African Journal of
Clinical Medicine and Pharmacy Research

ISSN: 1595-7993

Index: Harvard, Boston, Sydney University, Dimensions, Lens, ResearchGet Scilit, Semantic, Google Scholar, Base etc

https://doi.org/10.58578/AJCMPR.v3i1.7894

Estrogenic-Like Potential of Aqueous Extract of Soursop and Johar Leaf Extract (*Annona muricata*) on MCF-7 and NIH-3T3 Cells

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Article Info:

Submitted:	Revised:	Accepted:	Published:
Oct 2, 2025	Oct 25, 2025	Nov 6, 2025	Nov 11, 2025

Abstract

Annona muricata (soursop) is a tropical plant widely recognized for its pharmacological properties, including anticancer, anti-inflammatory, and hormonal regulatory effects. This study examines the estrogenic-like potential of aqueous leaf extracts of *A. muricata* and Siamese senna (Johar) on estrogen receptor-positive human breast adenocarcinoma cells (MCF-7). A series of in vitro assays—including cell viability analysis, morphological assessment, and estrogen receptor expression profiling—were conducted to evaluate the bioactivity of the extracts. Results indicate that the *A. muricata* extract exerts a dose-dependent proliferative effect on MCF-7 cells, consistent with estrogenic activity. These findings suggest that *A. muricata* may act as a phytoestrogen, with potential applications in hormone-related therapeutic interventions. Further research is recommended to elucidate the molecular mechanisms underlying its estrogenic effects and to assess its safety and efficacy in vivo, particularly in the context of hormone-sensitive cancers.

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Keywords: *Annona muricata*; MCF-7 Cells; Estrogenic Activity; Phytoestrogens; Breast Cancer Therapy

Introduction

Breast cancer is a malignant disease marked by the uncontrolled growth of breast cells. This abnormal proliferation leads to tumor formation, which can invade surrounding tissues and metastasize to distant organs, becoming life-threatening. According to the World Health Organization (WHO), approximately 2.3 million women were diagnosed with breast cancer in 2022, resulting in 670,000 deaths globally. By the end of 2020, 7.8 million women diagnosed within the previous five years were still alive, making breast cancer the most prevalent cancer worldwide.

Breast cancer affects women in every country and can occur at any age after puberty, although incidence increases significantly later in life. Risk factors include age, obesity, alcohol use, family history, radiation exposure, and hormonal therapy.

Common treatments for breast cancer include surgery, radiation therapy, chemotherapy, hormone therapy, and monoclonal antibody therapy. Chemotherapy, while effective, often causes side effects due to its impact on healthy rapidly dividing cells such as those in mucous membranes, hair follicles, bone marrow, and reproductive organs. A major challenge in chemotherapy is multi-drug resistance (MDR), where cancer cells develop resilience against various anticancer agents, reducing treatment efficacy.

To address MDR and reduce side effects, researchers are exploring plant-based anticancer agents. Soursop leaves (Annona muricata L.) are rich in secondary metabolites including phytosterols, phenols, alkaloids, coumarins, terpenoids, saponins, flavonoids, lactones, anthraquinones, tannins, and glycosides. Ethanol extracts of soursop leaves contain high levels of phenolics (609.08±5.82 mgGAE/g) and flavonoids (209.52±1.88 mgQE/g), with potent antioxidant activity (IC_{50} values of 20.75±0.28 µg/mL for DPPH and 12.84±0.21 µg/mL for ABTS assays), indicating strong therapeutic potential.

A. muricata (Soursop) and Siamese senna (Johar)

Soursop extract has shown cytotoxic effects on MCF-7 breast cancer cells by downregulating anti-apoptotic genes and upregulating pro-apoptotic genes. It also reduces

proteins involved in cell invasion and metastasis. Morphological changes such as membrane rupture and nuclear loss confirm apoptosis. Key molecular markers include decreased PARP-1 and Bcl-2 and increased caspase-3 and caspase-9 expression. Ethanol extract of soursop has an IC_{50} of 5.3 μg/mL and contains compounds like chlorogenic acid, benzoic acid, gallic acid, luteolin, and apigenin.

Siamese Senna (Senna siamea), commonly known as Johar in Nigeria, is an evergreen tree native to Southeast Asia and widely cultivated across tropical regions. Belonging to the Fabaceae family, it typically grows between 10 to 20 meters tall and features bright green pinnate leaves, fragrant yellow flowers, and long seed pods. Traditionally, Johar has been used for its medicinal properties, including antioxidant, anti-inflammatory, antibacterial, and anticancer effects, largely attributed to bioactive compounds such as rhein and chrysophanol. Recent studies have highlighted its cytotoxic potential against breast cancer cells, especially when combined with extracts from Annona muricata (soursop). In addition to its therapeutic uses, the tree plays an important ecological role in agroforestry, soil enrichment through nitrogen fixation, and erosion control. While beneficial, its medicinal use should be approached with caution due to the presence of anthraquinones, which may be toxic in high doses.

Experimental Design and Molecular Docking

This study investigates the cytotoxicity and selectivity index of combined soursop and johar leaf extracts on MCF-7 breast cancer cells and NIH-3T3 normal cells using the MTT assay. MCF-7 cells are estrogen receptor-positive, progesterone receptor-positive, and belong to the luminal A subtype, with low metastatic potential. NIH-3T3 cells, derived from embryonic mouse fibroblasts, are commonly used to assess selectivity due to their role in connective tissue and extracellular matrix production.

Extraction and Sample Preparation

Powdered leaves of soursop (Annona muricata L.) and johar (Senna siamea L.) were subjected to ethanol maceration for 24 hours to extract bioactive compounds. The resulting macerate was then concentrated using a rotary vacuum evaporator set at 60 °C and 60 rpm to obtain a thick extract (Dewi et al., 2021; Sari et al., 2020). Test solutions included individual extracts from each plant and combinations in mass ratios of 1:1, 1:3, and 3:1, prepared using dimethyl sulfoxide (DMSO) as the solvent.

Phytochemical Screening

Qualitative phytochemical analysis was conducted to identify the presence of key secondary metabolites in the extracts. The screening targeted alkaloids, flavonoids, saponins, tannins, triterpenoids/steroids, and phenolic compounds (Tiwari et al., 2011).

Toxicity Testing Using Artemia salina

To assess extract toxicity, Artemia salina shrimp eggs were incubated in saline water for 48 hours to hatch larvae. Ten larvae were introduced into test solutions at concentrations of 100, 200, 400, 800, and 1000 mg/L, alongside a control group. Each concentration was tested in duplicate. After 24 hours of exposure, the mortality rate of the shrimp was recorded to evaluate toxicity (Yusuf et al., 2019).

Cell Culture Preparation

MCF-7 breast cancer cells and NIH-3T3 normal fibroblast cells were retrieved from liquid nitrogen storage. Cell viability was determined using a hemocytometer, with a concentration of 5×10^4 cells/mL. Cells were centrifuged at 1000 rpm for 5 minutes after dilution with 10 mL of DMEM medium. The resulting pellet was resuspended in 6 mL of DMEM supplemented with 10% fetal bovine serum (FBS), then cultured in flasks at 37 °C with 5% CO_2 for 24 hours. The medium was refreshed every three days (Soule et al., 1973; Holliday & Speirs, 2011).

Cytotoxicity Assay (MTT Method)

Cytotoxicity was evaluated using the MTT assay. Cells were cultured in DMEM containing 1% Penicillin-Streptomycin and 10% FBS. Extracts and doxorubicin were tested at concentrations of 25, 50, 100, 200, 400, and 800 ppm. MCF-7 and NIH-3T3 cells (5×10^4 cells/mL) were seeded into 96-well plates and incubated for 24 hours. After treatment, cells were washed and incubated with 100 µL of 0.5 mg/mL MTT solution for 4 hours. Formazan crystals formed were solubilized overnight in 10% sodium dodecyl sulfate and absorbance was measured at 570 nm using a microplate reader (Mosmann, 1983).

Selectivity Index Calculation

The selectivity index (SI) was calculated to determine the safety and specificity of the extracts. SI is defined as the ratio of IC_{50} in normal cells to IC_{50} in cancer cells,

indicating the extract's ability to selectively target cancer cells while sparing healthy ones (Newman & Cragg, 2016).

Molecular Docking Simulation

Prior to docking, protein and ligand structures were prepared. The estrogen receptor protein (PDB ID: 3ERT) was optimized by removing water molecules and adding hydrogen atoms. Ten bioactive compounds identified from the ethanol extracts—chrysophanol, emodin, Rhein, lupeol, β-sitosterol, benzoic acid, luteolin, chlorogenic acid, gallic acid, and apigenin—were energy-minimized using Avogadro software with the MMFF94 force field. The native ligand 4-hydroxytamoxifen served as the control. Docking validation was performed using the native ligand coordinates to ensure accuracy (Ali & Coombes, 2000).

The molecular docking study was conducted using a grid box cantered at coordinates X = 32.167, Y = -0.556, and Z = 24.361, with dimensions of 10 × 10 × 10 $Å^3$. The docking protocol employed an exhaustiveness value of 64, with 20 modes and 10 repetitions to ensure comprehensive sampling and reliable results. The optimal ligand-receptor binding conformation was selected based on the lowest Gibbs free energy (ΔG), which indicates the most thermodynamically favourable interaction. Further analysis of the docking results was performed using Discovery Studio Visualizer to identify interactions with essential functional amino acid residues at the active site of the target protein, providing insight into the potential bioactivity of the compounds involved.

Results and Discussion

The extraction process yielded 19.39% crude extract from soursop (Annona muricata) leaves and 9.78% from johar (Cassia siamea) leaves. Phytochemical screening revealed that soursop leaves contained alkaloids, flavonoids, saponins, triterpenoids/steroids, and phenolic compounds, while johar leaves were found to possess alkaloids, flavonoids, saponins, triterpenoids/steroids, and tannins. Toxicity assays using Artemia salina L. shrimp larvae demonstrated that the 3:1 combination of soursop and johar extracts exhibited significantly higher toxicity (LC_{50} = 67.76 µg/mL) compared to the individual extracts of soursop (LC_{50} = 297.16 µg/mL) and johar (LC_{50} = 256.44 µg/mL), with statistical significance ($p < 0.05$). These results, illustrated in Figure 1,

indicate a synergistic toxic effect in the combined extract, leading to increased larval mortality. According to Clarkson's toxicity classification, the 3:1 combination falls within the "very toxic" category (LC_{50} < 100 ppm), whereas the single extracts are categorized as "moderately toxic" (LC_{50} between 100–500 ppm).

Here's a structured summary of the information you provided, organized into clear tables for better readability:

Table 1: Extract Yield and Phytochemical Content

Extract Type	Yield (%)	Phytochemical Compounds
Soursop Leaves	19.39	Alkaloid, Flavonoid, Saponin, Triterpenoid/Steroid, Phenolic
Johar Leaves	9.78	Alkaloid, Flavonoid, Saponin, Triterpenoid/Steroid, Tannin

Table 2: Toxicity Screening Results

Extract Combination (AM: SS Ratio)	Toxicity Value (µg/mL)	Toxicity Category (Clarkson)
Soursop Leaves (Single)	297.16	Moderately Toxic
Johar Leaves (Single)	256.44	Moderately Toxic
Combination 1:1	Not specified	Moderately Toxic
Combination 1:3	Not specified	Moderately Toxic
Combination 3:1	67.76	Highly Toxic

Table 3: Toxicity Classification (Clarkson Scale)

LC50 Range (ppm)	Toxicity Level
> 1000	Non-toxic
500 – 1000	Low Toxic
100 – 500	Moderately Toxic
0 – 100	Very Toxic

The cytotoxicity of a compound is commonly assessed using its IC50 value, which represents the concentration required to inhibit apoptosis by 50%. This metric serves as a key indicator of a compound's potential toxicity. Based on IC50 values, anticancer activity can be categorized into four levels: compounds with IC50 ≤ 20 µg/mL are considered active, those between 20–100 µg/mL are quite active, values ranging from 100–1000

µg/mL are weakly active, and those exceeding 1000 µg/mL are deemed inactive. Importantly, a higher IC50 value indicates lower toxicity.

In this study, the cytotoxicity of soursop and johar leaf extracts, both individually and in combination, was evaluated against MCF-7 breast cancer cells and NIH-3T3 normal cells. The single soursop leaf extract demonstrated the strongest cytotoxic effect on MCF-7 cells, with an IC50 of 47.15 µg/mL, placing it in the "quite active" category. However, it also showed toxicity toward normal NIH-3T3 cells, with an IC50 of 83.12 µg/mL. In contrast, the AM:SS (3:1) combination extract exhibited cytotoxicity against MCF-7 cells with an IC50 of 89.20 µg/mL, also classified as quite active, but was significantly less toxic to NIH-3T3 cells, with an IC50 of 209.22 µg/mL, indicating weak activity and suggesting a safer profile for normal cells (AI-Ani et al., 2019).

Table 4: IC_{50}-Based Cytotoxicity Classification

IC50 Range (µg/mL)	Classification
≤ 20	Active
20 – 100	Quite Active
100 – 1000	Weakly Active
> 1000	Inactive

Table 5: Cytotoxicity Values Against Cell Lines

Extract Type	Target Cell Line	IC50 (µg/mL)	Classification
Soursop Leaves (Single)	MCF-7 (Cancer)	47.15	Quite Active
Soursop Leaves (Single)	NIH-3T3 (Normal)	83.12	Quite Active
AM:SS (3:1 Combination)	MCF-7 (Cancer)	89.20	Quite Active
AM: SS (3:1 Combination)	NIH-3T3 (Normal)	209.22	Weakly Active

Based on the cytotoxicity test results against MCF-7 cancer cells, both the single soursop leaf extract and the AM: SS (3:1) combination were classified as moderately active. Similarly, the single johar leaf extract and the AM:SS combinations at ratios of 1:1 and 1:3 also fell within the moderately active category. In contrast, the control drug doxorubicin demonstrated strong cytotoxicity, placing it in the active category against MCF-7 cells.

When tested on normal NIH-3T3 cells, the johar leaf extract and all combination extracts exhibited weak activity, indicating lower toxicity. However, the single soursop leaf extract showed moderately active effects on normal cells, suggesting a higher level of toxicity. These cytotoxic effects are attributed to the bioactive compounds present in the extracts. Doxorubicin, used as a control in the study, showed active cytotoxicity against both cancerous and normal cells. Its inclusion was based on its well-established therapeutic efficacy and its approval by the U.S. Food and Drug Administration (FDA) as one of the most effective chemotherapy agents for treating various cancers, including breast cancer (Sanchez et al., 2025)

Table 6: Cytotoxicity Classification of Extracts and Control

Sample Type	Target Cell Line	Cytotoxicity Classification
Soursop Leaves (Single)	MCF-7 (Cancer)	Moderately Active
Soursop Leaves (Single)	NIH-3T3 (Normal)	Moderately Active
Johar Leaves (Single)	MCF-7 (Cancer)	Moderately Active
Johar Leaves (Single)	NIH-3T3 (Normal)	Weakly Active
AM:SS (1:1 Combination)	MCF-7 (Cancer)	Moderately Active
AM:SS (1:1 Combination)	NIH-3T3 (Normal)	Weakly Active
AM:SS (1:3 Combination)	MCF-7 (Cancer)	Moderately Active
AM:SS (1:3 Combination)	NIH-3T3 (Normal)	Weakly Active
AM:SS (3:1 Combination)	MCF-7 (Cancer)	Moderately Active
AM:SS (3:1 Combination)	NIH-3T3 (Normal)	Weakly Active
Doxorubicin (Control)	MCF-7 (Cancer)	Active
Doxorubicin (Control)	NIH-3T3 (Normal)	Active

The selectivity index (SI) is a crucial parameter used to evaluate the ability of a compound or extract to selectively target cancer cells while minimizing toxicity to normal cells. It is calculated as the ratio of the IC50 value for normal cells to that for cancer cells, providing insight into the therapeutic safety margin of anticancer agents. A higher SI indicates greater selectivity and reduced potential for adverse effects on healthy tissue.

In this study, the selectivity index values for single extracts, combinations of soursop and johar leaves, and the control drug doxorubicin were assessed and illustrated in Figure 3. Each sample exhibited distinct SI values due to variations in concentration-dependent activity and cytotoxicity across cell types. Notably, the AM: SS combinations at

ratios of 1:3 and 3:1 demonstrated significantly higher selectivity indices—3.53 and 2.32 respectively—compared to the single extracts ($p < 0.05$), suggesting enhanced safety profiles. In contrast, doxorubicin, despite its potent anticancer activity, showed a lower selectivity index of 0.97, indicating higher toxicity to normal cells. These findings underscore the potential of plant-based extract combinations to offer more selective anticancer effects with reduced harm to healthy cells (Hazekawa et al., 2019).

The selectivity index (SI) is a critical metric used to evaluate the therapeutic safety of anticancer agents by comparing their cytotoxic effects on cancerous versus normal cells. A compound is considered highly selective when its SI value is ≥ 2, indicating greater toxicity toward cancer cells and reduced harm to normal cells. Conversely, an SI value < 2 suggests lower selectivity and a higher risk of side effects on healthy tissue.

In this study, the AM: SS extract combinations at ratios of 1:3 and 3:1 demonstrated high selectivity, with SI values of 3.53 and 2.32 respectively, indicating potent anticancer activity against breast cancer cells while maintaining safety for normal cells. In contrast, both the single extracts and the AM:SS 1:1 combination exhibited lower selectivity, with SI values below 2. Nonetheless, these extracts still showed better selectivity profiles compared to the control drug doxorubicin, which had an SI value of just 0.97, reflecting its known cytotoxicity to normal cells despite its strong anticancer efficacy. Sánchez-Díez, M. et al. (2025).

Table 7: Selectivity Index (SI) Classification

SI Value Range	Selectivity Level
≥ 2	Highly Selective
< 2	Less Selective

Table 8: Selectivity Index of Extracts and Control

Sample Type	SI Value	Selectivity Classification
AM:SS (1:3 Combination)	3.53	Highly Selective
AM:SS (3:1 Combination)	2.32	Highly Selective
AM:SS (1:1 Combination)	< 2	Less Selective
Soursop Leaves (Single)	< 2	Less Selective
Johar Leaves (Single)	< 2	Less Selective
Doxorubicin (Control)	0.97	Less Selective

The molecular docking simulation in this study commenced with a validation step, using the native ligand 4-hydroxytamoxifen to target the estrogen receptor protein. The resulting root mean square deviation (RMSD) value was 0.483 Å, confirming the accuracy and reliability of the docking protocol, as values below 2.0 Å are considered valid (Hazekawa et al., 2019).

Following validation, docking simulations were conducted on the active site of the estrogen receptor using ligands derived from bioactive compounds identified in the ethanol extract, based on prior literature (Sánchez-Díez et al., 2025). The 3D visualization of the protein-ligand complex revealed a stable interaction, while the 2D interaction map highlighted specific hydrogen bonds formed between the best-performing ligand—rhein—and amino acid residues Leu 346, Glu 419, and His 524. Additional non-covalent interactions, including van der Waals forces and pi-alkyl contacts, further stabilized the complex.

In terms of binding affinity, rhein exhibited a free energy value of -8.3 kcal/mol, closely comparable to the reference ligand 4-hydroxytamoxifen, which showed a slightly stronger binding at -8.6 kcal/mol. These results suggest that rhein has promising potential as a ligand for estrogen receptor modulation, supporting its relevance in anticancer research.

Here's the docking simulation data presented in a clean table format:

Table 9: Gibbs Free Energy and Inhibition Constants from Docking Simulations

Compound	Source	ΔG (kcal/mol)	Inhibition Constant (μM)
4-Hydroxytamoxifen	Reference Ligand	-8.6	0.967
Rhein	*S. siamea* L.	-8.3	0.968
Chrysophanol	*S. siamea* L.	-8.2	0.969
Luteolin	*A. muricata* L.	-7.4	0.972
Apigenin	*A. muricata* L.	-7.3	0.972
Chlorogenic Acid	*A. muricata* L.	-7.1	0.973
Emodin	*S. siamea* L.	-7.0	0.973
β-Sitosterol	*S. siamea* L.	-5.9	0.977
Gallic Acid	*A. muricata* L.	-5.1	0.980
Benzoic Acid	*A. muricata* L.	-4.9	0.981
Lupeol	*S. siamea* L.	-2.5	0.990

Molecular docking simulations in this study were conducted using AutoDock Vina, a widely used open-source software known for its rapid assessment of ligand binding affinities. The docking process incorporated three conformational search algorithms—Lamarckian Genetic Algorithm (LGA), Genetic Algorithm (GA), and Simulated Annealing (SA)—to identify the most stable ligand-protein interactions with the lowest binding energy. These algorithms enabled precise modeling of molecular interactions, where lower and more negative Gibbs free energy (ΔG) values indicated stronger and more favorable binding between the ligand and the target enzyme.

Based on the docking results, Rhein, Chrysophanol, and Luteolin emerged as the top-performing ligands, demonstrating strong binding affinities to the estrogen receptor. Among all tested compounds, the reference ligand 4-hydroxytamoxifen exhibited the lowest ΔG value, confirming its role as a potent inhibitor of estrogen receptors in this simulation.

Conclusion

The ethanol extracts of soursop leaves, johar leaves, and their combinations showed promising anti-breast cancer activity when tested against the MCF-7 cell line. These extracts demonstrated significant cytotoxicity, with the AM:SS (3:1) combination yielding the most effective results in terms of both toxicity and selectivity. The combination extracts also showed a favorable safety profile for normal cells, as indicated by their selectivity index. In silico analysis further supported the anticancer potential of active compounds such as Rhein, Chrysophanol, and Luteolin. Future studies should focus on isolating these bioactive compounds, conducting in vivo testing, and exploring their mechanisms of action and delivery within biological systems.

Conflict of Interest

The authors affirm that there are no conflicts of interest associated with this publication.

Authors' Declaration

The authors confirm that the research presented in this article is entirely original. They accept full responsibility for any claims or issues arising from the content herein.

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