World Journal of
Pharmacy and Medical Science

ISSN: 3049-3501
Impact Factor: 5.157
CODEN (USA): WJPMAA

Research Article
2026 Volume 2, Issue 6
Page No.: 160-165

PHYTOCHEMICAL SCREENING, BIOASSAY-GUIDED FRACTIONATION, ANTIMICROBIAL EVALUATION, AND IN SILICO MOLECULAR DOCKING STUDIES OF BIOACTIVE FRACTIONS FROM *ANNONA SQUAMOSA* LEAVES

Dr. Sonali G. Banpure[1], Dr. Pramod L. Ingale[2], Pooja S. Shinde*[3], Rahul D. Kalyankar[4], Karan S. Karpe[5]

[1]Assistant Professor, Department of Pharmaceutical Chemistry, Shri Gajanan Maharaj Shikshan Prasarak Mandal's Dnyanvilas College of Pharmacy, Pune, India.
[2]Principal, Shri Gajanan Maharaj Shikshan Prasarak Mandal's Dnyanvilas College of Pharmacy, Pune, India.
[3,4,5]Student, Shri Gajanan Maharaj Shikshan Prasarak Mandal's Dnyanvilas College of Pharmacy, Pune, India.

Article Received: 24 April 2026 Article Review: 13 May 2026 Article Accepted: 04 June 2026

***Corresponding Author: Pooja S. Shinde**
Student, Shri Gajanan Maharaj Shikshan Prasarak Mandal's Dnyanvilas College of Pharmacy, Pune, India.
DOI: https://doi.org/10.5281/zenodo.20702661

How to cite this Article: Dr. Sonali G. Banpure, Dr. Pramod L. Ingale, Pooja S. Shinde, Rahul D. Kalyankar, Karan S. Karpe (2026). PHYTOCHEMICAL SCREENING, BIOASSAY-GUIDED FRACTIONATION, ANTIMICROBIAL EVALUATION, AND IN SILICO MOLECULAR DOCKING STUDIES OF BIOACTIVE FRACTIONS FROM *ANNONA SQUAMOSA* LEAVES. World Journal of Pharmacy and Medical Science, 2(6): 160-165.

ABSTRACT

Annona squamosa L. is a popular medicinal plant with appreciable biological effects and a diverse phytochemical profile. The present study aimed to bridge the gap between computational in silico prediction and experimental in vitro confirmation by evaluating the bioassay-guided fractionation, purification, and antibacterial activity of bioactive fractions of *A. squamosa* leaves. Four phytochemicals, anonaine, catechin, kaempferol, and rutin, which are commonly reported in *A. squamosa* leaves, were selected for molecular docking experiments against the microbial target proteins (1KZN, 1C14, 3JSU, and 6XYU). Rutin showed the highest binding affinity with a significant score of –10.36 kcal/mol followed by anonaine at –7.79 kcal/mol. Bioactive fractions were obtained by Soxhlet extraction with ethanol and purified using column and thin-layer chromatography (TLC). Phytochemical screening of the purified fraction demonstrated high concentrations of alkaloids, flavonoids, tannins, and phenolic compounds. The purified fraction exhibited a zone of inhibition of 19 mm in the antimicrobial assay against *Escherichia coli*, which was significantly higher than that of the crude extract (13 mm) and comparable to that of the standard antibiotic ciprofloxacin (28 mm). These findings highlight the potential of A. squamosa as a sustainable source for developing natural antibacterial agents and provide a molecular basis for its traditional use in treating infectious disorders.

KEYWORDS: Antimicrobial activity, *Annona squamosa*, Column chromatography, Molecular docking, TLC.

1. INTRODUCTION

1.1 Botanical and Ethnobotanical Profile

The tropical fruit tree *Annona squamosa L*., called custard or sugar apple, belongs to the Annonaceae family.[1,19] This deciduous tree originated in the Americas and has spread to tropical and subtropical regions of Africa, Southeast Asia, and India. It is an important crop and a key component of traditional medicine.[1,13] Every part of the plant, including the fruit, leaves, seeds, bark, and roots, is used to treat various illnesses.[1,13] Traditional medicine often uses leaf decoctions applied to the skin to treat boils and ulcers. The seeds are known for their strong insect-repelling and antiparasitic properties.

1.2 Pharmacological Significance

A. squamosa has been a leading candidate for drug development in recent decades. The plant produces many secondary metabolites, including annonaceous acetogenins, which are unique to the Annonaceae family.[4,13] Along with alkaloids, diterpenoids, and flavonoids, these substances support a wide range of

biological effects, such as anti-tumor, antioxidant, antidiabetic, and anti-inflammatory properties.[1,3] Leaves, rich in phenolic compounds and flavonoids, offer a natural alternative to synthetic medications by acting as antioxidants and antibacterial agents.[2,3]

1.3 Problem Statement and Research Objectives

Although we have a good understanding of the qualitative mapping of metabolites in *A. squamosa*, there is still a major gap in validating these compounds against specific microbial targets and improving downstream processes for high-purity fractionation.[4] This study combined experimental fractionation and biological testing with computational molecular docking to predict the binding mechanisms. The main objectives of this study are as follows:

- Systematic extraction and fractionation of bioactive substances from leaves.
- Computation of interactions between microbial proteins and phytochemicals.
- Experimental assessment of purified fractions against *Escherichia coli*.

2. LITERATURE REVIEW

2.1 Phytochemical Diversity

The wide variety of useful therapies provided by *A. Squamosa* arises from the presence of a complex mixture of chemicals. In 2016, over 80 different acetogenins were isolated from various parts of A. Squamosa as well as 33 diterpenes, 19 different alkaloids and 13 cyclopeptides.[1,13]

Acetogenins: are lipophilic polyketides often found in the seeds of annonaceous plants and are characterized by the presence of a long aliphatic chain and an-lactone end group.[4] Compounds such as squamocin and annonacin have been found in large quantities.[4]

Alkaloids and Flavonoids: The leaves are rich in alkaloid compounds (including anonaine) and flavonoid compounds (such as rutin and kaempferol).[1,2] These compounds have been observed to exert a high scavenging effect on free radicals and damage the bacterial cell membrane.[2,3]

2.2 Antimicrobial Mechanisms

The bioactive phytoconstituents present in *A. Squamosa* are flavonoids, alkaloids, acetogenins, tannins, and phenolic constituents. Investigations conducted on *A. Squamosa* have indicated that it possesses antibacterial activities in leaf extract.[3,9] The presence of phytochemicals alters the stability of the bacterial cell wall and cell membrane, causing leakage of cell components and preventing bacterial multiplication.[3,6] The presence of multiple active chemicals in the ethanolic leaf extract could account for its broad-spectrum antibacterial activity against pathogenic organisms such as *Escherichia coli*.[3,9] In the present study, the crude ethanolic extract of *A. Squamosa* exhibited the least antibacterial activity, whereas the purified fraction obtained from column chromatography showed the highest antibacterial activity. This indicates that using a purification column, the active antimicrobial constituents were concentrated more in the fraction, thus enhancing its effectiveness.[5,9]

3. METHODOLOGY

3.1 In Silico Molecular Docking

Molecular docking is a computational approach that predicts the interactions of biologically active compounds (ligands) with target proteins or enzymes at the molecular level. In research on *Annona squamosa* (sugar apple), this technique is vital for determining which phytochemical compounds from the plant have therapeutic value before costly and time-consuming experiments are carried out in the laboratory.[15,20]

Molecular docking was performed using the bioinformatics software and databases listed below.

- **RCSB PDB (Protein Data Bank) -** This was used to download the 3D crystal structure of microbial targets (PDB IDs: 1KZN, 1C14, 3JSU and 6XYU) in PDB format.
- **Swiss Target Prediction-** This was used to predict the potential microbial targets of chosen phytochemicals.
- **Marvin Sketch (ChemAxon) -** Used to draw a 2D structure of ligands and to calculate the chemical properties of the ligands.
- **Marvin View -** Used to view and convert ligand structures into 3D format.
- **AutoDock tool -** This software was used to simulate molecular docking to determine the binding affinity (kcal/mol).
- **Biovia Discovery Studio -** This software was used to visualize and analyze the protein-ligand interactions and generate 2D interaction diagrams illustrating hydrogen bonding and hydrophobic contacts.
- **Ligands -** Anonaine, Catechin, Kaempferol and Rutin (common phytochemicals found in A. Squamosa leaves).
- **Receptors -** Microbial targets 1KZN, 1C14, 3JSU, 6XYU (downloaded from RCSB PDB).

For each interaction between the ligand and receptor, the parameters for hydrogen bonding and hydrophobic interaction stability were optimized.[1,15] The active site of each protein was centered using a grid box, and an exhaustiveness value of 10 was chosen. The Lamarckian genetic algorithm was used for the binding affinity determination, and the best binding pose was chosen based on the lowest binding energy. Biovia Discovery Studio was used to visualize the docked complexes and generate the 2D interaction diagram of the hydrogen bonding and hydrophobic contacts.

3.2 Plant Material Collection and Preparation

The leaves of *Annona squamosa* were obtained from a confirmed location in Pune (Maharashtra, India) and

were certified by a taxonomist. The collected leaves were cleaned and then shadow-dried at room temperature for 7-10 days to prevent thermal degradation of heat-labile secondary metabolites before grinding in a mechanical grinder to obtain a fine powder.[3,11] The powdered plant material was stored in an airtight container for further analysis.

3.3 Preliminary Screening and Extraction

250g of the leaf powder was extracted continuously in Soxhlet using ethanol as solvent.[4,8] The extraction was performed over 48 hours to ensure maximum yield of biologically active components. The resulting extract was concentrated using a rotary evaporator at 45^0C and reduced pressure. Preliminary phytochemical analysis for alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, phenolic compounds was conducted.[2,7]

3.4 Chromatographic Fractionation and Purification

The crude ethanolic extract was subjected to column chromatography using silica gel (60-120 mesh) as stationary phase and the fractions were separated on gradient elution starting from n-hexane and increasing amount of ethyl acetate.[4,8] Each fraction was monitored by TLC on pre-coated silica gel plates and using solvent system consisting of n-hexane: ethyl acetate (8:2). The spots with clear visible results under UV light and with similar Rf values were combined and purified again by recrystallization.[4,10]

3.5 In Vitro Antibacterial Screening

Antibacterial activity of crude extract and purified fraction was performed using agar well diffusion method with *Escherichia coli* (ATCC 25922).[9,16]

1. **Preparation of Inoculum:** *E.coli* was grown in nutrient broth for 24 hours at 370C.
2. **Well Diffusion:** Nutrient agar plates were prepared and wells of 6 mm diameter were made using a sterile cork borer.
3. **Application of Samples:** Wells were filled with the crude extract (100mg/mL), purified fraction (50mg/mL), and Ciprofloxacin (5g/mL) as positive control.
4. **Inhibition Zone Measurement:** After incubation at 370 C for 24 hours, zones of inhibition were measured using a ruler in millimeters.

4. RESULTS

4.1 Molecular Docking Interactions

Based on the molecular docking studies, there was clear ranking of binding efficacy, and all four phytochemicals have significant binding towards the bacterial targets.[1,15] Binding energies are shown in the **Table 1** for all the ligands and targets.

Rutin exhibited high binding affinity -10.36 Kcal/mol with protein 6XYU due to formation of hydrogen bonding and hydrophobic interaction between the protein and the ligand creating a stable protein-ligand complex.[1,15] Anonaine showed moderate binding energy -7.79 Kcal/mol with protein 1KZN. The binding energies of kaempferol and catechin showed similar values -7.74 Kcal/mol and -7.42 Kcal/mol respectively.

Figures 1-4 show the 2D interactions diagrams for docked complex showing hydrogen bonds and hydrophobic interactions with target proteins drawn with Biovia Discovery studio software.

Table 1: Binding Energies from Molecular Docking.

Ligand	Target Protein	Binding Energy (kcal/mol)
Rutin	6XYU	–10.36
Anonaine	1KZN	–7.79
Kaempferol	3JSU	–7.74
Catechin	1C14	–7.42

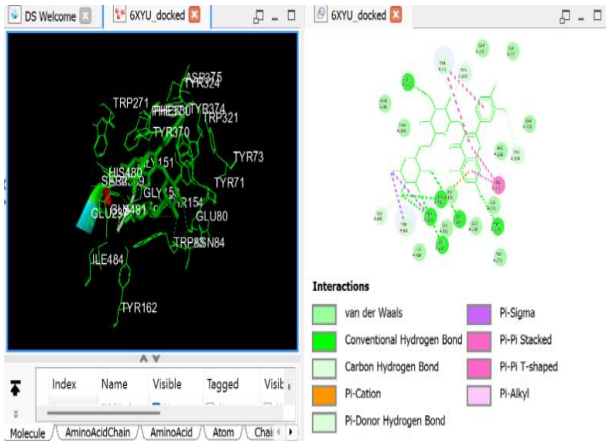

Fig. 1: 2D docked image of Rutin.

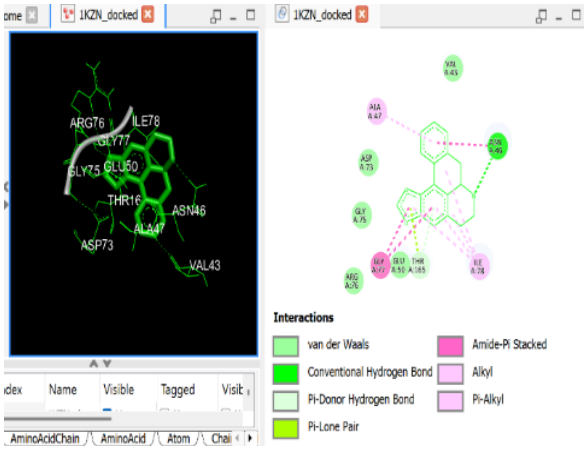

Fig. 2: 2D docked image of Anonaine.

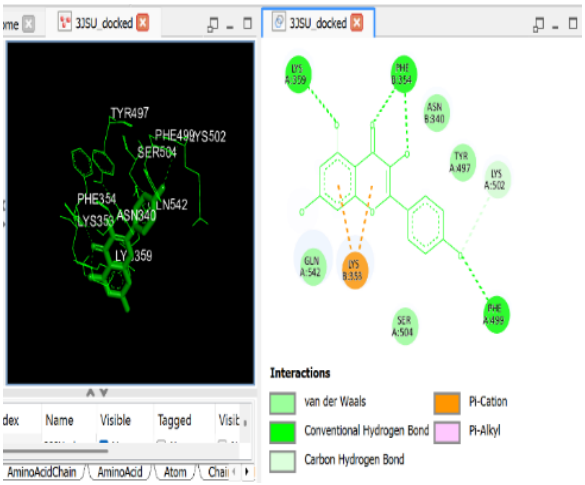

Fig. 3: 2D docked image of Kaempferol.

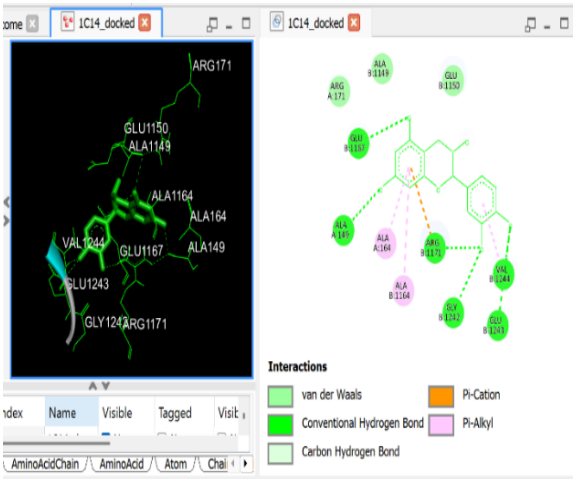

Fig. 4: 2D docked image of Catechin.

4.2 Phytochemical Screening

The qualitative study of the ethanolic leaf extract showed an array of phytochemicals as described in **Table 2.**[2,7] Alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids and phenolic compounds were detected and are responsible for the biological activities exhibited by the leaf extract.

Table 2: Phytochemical Screening of *A. squamosa* Leaf Extract.

Phytochemical Constituent	Test	Observation	Result
Alkaloids	Wagner's Test	Formation of precipitate	Present
Flavonoids	Alkaline Reagent Test	Yellow coloration	Present
Tannins	Ferric Chloride Test	Blue-black coloration	Present
Saponins	Froth Test	Stable froth formation	Present
Glycosides	Keller-Kiliani Test	Brown ring formation	Present
Terpenoids	Salkowski Test	Reddish-brown interface	Present
Phenolic Compounds	Ferric Chloride Test	Dark coloration	Present

4.3 TLC and Purification Analysis

The resolution and purity of column chromatographic fractions from the ethanolic leaf extract of *Annona squamosa* was checked by TLC. The stationary phase used was TLC plates pre-coated with silica gel and the mobile phase was n-hexane: ethyl acetate (8:2) this is an effective system for separating phytochemicals according to polarity.[4,8]

Different spots were appeared at the development of TLC plate under UV light. A clean resolution of spots with Rf values ranging between 0.19 and 0.76 showed the presence of number of phytoconstituents present in the purified fraction.[4,10] The spots formed were clear, sharp and showed good chromatographic separation and correct chemical migration on the applied solvent system as clearly visualized in **Figure 5 and Table 3.**

Table 3: Thin Layer Chromatography Results.

Spot No.	Distance Travelled by Solute (cm)	Distance Travelled by Solvent (cm)	Rf Value
1	1.1	5.8	0.19
2	1.9	5.8	0.33
3	2.8	5.8	0.48
4	3.7	5.8	0.64
5	4.4	5.8	0.76

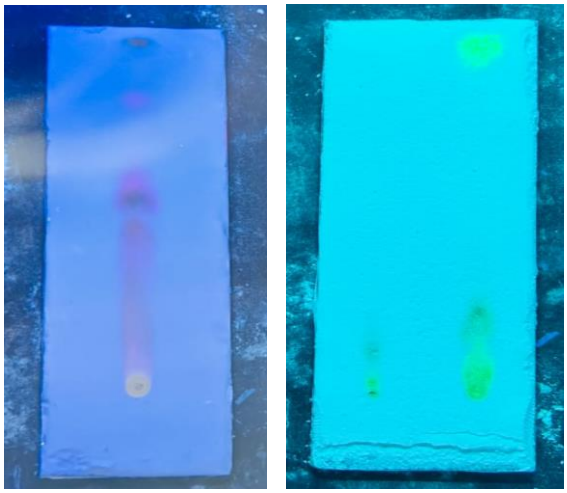

Fig. 5: TLC chromatogram of *Annona squamosa* leaf extract fraction.

The Rf values obtained 0.19–0.76 indicate good separation of compounds with varying polarities, confirming successful fractionation of the crude extract.

4.4 Antimicrobial Activity Results

Validation of this purification method by comparison with an experimental comparison using *Escherichia coli* revealed that purification increased the biological

efficacy of phytochemicals considerably.[9,16] **Table 4** shows the zones of inhibition displayed by crude extract, purified fraction, and positive control (ciprofloxacin). The purified fraction at 50 mg/mL gave a zone of inhibition of 19 mm. This compared with a zone of inhibition of 13 mm at 100 mg/mL for the crude extract gave a 46% increase in activity. This increased activity at half the dose is indicative of the concentrating effects of purification on active compounds.[5,9] The greatest zone of inhibition recorded was by the standard antibiotic ciprofloxacin at 5 g/mL (28 mm).

Table 4: Antimicrobial Activity by Agar Well Diffusion Method.

Sample Tested	Concentration	Zone of Inhibition (mm)
Crude Ethanolic Extract	100 mg/mL	13 ± 0.5
Purified Fraction	50 mg/mL	19 ± 0.6
Ciprofloxacin	5 µg/mL	28 ± 0.4

All tests have been done three times and expressed as standard deviation.

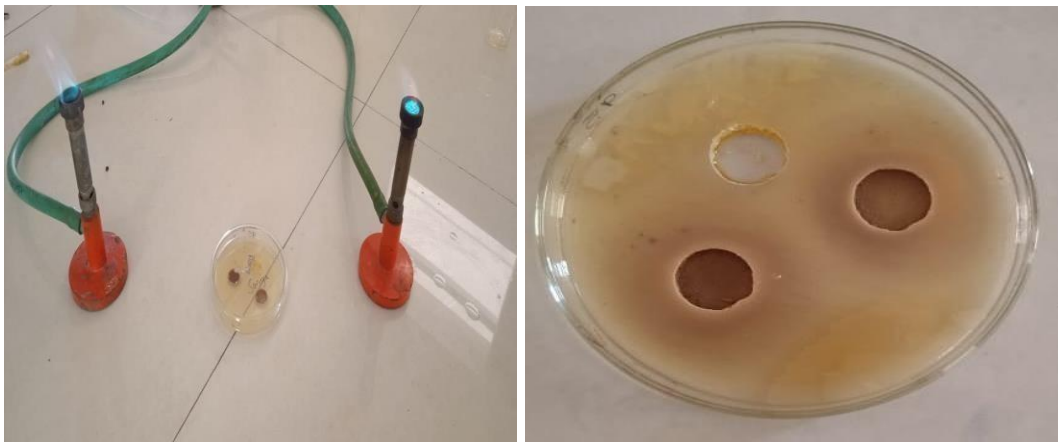

Fig. 6: Agar well diffusion Method.

Figure 6 illustrates the agar well diffusion plates with clear zone of inhibition around the wells with crud extract, purified fraction and ciprofloxacin.

5. DISCUSSION

5.1 Correlation between In Silico and In vitro study

The result of docking where rutin shows very high binding affinity (-10.36 Kcal/mol) to target protein 6XYU is a very significant finding of this study.[1,15] This score suggests highly stabilized complexes formed by hydrogen bonds and hydrophobic interaction that is likely to interfere with critical microbial enzymes.

The purified fraction having highly flavonoids especially the rutin showed significant zone of inhibition (19 mm) against *E.coli*[2,3], which supports the result obtained by docking study. And is nearly 50% effective to the crude extract (13 mm) but the dosage is less (50 mg/mL as compared to 100 mg/mL).[5,9]

5.2 Mechanistic Explanation

The potent antimicrobial activities are likely due to the presence of both alkaloid anonaine and flavonoid rutin.[2,3] Alkaloids are known to interfere with cellular metabolism while flavonoids disturb the bacterial cell wall integrity resulting in the release of cellular contents into the medium.[3,6] This has been proved by the phytochemical screening and TLC profile of the leaf extract in the present study.[4,10]

5.3 Comparison with Literature

Our finding is in consistent with the results of previous studies that stated *Annona squamosa* leaves is a source of potent inhibitors of metabolic enzyme like-amylase and-glucosidase.[1,3] Zone of inhibition (19 mm) shown by the purified fraction against the bacteria is similar to previous research works which shows the activity of the leaf extract and acetogenins of Annonaceous species against Gram-positive and Gram-negative bacteria.[5,11] Though the purified fraction is showing significant antibacterial activity but is lesser potent compared to the standard antibiotic, Ciprofloxacin (28 mm) so, need to study for increase of the therapeutic efficacy.[12]

6. CONCLUSION

In this work, a combined approach of molecular docking and purification of biologically active fraction has shown the therapeutic potential of *Annona squamosa* leaves.[1,15] Antimicrobial activity against E. Coli is increased significantly upon purification of biologically active fraction. Molecular docking results strongly supported the observed activity, where rutin showed a very high binding affinity (-10.36 Kcal/mol).[1,15]

Hence, *Annona squamosa* leaves is a suitable plant source for the development of novel natural antibacterial agents, and the research work provides a scientific basis to the traditional use of *A. Squamosa* in diseases.[13,15] Toxicological study of the purified fraction needs to be

carried out and further investigation should be done on the nanoformulations.[12,15]

7. ACKNOWLEDGEMENTS

The authors express their sincere gratitude to the Department of Pharmaceutical Chemistry, Dnyanvilas College of Pharmacy, Pune, Maharashtra, India, for providing the necessary laboratory and research facilities to conduct this study. The authors are deeply grateful to their research guide for invaluable guidance on the molecular docking methodology and continuous support throughout the research work. The authors also thank the laboratory technician for providing essential laboratory materials and technical assistance. Appreciation is extended to the staff of the Chemistry and Microbiology laboratories for their support in antibacterial testing, chromatographic studies, and other experimental procedures. The authors are thankful to their colleagues for their valuable suggestions and constructive feedback during the preparation of this manuscript. Finally, the authors acknowledge the encouragement and support received from their families throughout the course of this work.

8. REFERENCES

1. Ma C, Chen Y, Chen J, Li X, Chen Y, A review on *Annona squamosa* L. phytochemicals and biological activities, *The American Journal of Chinese Medicine*, 2017; 45(5): 933–964.
2. Al-Nemari R, Bacha AB, Al-Senaidy A, Selective cytotoxic effects of *Annona squamosa* leaves against breast cancer cells via apoptotic signaling proteins, *Plants*, 2020; 9(1): 1–14.
3. Kumar M, Changan S, Tomar M, Hassan I, Custard apple (*Annona squamosa* L.) leaves: nutritional composition, phytochemical profile, and health-promoting biological activities, *Biomolecules*, 2021; 11(4): 614.
4. Bonneau L, Valentin A, Kostani M, Acetogenins from *Annona* species: isolation and biological activity, *Natural Product Research*, 2017; 31(14): 1623–1638.
5. Nasser M, Al-Harrasi A, Al-Rawahi A, Broad-spectrum antibacterial activity of Annonaceous acetogenins against Gram-positive and Gram-negative bacteria, *Saudi Pharmaceutical Journal*, 2017; 25(6): 845–852.
6. Pinto NCC, Silva JB, Menegati LM, Cytotoxicity and bacterial membrane destabilization induced by *Annona squamosa* L. extracts, *Anais da Academia Brasileira de Ciências*, 2017; 89(3 Suppl.): 2053–2073.
7. Nguyen MT, Nguyen VT, Le VM, Assessment of preliminary phytochemical screening, polyphenol content, flavonoid content, and antioxidant activity of custard apple leaves (*Annona squamosa* Linn.), *IOP Conference Series: Materials Science and Engineering*, 2020; 736: 062012.
8. Sahai M, Pandey A, Tripathi N, Soxhlet extraction of bioactive compounds from medicinal plants: method optimization and comparative studies, *Journal of Chromatographic Science*, 1994; 32(8): 345–351.
9. El-Chaghaby GA, Ahmad AF, Ramis ES, Evaluation of the antioxidant and antibacterial properties of various solvent extracts of *Annona squamosa* L. leaves, *Arabian Journal of Chemistry*, 2014; 7(2): 227–233.
10. Fujimoto Y, Takeda K, Nakamura H, Recrystallization techniques for the purification of plant secondary metabolites, *Journal of Natural Products*, 1994; 57(5): 678–684.
11. Verma RS, Joshi N, Padalia RC, Singh VR, Goswami P, Chauhan A, Characterization of the leaf essential oil composition of *Annona squamosa* L. from the foothills of north India, *Medicinal and Aromatic Plants*, 2016; 5(5): 1–5.
12. Quilez J, Martinez M, Garcia L, Optimization of dosage and delivery systems for natural antibacterial compounds, *Drug Delivery and Translational Research*, 2018; 8(3): 567–580.
13. Al-Kazman BSM, Harnett JE, Traditional uses, phytochemistry, and pharmacological activities of Annonaceae, *Molecules*, 2022; 27(11): 3462.
14. Safira A, Widayani P, An-Najaaty D, Rani CAM, Septiani M, Putra YAS, Solikhah TI, Khairullah AR, Raharjo HM, A review of an important plant: *Annona squamosa* leaf, *Pharmacognosy Journal*, 2022; 14(2): 456–463.
15. Ashaimaa YM, Siddiqui SA, Elhawary EA, Guo K, Anwar S, Xu B, Phytochemical constituents, bioactivities, and applications of *Annona squamosa* L.: a narrative review, *Food Chemistry*, 2024; 459: 140363.
16. Porwal M, Kumar A, Neuroprotective effect of *Annona squamosa* and (-)-anonaine in decreased GABA receptors of epileptic rats, *Journal of Applied Pharmaceutical Science*, 2015; 5(1): 18–23.
17. Ko GA, Kang HR, Moon JY, *Annona squamosa* L. leaves inhibit alpha-melanocyte-stimulating hormone (α-MSH)-stimulated melanogenesis via p38 signaling pathway in B16F10 melanoma cells, *Journal of Cosmetic Dermatology*, 2020; 19(7): 1785–1792.
18. Hidayati NLD, Nopianti T, Rahayuningsih N, Dewi Y, Suryani Y, Antifertility activities of ethanol extract of sugar apple leaves (*Annona squamosa* L.) in the reproductive system: spermatogenesis and sperm quality study, *Pharmaciana*, 2018; 8(2): 339–348.
19. Magadula JJ, Oteng-Yeboah AA, Singh R, Ethnobotanical and phytochemical studies on African *Annona* species, *Journal of Ethnopharmacology*, 2009; 125(2): 173–180.
20. Dash S, Sahoo N, Pattnaik G, Das C, Pattanaik S, Bhar K, Kar B, GC-MS profiling, antimicrobial activity of *Annona squamosa*: an in-silico and in-vitro approach, *Tropical Journal of Natural Product Research*, 2023; 7(8): 3691–3700.