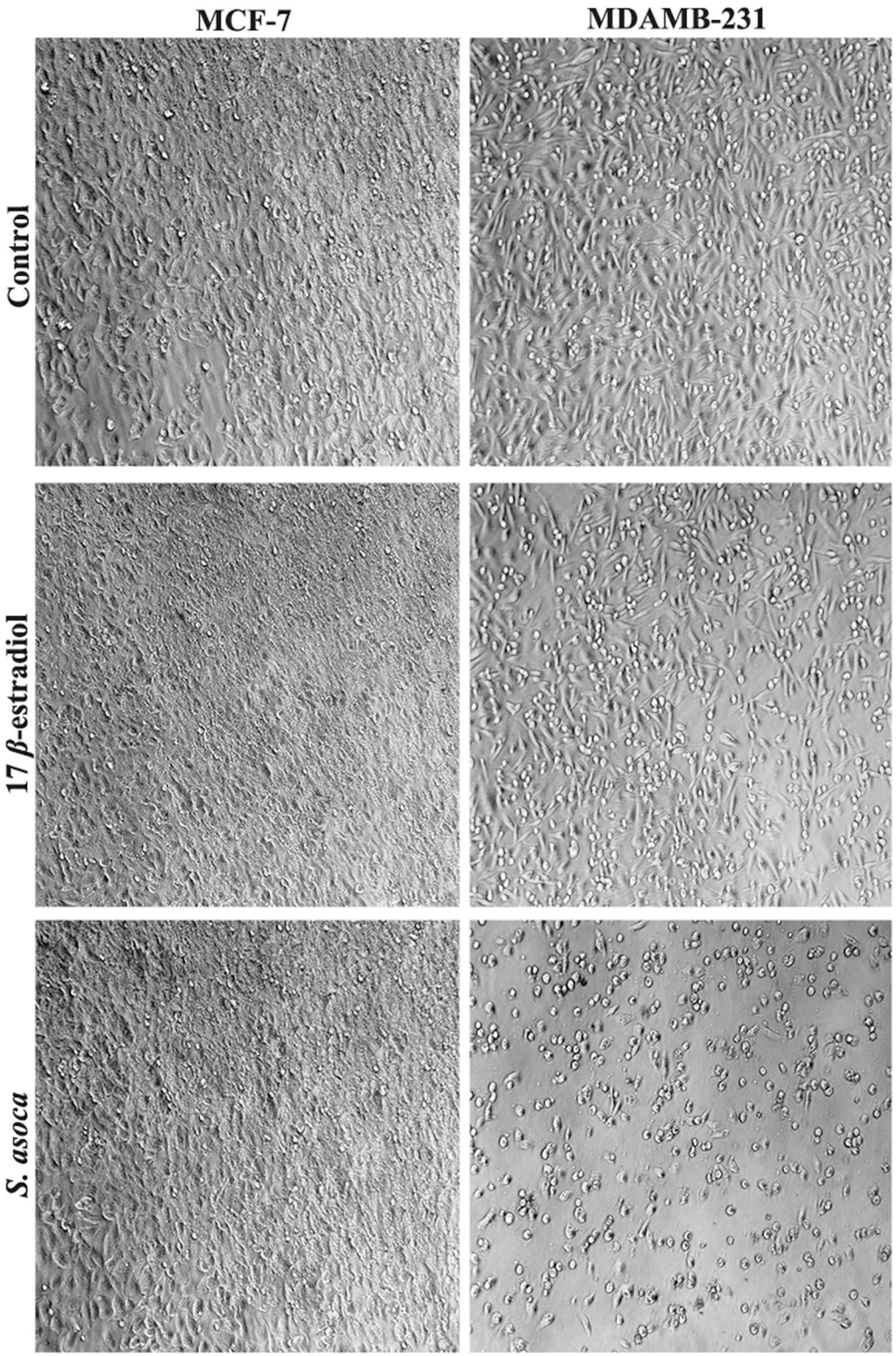


Fig. 3 Change in cell volume of MCF-7 and MDAMB-231 breast cancer cells following exposure to extract and 17 β-estradiol (1000 pM) in the estrogen-screen assay

cell viability of MDAMB-231 cells in a concentration-dependent manner. Consequently, the estrogen-screen assay highlights the estrogenic impact of *S. asoca* crude extract on MCF-7 and its anti-estrogenic/antiproliferative effect on MDAMB-231 cells.

Chemical profiling

Various techniques like UV–Vis spectroscopy, fourier-transform infrared spectroscopy (FTIR) and LC–MS were employed to evaluate the chemical profile of *S. asoca*. The absorption spectrum of UV-Spectrophotometric analysis of *S. asoca*, showed prominent peaks at 232, 275 and 449 nm which is in good correlation with the reported data [41]. These prominent peaks may have arisen from the phytoestrogens. Henceforth, the UV-spectra of standards (quercetin, kaempferol, β-sitosterol) were cross-checked and found that all of the suspected phytoestrogens have three peaks between 230 and 290 nm in the UV range and a single peak in a visible area (387–390 nm) (Fig. 4A). Accordingly, there is a likelihood of superpositioning of these distinct peaks in the *S. asoca* crude extract. The biological activity of any molecule is influenced by its functional groups which play a key role in determining the overall physicochemical properties. In FTIR, the results show functional groups such as alcohol, phenol, ester, alkane, aromatic and alkene in the extract. The functional groups identified in the extract are shown in Fig. 4B and Table 1.

The LC–MS analysis identified some of the important compounds such as caffeic acid, catechin, quercetin, kaempferol, gallic acid, rutin, β-sitosterol, p-coumaric acid, luteolin etc. (Fig. 5). Phytoestrogenic compounds like β-sitosterol, kaempferol, and quercetin present in the extract are presumed to contribute to the proliferative/antiproliferative effects of the *S. asoca* crude methanol extract on MCF-7, MDAMB-231, and SKBR3 cancer cell lines.

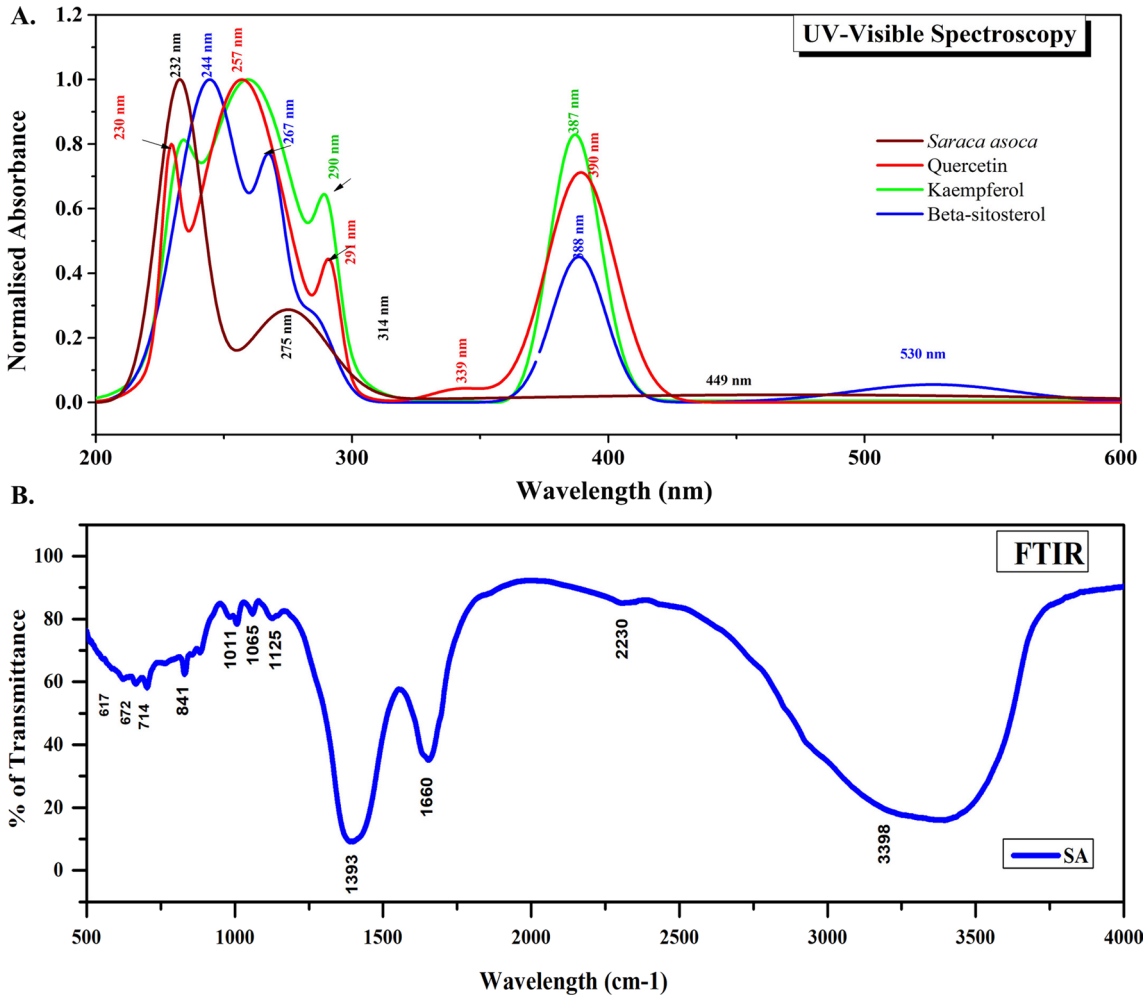


Fig. 4 **A** UV–visible spectrum of *S. asoca* crude methanol extract and phytoestrogen standards, **B** FTIR spectrum of *S. asoca* crude extract

Table 1 FTIR interpretation of compounds of *S. asoca* crude extract

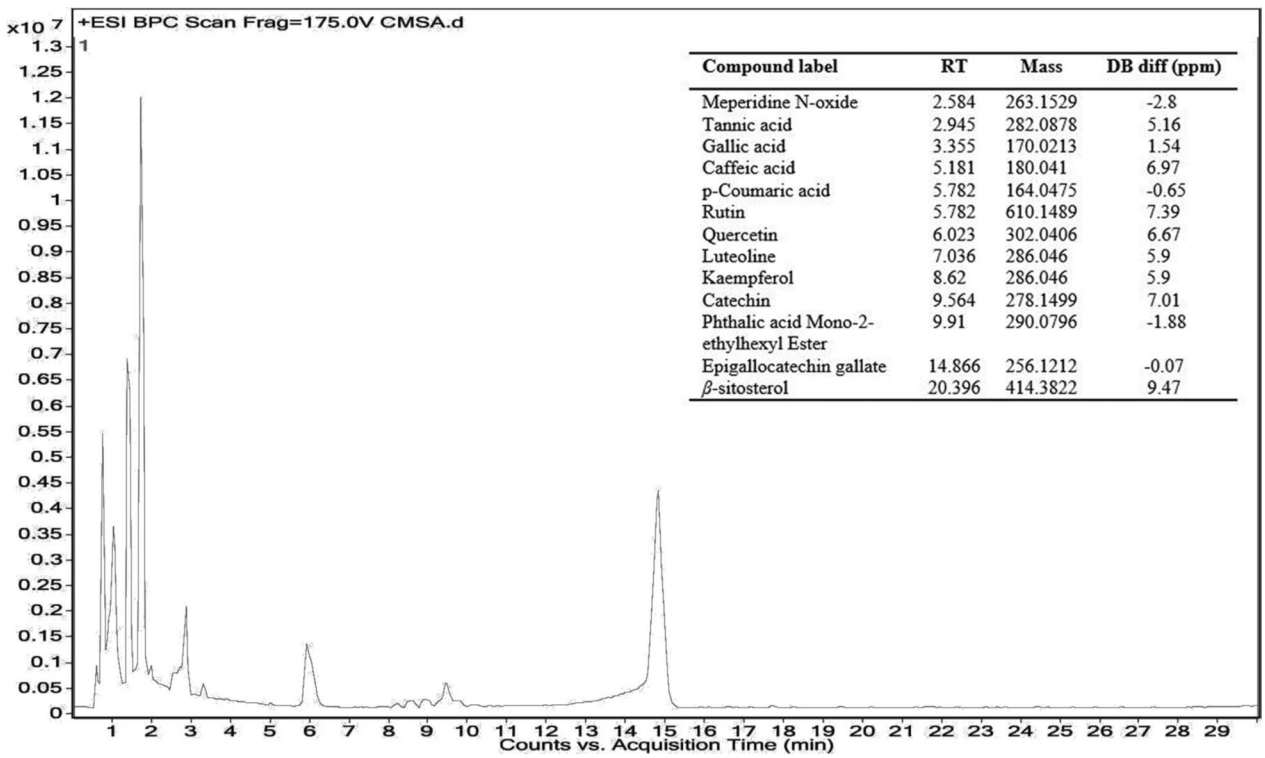
Wave number cm^{-1}	Bond assigned	Functional groups
617	C–H vibration	Alkanes, alkenes
672	C–H and C–C stretching	Alkenes, alcohol, phenol
714	C=C and N–H stretching	Alkenes, amines
841	C–C and C–H stretching	Amides, aldehydes
1011	C–O stretching	Alcohol
1065	C–O stretching	Alcohol, aromatic
1125	C–O stretching	Alcohol
1393	C–O and C–H stretching	Phenol, aldehydes
1660	C–C stretching	Phenols
2230	C=C stretching	Conjugated alkene
3398	O–H stretching	Alcohol

Molecular docking

The studies suggest that certain phytoestrogens act as natural agonists for ERβ, making them promising drug candidates for their ability to modulate the cell cycle, influence epigenetic events, and induce apoptosis. Interestingly, in the current investigation, the extract exhibits specific cytotoxicity towards ERβ expressing cells, not affecting ERα. This raises the intriguing possibility that the phytoestrogens in the plant may act as agonists for ERβ. To explore potential interactions between phytoestrogens and ERα/β, molecular docking was performed using Schrodinger Maestro software.

Interaction of phytoestrogens with ERα

The docking of the inbuilt ligand, estradiol into the 3D structure of ERα was done using a glide dock. The amino acid residues in the active site of 3ERT are Trp383, Leu384, Leu387, Met388, Gly390, Lbu391, Val392, Arg394, Met342, Met343, Leu345, Leu346, Thr347, Asn348, Leu349, Ala350, Asp351, Glu353, Leu354, Leu327, Phe404, Leu402, Leu428, Phe425, Ile424, Val422, Met421, Gly420, Glu419, Val418, Met517, Ser518, Lys520, Gly521, Met522, Glu523, Hie524, Leu525, Met528, Lys529, Cys530, Val533, Leu536, Leu539. The estradiol was docked into the active site region and interactions were made with the residues by hydrogen bonding with GLU353 and ARG394 and electrostatic bonding with ASP351. The inbuilt ligand shows a docking score of −12.17 and binding energy of −125.19 kcal/mol. The quercetin was docked into the active site region making interactions with the residues by hydrogen bonding with ASP351. The docking score and binding energy were found to be −6.945 and −47.026 kcal/mol which was more compared to kaempferol (−6.93). Tamoxifen shows



Compound label	RT	Mass	DB diff (ppm)
Meperidine N-oxide	2.584	263.1529	-2.8
Tannic acid	2.945	282.0878	5.16
Gallic acid	3.355	170.0213	1.54
Caffeic acid	5.181	180.041	6.97
p-Coumaric acid	5.782	164.0475	-0.65
Rutin	5.782	610.1489	7.39
Quercetin	6.023	302.0406	6.67
Luteoline	7.036	286.046	5.9
Kaempferol	8.62	286.046	5.9
Catechin	9.564	278.1499	7.01
Phthalic acid Mono-2-ethylhexyl Ester	9.91	290.0796	-1.88
Epigallocatechin gallate	14.866	256.1212	-0.07
β-sitosterol	20.396	414.3822	9.47

Fig. 5 LC–MS spectrum of *S. asoca* crude methanol extract

a docking score of −10.512 and *β*-sitosterol did not dock with the binding pocket of ER*α*. The 3D interaction picture of the study is shown in Fig. 6A and their docking score and binding energy are tabulated in Table 2.

The 2D image (Fig. 6A) reveals the type of interaction between the ligands and amino acids in the active sites of ER*α*. The inbuilt ligands estradiol, tamoxifen, kaempferol and quercetin form p bonds from their aromatic ring to Phe 404 but the number and nature of hydrogen bonds vary with ligands. Estradiol, kaempferol and quercetin form three hydrogen bonds with Glu353, Hie524 and Arg394, and tamoxifen only once with Asp351.

Interaction of phytoestrogens with ER*β*

The amino acid residues in the active site of ER*β* (PDBID: 3OLL) are Val280, Met295, Ser297, Leu298, Thr299, Leu301, Ala302, Asp303, Glu305, Trp335, Met336, Leu339, Met340, Gly342, Leu343, Met344, Arg346,

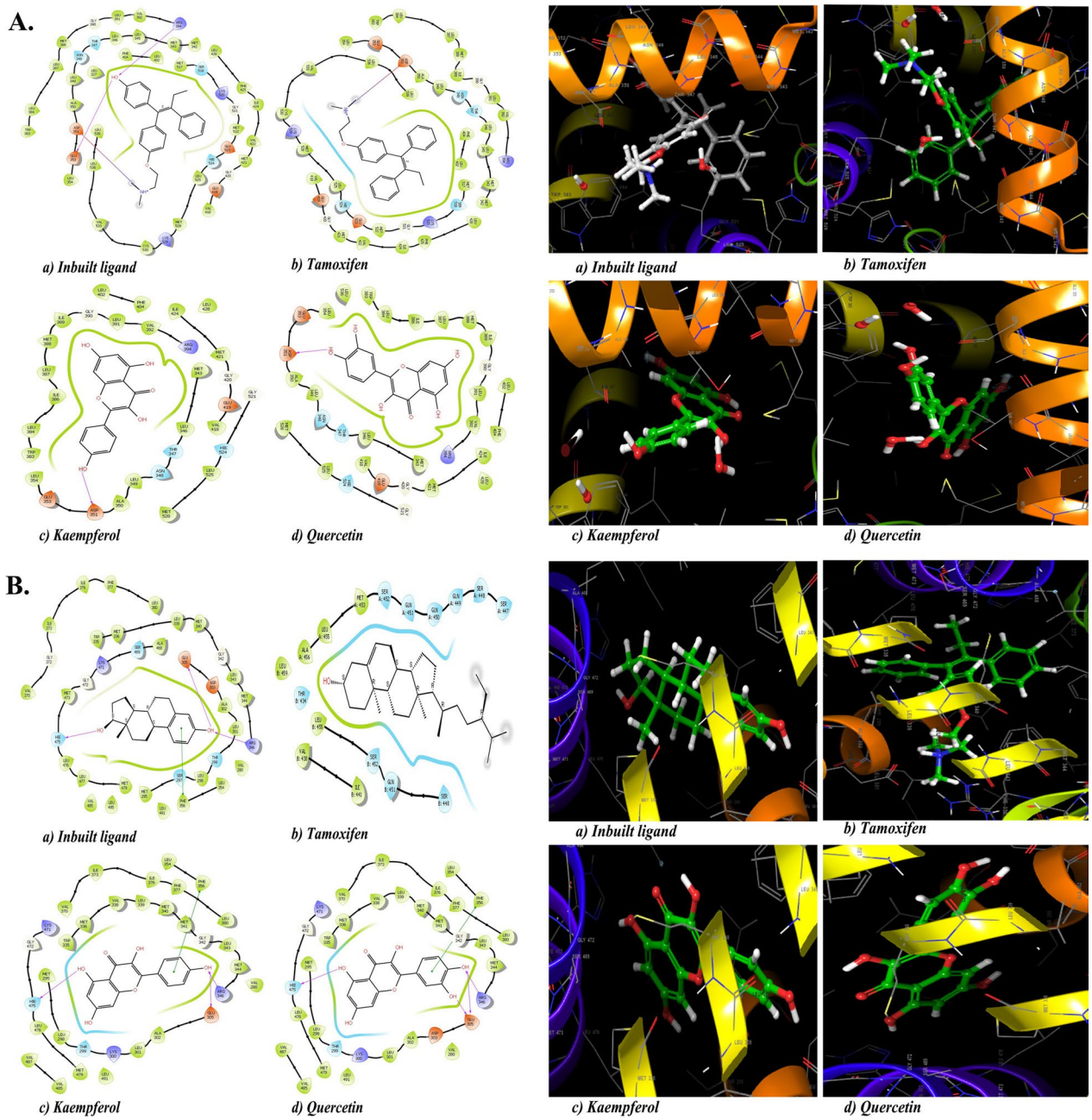


Fig. 6 **A** 2D and 3D image of the interaction between ER*α* and ligands, **B** 2D and 3D image of the interaction between ER*β* and ligands

Table 2 Docking score and binding energy of estrogen receptors and ligands

Molecule	ER*α*		ER*β*	
	Docking score	Binding energy (kcal/mol)	Docking score	Binding energy (kcal/mol)
Inbuilt ligand-Estradiol	−12.17	−125.19	−10.5	−85.248
Quercetin	−6.945	−47.026	−9.220	−66.945
Kaempferol	−6.93	−45.07	−8.478	−58.435
Tamoxifen	−10.512	−103.53	−8.023	−22.203

Leu354, Phe356, Val370, Gly372, Ile373, Ile376, Phe377, Leu380, Ala468, Ser469, Lys471, Gly472, Met473, Hie475, Leu476, Leu477, Met479, Val485, Leu491, Leu495.

The inbuilt ligand was bound deep into the active site area, making hydrogen bonding interactions with Hie475, Arg346, Glu305 and π–π stacking interactions with Phe356. The inbuilt ligand shows a docking score of −10.5 and binding energy of −85.248 kcal/mol. The docking of ERβ with quercetin showed the highest docking score of −9.220 and binding energy of −66.945 kcal/mol. Quercetin makes hydrogen bond interactions with Arg346, Glu305, Hie475 and π–π stacking with Phe356. Quercetin shows the highest affinity followed by kaempferol (−8.478) and tamoxifen (−8.023). Here also, β-sitosterol did not dock with the binding pocket of ER β. The 3D and 2D figures of other ligands are given in Fig. 6B. The docking score and binding energy of other ligands are given in Table 2.

The 2D image (Fig. 6B) reveals the type of interaction between the ligands and amino acids in the active sites of ERβ. The inbuilt ligands estradiol, tamoxifen, kaempferol and quercetin form p bonds from their aromatic ring to Phe 356 but the number and nature of hydrogen bonds vary with ligands. Estradiol, kaempferol and quercetin form three hydrogen bonds with Glu305, Hid475 and Arg346, and tamoxifen only once with Asp351.

Discussion

The study emphasizes the considerable antiproliferative potential of crude extract from *Saraca asoca* against breast cancer cells, particularly targeting triple negative breast cancers. Specifically, the plant demonstrated selective cytotoxicity towards breast cancer cells expressing ERβ, while sparing those expressing ERα. The estrogen-screen assay conducted on MDAMB-231 breast cancer cells revealed a pronounced antiproliferative effect, in contrast to MCF-7 cells, which exhibited a proliferative response to estrogen and the extract. The LC-MS analysis identified phytoestrogens such as β-sitosterol, quercetin, kaempferol, and others in the plant. In in-silico docking analysis, these phytoestrogens showed higher binding affinity towards ERβ compared to ERα receptors, except for β-sitosterol. Therefore, the observed preferential cytotoxicity of *S. asoca* towards ERβ expressing breast cancers holds significant clinical relevance, given that triple negative breast cancers represent the most aggressive form of cancer with limited treatment options.

Triple-negative breast cancer (TNBC) is characterized by the absence of expression of ERα, progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) [42]. In recent research on the immunological profile of these TNBC cell lines, the presence of estrogen receptor isoform ERβ, which acts as an opponent of ERα, in 50–80% of TNBCs was found [19]. In cytotoxic assays conducted in this study, Asoka exhibited significant cytotoxicity towards various cancer cells of both murine and human origin. This was evidenced by a concentration-dependent increase in the percentage of dead cells, particularly in DLA and EAC murine cancer cells. Notably, previous research has already reported comparable cytotoxic effects against mouse tumor cells [43]. Interestingly, despite the use of high concentrations, the extract did not induce cytotoxic effects on breast cancer cells expressing ERα, such as MCF-7, as observed in the MTT assay. However, the extract did exhibit cytotoxic effects on breast cancer cell lines expressing ERβ, such as MDAMB-231 and SKBR3. Prior investigations have suggested the antiproliferative effects of *S. asoca* on MDAMB-231 and also on MCF-7 cell lines [11]. However, in our study, MCF-7 cell lines did not exhibit any cytotoxicity even at higher concentrations of the extract. This interesting contrast in cytotoxicity between ERα and ERβ expressing breast cancer cells in our study corresponds with earlier findings which mention ERα activation leading to cell proliferation, while ERβ activation exhibiting an antiproliferative effect [15, 18].

The proliferative/anti-proliferative effect of the *S. asoca* crude extract was evaluated through an estrogen-screen assay on various breast cancer cell lines, with 17β-estradiol as a reference. This bioassay determines the increase or decrease in cell number in response to estrogen, resembling an increase in mitotic activity within reproductive system-associated tissues [44]. Our investigation revealed that Asoka demonstrates a moderate

anti-proliferative effect on the MDAMB-231 cell line expressing ERβ, while it induces proliferation in the MCF-7 cell line expressing ERα. The presence of phytoestrogens such as quercetin, kaempferol, and β-sitosterol, among others, was reported in Asoka by LC-MS. Additionally, there are reports of flavonoids like quercetin, chrysin, and 3-hydroxyflavone with known anti-proliferative properties [45]. Therefore, it is likely that the phytoestrogens in Asoka may act as agonists for ERβ due to their specific affinity for ERβ expressing cells.

The results of our docking experiments also have demonstrated the potent affinity of phytoestrogens towards ERβ in comparison to the ERα. Both quercetin and kaempferol exhibited significantly higher docking scores and binding energies than tamoxifen, a commonly used chemotherapeutic medication. Remarkably, quercetin's docking score was close to that of estradiol, the endogenous ligand for estrogen receptors. Furthermore, there have been reports highlighting the robust binding affinity of *S. asoca* flavonoids to human estrogen receptors. In a molecular simulation research involving Asoka flavonoids and estrogen receptors, the binding scores indicate their exceptional ability to form strong interactions with these receptors. Molecular orbital analysis and pharmacokinetic parameters further support their efficacy [46]. Notably, our study revealed high binding affinity values of phytoestrogens binding with estrogen receptors which surpassed that reported in a previous investigation on Asoka flavonoids [47]. This increased affinity of phytoestrogens, particularly for ERβ, holds significant implications, as it appears to underpin their antiproliferative effects. This interaction with estrogen receptors, notably ERβ, may give rise to a wide array of biological responses [22].

Conclusion

The study highlights the preferential cytotoxicity of the *S. asoca* crude methanol extract towards ERβ expressing cells, particularly to triple negative breast cancers, a highly aggressive and therapeutically challenging type. The observed antiproliferative effects on breast cancer cells may be attributed to the action and interaction of phytoestrogens present in Asoka with ERβ. Notably, quercetin and kaempferol among the identified phytoestrogens exhibit high docking into the binding sites of active amino acids in the ERβ. These findings suggest that the phytoestrogens in Asoka may act as agonists to ERβ, offering promising prospects for the development of targeted therapies for triple negative breast cancer, thereby opening new avenues in the currently limited treatment landscape.

Abbreviations

MTT 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
ERβ Estrogen Receptor β
DLA Daltons Lymphoma Ascites
EAC Ehrlich's Ascites Carcinoma

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Author contributions

Chennattu M Pareeth has done experiments and wrote the paper, K P Safna Hussan, Davis Anu, Nair Meera discussed and designed experiments and done dry lab calculations. Deepu Mathew, Ravishankar Valsalan, Mohamed Shahin Thayyil—Resources, Software, and Supervision of the in-silico (molecular docking) works. Kannoor M Thara, Achuthan C Raghavamenon, Thekkekara D Babu—Supervision, finalize the design, experiments and corrections.

Availability of data and material

Available upon request to the corresponding author.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors have no objection to publishing the data.

Competing interests

The authors declare that they have no competing interests.

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References

1. Pradhan PK, Joseph LS, Gupta V, Chulet R, Arya H, Verma R, Bajpai A (2009) *Saraca asoca* (Ashoka): A review. J Chem Pharm Res 1:62–71
2. Biswas TK, Debnath PK (1972) Aśoka (*Saraca indica* Linn)—A cultural and scientific evaluation. Indian J Hist Sci 7:99–114
3. Seetharam YN, Sujeeth H, Jyothishwaran G, Barad A, Sharanabasappa G, Parveen S (2003) Antibacterial activity of *Saraca asoca* bark. Indian J Pharm Sci 65:658–659
4. Yadav NK, Saini KS, Hossain Z, Omer A, Sharma C, Gayen JR, Singh P, Arya K, Singh R (2015) *Saraca indica* bark extract shows *in vitro* antioxidant, antibreast cancer activity and does not exhibit toxicological effects. Oxid Med Cell Longev 2015(2015):205360
5. Sasmal S, Majumdar S, Gupta M, Mukherjee A, Mukherjee PK (2012) Pharmacognostical, phytochemical and pharmacological evaluation for the antipyretic effect of the seeds of *Saraca asoca* Roxb. Asian Pac J Trop Biomed 2:782–786
6. Kumar S, Narwal S, Kumar D, Singh G, Narwal S, Arya R (2012) Evaluation of antihyperglycemic and antioxidant activities of *Saraca asoca* (Roxb.) De Wild leaves in streptozotocin induced diabetic mice. Asian Pac J Trop Dis 2:170–176
7. Nayak S, Sahoo AM, Chakraborti CK (2011) Anthelmintic activity study of *Saraca indica* leaves extracts. Int J Appl Biol Pharm Technol 2:37
8. Varghese CD, Satish, Nair CPR, Panikkar KR (1992) Potential anticancer activity of *Saraca asoca* extracts toward transplantable tumors in mice. Indian J Pharm Sci 54:37

9. Asokan A, Thangavel M (2014) *In vitro* cytotoxic studies of crude methanolic extract of *Saraca indica* bark extract. IOSR J Pharm Biol Sci 9:26–30
10. Dharshini AD, Elumalai P, Raghunandhakumar S, Lakshmi T, Roy A (2021) Evaluation of anti-cancer activity of *Saraca asoca* flower extract against lung cancer cell line. J Pharm Res Int 33:423–431
11. Yadav NK, Saini KS, Hossain Z, Omer A, Sharma C, Gayen JR, Singh P, Arya KR, Singh RK (2015) *Saraca indica* bark extract shows *in vitro* antioxidant, antibreast cancer activity and does not exhibit toxicological effects. Oxid Med Cell Longev 2015(2015):205360
12. Mukhopadhyay MK, Shaw M, Nath D (2017) Chemopreventive potential of major flavonoid compound of methanolic bark extract of *Saraca asoca* (Roxb.) in benzene-induced toxicity of acute myeloid leukemia mice. Pharmacogn Mag 13:S216
13. Varghese CD, Nair SC, Panikkar B, Panikkar KR (1993) Effect of Asoka on the intracellular glutathione levels and skin tumor promotion in mice. Cancer Lett 69:45–50
14. Bendigeri S, Das G, Shrman K, Kumar S, Khare R, Sachan S, Saiyam R (2019) Phytochemical analysis of *Saraca asoca* bark and *Azadirachta indica* seeds. Int J Chem Stud 7:126–131
15. Paterni I, Granchi C, Katzenellenbogen JA, Minutolo F (2014) Estrogen receptors alpha (ERα) and beta (ERβ): subtype-selective ligands and clinical potential. Steroids 90:13–29
16. Song D, He H, Indukuri R, Huang Z, Stepanauskaite L, Sinha I, Haldosén L-A, Zhao C, Williams C (2022) ERα and ERβ homodimers in the same cellular context regulate distinct transcriptomes and functions. Front Endocrinol 13:930227
17. Sellitto A, D'Agostino Y, Alexandrova E, Lamberti J, Pecoraro G, Memoli D, Rocco D, Coviello E, Giurato G, Nassa G (2020) Insights into the role of estrogen receptor β in triple-negative breast cancer. Cancers 12:1477
18. Zhou Y, Liu X (2020) The role of estrogen receptor beta in breast cancer. Biomark Res 8:39
19. Wisinski KB, Xu W, Tevaarwerk AJ, Saha S, Kim K, Traynor A, Dietrich L, Hegeman R, Patel D, Blank J, Harter J, Burkard ME (2016) Targeting estrogen receptor beta in a phase 2 study of high-dose estradiol in metastatic triple-negative breast cancer: A Wisconsin Oncology Network Study. Clin Breast Cancer 16:256–261
20. Saha Roy S, Vadlamudi RK (2012) Role of estrogen receptor signaling in breast cancer metastasis. Int J Breast Cancer 2012(2012):654698
21. Chantzi **NI**, Palaiologou M, Stylianidou A, Goutas N, Vassilaros S, Kourea HP, Dhimolea E, Mitsiou DJ, Tiniakos DG, Alexis **M**N (2014) Estrogen receptor β2 is inversely correlated with Ki-67 in hyperplastic and noninvasive neoplastic breast lesions. J Cancer Res Clin Oncol 140:1057–1066
22. Kuiper GGJM, Lemmen JG, Carlsson BO, Corton JC, Safe SH, Van Der Saag PT, Van Der Burg B, Gustafsson J-AK (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139:4252–4263
23. Bilal I, Chowdhury A, Davidson J, Whitehead S (2014) Phytoestrogens and prevention of breast cancer: The contentious debate. World J Clin Oncol 5:705–712
24. Harris DM, Besselink E, Henning SM, Go VLW, Heber D (2005) Phytoestrogens induce differential estrogen receptor alpha- or beta-mediated responses in transfected breast cancer cells. Exp Biol Med 230:558–568
25. Mersereau JE, Levy N, Staub RE, Baggett S, Zogric T, Chow S, Ricke WA, Tagliaferri M, Cohen I, Bjeldanes LF (2008) Liquiritigenin is a plant-derived highly selective estrogen receptor β agonist. Mol Cell Endocrinol 283:49–57
26. Middleton E, Kandaswami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacol Rev 52:673–751
27. Sareddy GR, Vadlamudi RK (2015) Cancer therapy using natural ligands that target estrogen receptor beta. Chin J Nat Med 13:801–807
28. Schüler-Toprak S, Häring J, Inwald EC, Moehle C, Ortmann O, Treeck O (2016) Agonists and knockdown of estrogen receptor β differentially affect invasion of triple-negative breast cancer cells *in vitro*. BMC Cancer 16:1–13
29. Hinsche O, Girgert R, Emons G, Gründker C (2015) Estrogen receptor β selective agonists reduce invasiveness of triple-negative breast cancer cells. Int J Oncol 46:878–884
30. Meera N, Divya MK, Silpa P, Pareeth CM, Raghavamenon AC, Babu TD (2021) Amelioration of sodium fluoride induced oxidative stress by *Cynometra travancorica* Bedd in mice. J Complement Integr Med 19:243–249
31. Harborne AJ (1998) Phytochemical methods a guide to modern techniques of plant analysis. Springer, Dordrecht
32. Das PK, Nath V, Gode KD, Sanyal AK (1964) Preliminary phytochemical and pharmacological studies on *Cocculus hirsutus*, Linn. Indian J Med Res 52:300–307
33. Kaur C, Kapoor HC (2002) Anti-oxidant activity and total phenolic content of some Asian vegetables. Int J Food Sci Technol 37:153–161
34. Chang C-C, Yang M-H, Wen H-M, Chern J-C (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 10:178
35. Moldeus P, Hogberg J, Orrenius S, Fleischer S, Packer L (1978) Methods in enzymology, vol 52. Academic Press, New York, pp 60–71
36. Prinz H (2010) Hill coefficients, dose–response curves and allosteric mechanisms. J Chem Biol 3:37–44
37. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
38. Han D-H, Denison MS, Tachibana H, Yamada K (2002) Relationship between estrogen receptor-binding and estrogenic activities of environmental estrogens and suppression by flavonoids. Biosci Biotechnol Biochem 66:1479–1487
39. Coates J (2000) Interpretation of infrared spectra, a practical approach. Encycl Anal Chem 12:10815
40. Hussan KPS, Thayyil MS, Ahamed TS, Muraleedharan K (2020) Biological evaluation and molecular docking studies of benzalkonium ibuprofenate. In: Computational Biology and Chemistry. IntechOpen
41. Sharma S, Tiwari G, Tiwari R (2021) Development and validation of UV-Spectrophotometric method for the estimation of *Saraca asoca*, *Bauhinia variegata* Linn, and *Commiphora mukul* in standardized polyherbal formulation. J Pharm Sci Res 13:525–528
42. Takano EA, Younes MM, Meehan K, Spalding L, Yan M, Allan P, Fox SB, Redfern A, Clouston D, Giles GG (2023) Estrogen receptor beta expression in triple negative breast cancers is not associated with recurrence or survival. BMC Cancer 23:1–12
43. Varghese CD, Nair C, Panikkar KR (1992) Potential anticancer activity of *Saraca asoca* extracts toward transplantable tumors in mice. Indian J Pharm Sci 54:37–40
44. Soto AM, Sonnenschein C (1985) The role of estrogens on the proliferation of human breast tumor cells (MCF-7). J Steroid Biochem 23:87–94
45. Resende FA, de Oliveira APS, de Camargo MS, Vilegas W, Varanda EA (2013) Evaluation of estrogenic potential of flavonoids using a recombinant yeast strain and MCF7/BUS cell proliferation assay. PLoS ONE 8:e74881
46. Sherin DR, Manojkumar TK (2017) Flavonoids from *Saraca asoca*-ideal medication for breast cancer-A molecular simulation approach. Biomed J Sci Tech Res 1:1761–1763
47. Suganya J, Radha M, Naorem DL, Nishandhini M (2014) *In silico* docking studies of selected flavonoids-natural healing agents against breast cancer. Asian Pac J Cancer Prev 15:8155–8159

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