RESEARCH ARTICLE

Isolation, *In-silico* Studies, and Biological Evaluation of Higenamine from *Annona squamosa* L. against Breast Cancer

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ABSTRACT

The efficacy of *Annona squamosa L.* in cancer treatment has been documented, inspiring the authors to investigate the plant further for potential novel anticancer compounds. Therefore, the current study aimed to isolate and characterize Higenamine from leaves extract of *A. squamosa L.* and studied for the *in-vitro* cancer cell line and molecular docking. The high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) method development of *A. squamosa L.* leaves extract furnished Higenamine of which characterization was established by HPLC, infrared radiation (IR), liquid chromatography-mass spectrometry (LC-MS) and its acute toxicity study and cytotoxic properties using MCF-7 cell lines by MTT assay were assessed. Molecular docking was done using Auto Dock Vina software to validate the anticancer activity. The extraction batches by ultrasonication and maceration methods showed good results using 80% methanol. Acute toxicity studies, after a single dose (2000 mg/kg) administration of methanolic extract don't show clinical symptoms of toxicity and mortalities. From the extract, Higenamine was eluted and the dried fraction showed 66% of enrichment of Higenamine. The developed HPLC and TLC methods showed Higenamine (HGN) peak at 6.21 minutes band at Rf 0.61, respectively. The isolated compound was identified as Higenamine with FTIR spectroscopic analysis which revealed various characteristic band values with functional groups. HPLC-MS showed m/z at 272.1 ($M+H^+$) corresponding to the molecular formula $C_{16}H_{17}NO_3$. The IC_{50} value for Higenamine was 42.39 µg/mL were observed in MCF-7 breast cancer which was better than 5-Fluorouracil having 39.22 µg/mL. *In-silico* studies explored that higenamine showed good binding affinity to breast cancer PDBIDs and forms a stable complex. Furthermore, absorption, distribution, metabolism, and excretion (ADME) properties of isolated compound was calculated using the Swiss ADME online tool in which higenamine was found to have a good pharmacokinetic profile as compared with 5-fluorouracil and doxorubicin. Higenamine has been isolated and identified as a potential anticancer agent. Further research can explore its effectiveness through pharmaceutical formulation and in vitro cell line studies.

Keywords: Molecular docking, *Annona squamosa L.*, HPLC, Cytotoxicity, Higenamine.

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INTRODUCTION

Cancer stands out as a profoundly intricate disease when compared to numerous other human ailments. As a consequence of its complexity, it offers a multitude of potential molecular targets for therapeutic development. Breast cancer is recognized as one major prevalent cancer affecting women worldwide which sadly remains a leading cause of global mortality. Despite the existence of potent drugs, the current treatments for breast cancer often exhibit adverse effects and prove ineffective for some patients. This inefficacy is often attributed to acquired resistance caused by cancer-related mutations and the presence of minor, heterogeneous subpopulations. These factors collectively contribute to the challenges in effectively treating breast cancer and necessitate further research and innovative therapeutic approaches.[1,2]

In 2022, the United States is projected to witness 287,850 of (invasive) breast cancer and 51,400 cases of ductal carcinoma (in situ). Additionally, breast cancer is anticipated to cause approximately 43,250 deaths in the same year. Among these figures, a significant portion, approximately 83% of invasive breast cancer diagnoses, is observed in women above 50 years. This age group also accounts for 91% of breast cancer-related deaths. Notably, 50% of breast cancer deaths arise in women 70 years age or older, highlighting the increased vulnerability of this age group to the disease. These statistics underscore the need for continued efforts in early detection, improved

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treatments, and increased awareness to combat breast cancer's impact on women's health, especially among older populations. The average age at diagnosis for female breast cancer across all populations is 62 years. However, there are slight variations in this age among different ethnic groups: Hispanic women are diagnosed at around 57 years, Asian/Pacific islander (API) and black women at approximately 58 and 60 years, respectively, while White women are diagnosed at a median age of 64 years. These differences can be attributed, in part, to variations in population age distributions.

When considering breast cancer-related deaths, the median age varies as well. For White women, the median age of breast cancer death is 70 years, while for Hispanic women, it is 62 years, and for API and Black women, it is 63 years. While this type of cancer predominantly affects females but there are still cases in men observed. It is estimated that in 2022, approximately 2,710 breast cancer cases and 530 deaths will be reported in men. It is important to note that unless specifically mentioned, the information presented here pertains to female breast cancer.[3]

Many medicinal plants have garnered the attention of researchers due to their potential therapeutic effects against a range of diseases, with a special focus on their anti-carcinogenic properties. These plants are abundant in nature and offer the advantage of being less toxic or non-toxic compared to the synthetic counterparts currently used in cancer treatment. Their natural availability and potential efficacy make them promising candidates for further exploration in the quest for safer and more effective cancer therapies.[4,5] The origins of Ayurvedic medicine unveil a treasure trove of natural components with powerful anti-cancer, anti-angiogenic, and anti-proliferative properties. Phytochemicals found in these natural substances play a vital role in various stages of carcinogenesis, from initiation to development and progression. They exhibit the ability to suppress or even reverse the early stages of cancer and inhibit the invasive potential of premalignant cells. Additionally, these phytochemicals modulate cell proliferation and apoptosis signaling pathways in transformed cells, contributing to their potential as valuable candidates for cancer treatment and prevention.[6,7]

Figure 1: The plant of *A. squamosa* L.

A. squamosa L. called as custard apple is an evergreen tree with a height ranging from 3–8 meters. Its leaves are oblong-lanceolate or lanceolate, measuring 6–17 cm in length and 3 to 5 cm in width, arranged alternately on short petioles. The tree's bark is thin and gray, while its flowers are greenish, fleshy, and drooping, appearing extra-axillary. The fruit of *A. squamosa L.* exhibits a diverse range of shapes, usually spanning 5–10 cm in diameter. Its surface is adorned with numerous rounded protuberances. The seeds, characterized by their oblong shape, smooth texture, and shiny appearance, measure between 1.3–1.6 cm in length. These seeds display shades of blackish or dark brown coloration.[8,9]

In traditional medicine, different ethnic communities have been utilizing all parts of *A. squamosa L.* for the treatment of diverse chronic ailments. These include cancerous tumors, insect bites, and various skin complaints. The plant has a rich medicinal heritage and widespread usage in traditional remedies highlights its potential as a valuable natural resource in combating various health challenges. The medicinal plants naturally contain phytochemicals that act as defense mechanisms and shield against a variety of ailments. Phytochemicals can be categorized into primary and secondary metabolites. Primary constituents encompass chlorophyll, proteins, and common sugars, while the latter compounds consist of terpenoids, alkaloids, and phenolic constituents.[10,11] Thorough phytochemical assessments of various components of the *A. squamosa L.* plant in Figure 1 have revealed the existence of diverse phytochemicals. These include alkaloids, cyclopeptides, diterpenes, annonaceous acetogenins, and essential oils.[8]

This research endeavor focuses on the isolation and characterization of the higenamine alkaloid from the leaves of *A. squamosa L.* The study delves into its cytotoxic effects, specifically targeting the MCF-7 associated with breast cancer. The findings are further substantiated through molecular docking analysis utilizing specific PDBIDs.

MATERIALS AND METHODS

Chemicals

Higenamine standard from Sigma Aldrich (Bangalore, India), Acetonitrile from Rankem, HPLC grade Water from Thermo Fisher and OPA from Loba chemicals. Dichloromethane was procured from Thermo fisher, methanol was procured from Merck.

Collection and Authentication of Plant

The leaves of *A. squamosa L.* were assemblaged from the western region of Satara, Maharashtra, India and verified by Dr. Priyanka A. Ingle from BSI (No. BSI/WRC/IDEN.CER. /2020/H3/107 dated. 18/12/2020). They were dried in the shade for several days at room temperature and then ground as

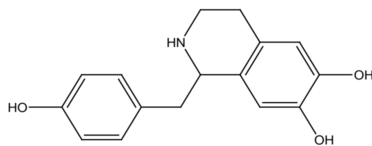


Figure 2: Structure of higenamine

powder. Subsequently, the powdered samples were stowed in an airtight container at room temperature for future utilization.

Extraction of Leaves of *A. squamosa L.*

Ultrasound-assisted extraction

Among the latest extraction methodologies, UAE stands out as a contemporary approach that provides a notable yield of active compounds while maintaining ease of handling, energy efficiency, and strong reproducibility. Predominantly applied in solid/liquid systems, UAE exerts its influence by disrupting the cellular structure of plant materials. This disruption results in improved mass transfer through cell membranes, thereby amplifying the accessibility of solvents to the targeted analytes. Notably, the effectiveness of UAE is profoundly influenced by the choice of solvents and the temperature settings, both of which emerge as critical determinants in optimizing the extraction process. The extracting solvents like 95% ethanol with 1:20 and 80% methanol 1:10 were used. In the second method ultrasonication along with maceration was done for good yield.[12]

Maceration

This is a very simple extraction method that could be used for the extraction of thermolabile components. After two times ultrasonication followed by maceration with various batch no. represented in the following Table 1.[13]

Acute toxicity studies[14]

- *Experimental animals*

Wister rats were provided from the National Institute of Biosciences 69, Pune.412205.1091/GO/BT/S/07/CPCSEA. Wistar rats are one of the recommended species as test system for preclinical toxicity studies.

Single dose toxicity study of leaves extract in rat according to OECD423 (Limit test)

The present investigation in wistar albino rat is to evaluate the preclinical safety of herbal extract. The study design involves one group consisting of six animals. The test item has been administered orally once on day one of an experiment at a dose of 2000 mg/kg. Firstly acquintation of animals to laboratory conditions for seven days was done. The 7 weeks old Wistar rat at the commencement of treatment was taken with the weight variation of the animals not exceeding ± 20% of the mean body weight in each sex. They were exposed to the test item which was diluted by 0.5% CMC solution given orally once on the first day. The response of animals was observed twice daily for 14 days for toxicity, particularly for lethality, if any, during the 14 days. The single-dose toxicity study design involves consisting six animals where body weight and feed intake were monitored weekly once during the experimental period. The experiment concluded with the sacrifice of all animals on the 15th day.

The animal husbandry process involved housing the animals in sterilized polycarbonate cages within controlled conditions. The animal rooms adhered to standard husbandry protocols. The room temperature was kept at 22 ± 3°C, with a relative humidity range of 30 to 70%. A photoperiod of 12 hours of light followed by 12 hours of darkness was maintained.

The animals were nourished with a pelleted feed comprising the standardized composition of essential macro and micronutrients (as outlined in Appendix III). Ample purified water, obtained through an Aquaguard system, was made available to the animals. Each animal was distinguished by a unique tail marking. To ensure clarity, every cage was labeled with specific cage tags detailing the study number, animal identifiers, dosage, group, administration route, species, and gender, as well as the commencement and conclusion dates of the experiment.

The animals were closely monitored at regular intervals (30 minutes, 1 hour, 2 hours, and 4 hours) following exposure to the test item. Additionally, observations were made twice daily for 14 days to document any signs of toxicity and mortality. Throughout the experimental period, the animals' food intake and body weight were measured weekly. Any clinical signs displayed by the animals were carefully recorded over the course of 14 days.[15]

High-Performance Liquid Chromatography[16]

The diluent as water with pH adjusted to 2.5 with orthophosphoric acid (OPA) was used. The standard solution was prepared by accurately weighing quantity 1.0 mg of higenamine reference standard and transferring to 10 mL volumetric flask in which 5 mL diluent was added and sonicated. Further, diluted to make up the volume using diluent to achieve 100 µg/mL. The sample was prepared by accurately weighing the quantity around 2 mg of dried column fraction and dissolved in 1-mL of Diluent. The solutions were sonicated to dissolve the fraction and filtered the solution through 0.22 µ nylon filters and 20 µL of sample was injected to HPLC.

Procedure

The analysis was carried out on injection valve by 20 µL, a photo-diode array detector (set at 225 nm) sensitivity was 0.001 and Hypersil gold (250 x 4.6 mm, 5 µ) column. The HPLC solvents were water (pH 2.5 with OPA): ACN with 90:10 proportion. The analytes were eluted using a gradient approach with a flow rate of 1-mL/min. Chromatograms were recorded on software. The HPLC instrument was operated at column temperature (27°C). Each diluted sample 100 µg/mL was injected into the column with run time 20 minutes having flow rate 1-mL/min for three times and the average peak area was reported.

Fractionation

The extract with 1:10 ratio of raw material: extracting solvent gave a good yield as compared to 1:5 and 1:20 which did

not show any significant increase in yield. Hence 1:10 ratio was selected for extraction. The extract obtained from 80% methanol was suspended in dichloromethane (DCM) and partitioned with 1M hydrochloric acid separately. The DCM and aqueous layer were separated and water layer was again washed with hexane, ethyl acetate, and DCM. The water layer was dried on a rotary evaporator (25.5g). The 10 mg water fraction was dissolved in 0.1% OPA for HPLC analysis. DCM and water fractions were checked for the presence of Higenamine on HPLC. Further water fraction was again washed with DCM and subjected to column chromatography in which silica gel as the stationary phase and a mobile phase consisting of mixtures of water (pH 2.5 with OPA): ACN 90:10 for enrichment. The elution was started with 3% methanol in DCM followed by 5, 7 and 10% methanol. Higenamine was seen eluting in 7 and 10% methanol. Fractions were dried and checked on HPLC for enrichment.

Thin Layer Chromatography[17]

For sample preparation, methanol was used as a diluent. Precoated silica plates were cut in desired dimensions. Sample was spotted on the plate using a capillary. The chamber saturation time was optimized as 15 minutes. Plate was run in the developed mobile phase as Dichloromethane: Methanol: OPA (6.5:3.5:0.1) and dried. After complete drying the plate was derivatized by dip method using an anisaldehyde-sulphuric acid reagent. The plate was dried at 100°C till color developed. The rf of the sample was measured by comparing it with the reference standard spot.

Fourier Transform Infrared Spectroscopy Analysis[18,19]

The isolated component was analyzed for FTIR spectra in range of 400 to 4000 cm^{-1} with Bruker Alpha Platinum ATR.

Mass Spectroscopy

The spectrum was acquired using an Agilent 1260 Infinity HPLC-MASS Analyzer 6460 Triple Quad LC/MS, situated at the Food Testing Laboratory in Pune, India.

ADME Prediction

The chosen ligand structures (SMILES notation) underwent drug-likeness assessment through the SwissADME server (accessible at http://www.swissadme.ch/). Ligand selection was carried out based on specific criteria, including a high predicted gastrointestinal absorption, no blood-brain barrier (BBB) permeability, and zero violations of Lipinski's rule of five (Ro5). The resulting ligand library was constructed using structures that met Lipinski's Ro5 criteria and exhibited drug-like attributes.[20]

Molecular Docking

The investigations were performed to elucidate interactions occurring between the phytoconstituent Higenamine, extracted from *A. squamosa L.,* and a specific protein associated with breast cancer. The molecular docking process encompassed the creation of three-dimensional structures for the phytoconstituents, referred to as ligands. Subsequently, these ligands were subjected to preparation and energy minimization. The protein of interest was acquired from RCSB (PDB IDs: 2J6M, 3ERT, 3TJM, and 4OAR) and subsequently refined using the MMCF-2 force field. To ensure the ligand-free state of the complex receptors, all heteroatoms were eliminated from the protein's crystal structure prior to docking, a step facilitated by PyMOL 2.3.4 software. The Auto Dock Tools (version 4.2.6) software was employed for protein and ligand molecules. The protein molecule underwent removal of water molecules, addition of polar hydrogens, Kollman charges, while the ligands, being small molecules, received Gasteiger charges. The protein's binding site was defined using a grid box, and the molecular docking process was conducted employing AutoDock Vina (version 1.1.2). The docking computations using AutoDock Vina were carried out on an IBM system, operating on the Microsoft Windows 10 platform. The outcomes were assessed based on the binding affinity exhibited between the ligand and the protein. The protein-ligand binding conformations were visualized and analyzed utilizing Discovery Studio Visualizer 2020 software. The 2D structures of the individual components were created using ChemDraw Professional 16.0.[21,22]

Cell Lines

The MCF-7 human breast cancer cell lines were sourced from the Biocyte Institute of Research and Development in Sangli. These MCF-7 cells were cultivated in DMEM-F12 medium, which was enriched with hydrocortisone (0.5 µg/mL), and human epidermal growth factor (hEGF) (20 ng/mL), and insulin (10 µg/mL). The culture media were prepared by supplementing with high glucose DMEM (Catalog No: 11965-092), Fetal Bovine Serum (FBS) sourced from Gibco (Catalog No: 10270106), and Antibiotic-Antimycotic 100X solution obtained from Thermo Fisher Scientific (Catalog No: 15240062) as described in previous studies.[26]

MTT Assay

Cells were incubated at a concentration of 1×10^4 cells/mL in a culture medium at 37°C and 5% CO_2 for 24 hours. In microplates (96 wells) with tissue culture-grade wells, cells were seeded at a concentration of 70 µL (10^4 cells/well) in 100 µL culture medium. Additionally, 100 µL of compounds (at concentrations of 10, 40, and 100 µg) were added to separate wells. Control wells contained DMSO (0.2% in PBS) and the cell line. All samples were incubated in triplicate. To determine cell survival and the percentage of live cells after incubation, control wells were maintained.

Following a 24-hour incubation period at 37°C and 5% CO2 in a Thermo Scientific BB150 CO2 incubator, the medium was removed, and 20 µL of MTT reagent (5 mg/mL in PBS) was added. Cells were then incubated for an additional 4 hours at 37°C in a CO_2 incubator. Formazan crystal formation, indicative of viable cells, was observed under a microscope. The yellowish MTT was reduced to a dark-colored formazan only by viable cells. After complete medium removal, 200 µL of DMSO was added (kept for 10 minutes) and incubated at 37°C, wrapped with aluminum foil.

Table 1: %Yield of various extraction methods for *A. squamosa* L. leaves

Batch no.	*Raw material Qty (g)*	*Extracting solvent*	*Raw material: Solvent*	*Extraction method*	*Yield (g)*	*% Yield*
AS/001/23	10	95% ethanol	1:20	Ultrasonication reflux (60°C, 8 hours)	0.817	7.46
AS/002/23	25	80% methanol	1:10	Ultrasonication+ Maceration	7.64	30.08
AS/003/23	50	100% methanol	1:5	Maceration	3.917	7.834
AS/004/23	10	Water	1:10	Maceration	1.35	13.5
AS/005/23	10	Methanol: 1M HCL (50: 50)	1:10	Maceration	0.951	9.51
AS/006/23	10	Hexane followed by Ethyl acetate	1:10	Maceration	Discarded	-
AS/007/23	50	100% methanol	1:10	Maceration	5.06	10.12
AS/008/23	200	80% methanol	1:10	Maceration	59.32	29.66

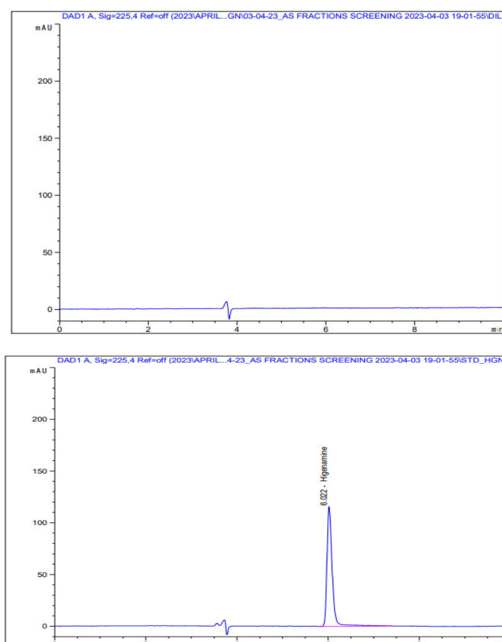


Figure 3: HPLC chromatogram of A. Diluent used B. Higenamine standard

To assess the results, triplicate samples were analyzed by measuring the absorbance of each sample using an Elisa microplate reader (Benesphera E21) at a wavelength of 570 nm. Control wells only contained media without the tested compound. The anticancer drug 5-Fluorouracil served as a positive control. The inhibition of cellular growth by the tested sample was calculated as the percent inhibitory activity and expressed as the IC_{50} value (the concentration of the tested sample required to inhibit 50% of cell growth).

%inhibition = (1 – [A1/A0] ×100) where; A0 is the absorbance of the control and A1 is the absorbance of the extracts.[24-27]

RESULTS AND DISCUSSION

Extraction of Leaves of *A. squamosa L.*

The extraction batches using conventional methods such as a combination of ultrasonication and maceration showed the highest yield (30.08%) in 80% methanol. The extraction by maceration with 1:10 ratio of raw material to solvent using 80% methanol showed 29.66% yield. In addition to the advantages of ultrasonication, it has disadvantages such as disrupting the structure of molecules. Hence bulk batch was done with 80% methanol by maceration. The Higenamine was present in water fraction yielded 25.5 g (42%) from 80% methanolic extract as explained in Table 1.

Acute Toxicity Studies

Previous reported acute toxicity study on *A. squamosa L.* extract showed no any significant toxicity, hence limit test was carried with dose 2000 mg/kg. Throughout the study period, as outlined in Table 2, there were no occurrences of preterminal deaths. Clinical observations remained consistent, encompassing a thorough assessment of various aspects such as skin, fur, eyes, mucous membranes, respiration, salivation, diarrhea, behavioral patterns, as well as the autonomic and central nervous systems. Special attention was given to detect any signs of tremors, convulsions, or coma. Notably, there were no significant alterations noted in the physical, physiological, and behavioral profiles of the animals, as highlighted in Table 3.

The animals displayed regular food intake with no noteworthy changes, and there were no substantial effects observed in any of the animals over the course of the study. Similarly, the animals exhibited normal body weight gain, and no significant effects were observed during the study period, as indicated in Table 4.

Ultimately, no clinical symptoms of toxicity or mortalities were identified among the animals in all treated groups following a single dose administration (2000 mg/kg) of the test item.

High-Performance Liquid Chromatography

The chemical investigation of alkaloid part was reported in previous studies, but HPLC method development for Higenamine was done firstly in this research. The elution was started with 3% methanol in DCM followed by 5, 7, and 10% methanol. Higenamine was seen eluting in 7% and 10% methanol. Figure 2 shows a chromatogram of diluent and

Table 2: Observations for preterminal deaths

Sex	*Animal No.*	*Day*													
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Females	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Males	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0-Normal: X-Not survived

Table 3: Clinical signs observed in acute toxicity studies

S.No.	*General signs*	*1*	*2*	*3*
1	Assessments of posture	0	0	0
2	Signs of Convulsion (Limb paralysis),	0	0	0
3	Body tone	0	0	0
4	Lacrimation	Y	Y	Y
5	Salivation	Y	Y	Y
6	Tremor	0	0	0
7	Change in skin color	Y	Y	Y
8	Piloerection	Y	Y	Y
9	Defaecation	0	0	0
10	Sensitivity response	0	0	0
11	Locomotion	0	0	0
12	Muscle gripness	0	0	0
13	Rearing	0	0	0
14	Food intake	0	0	0

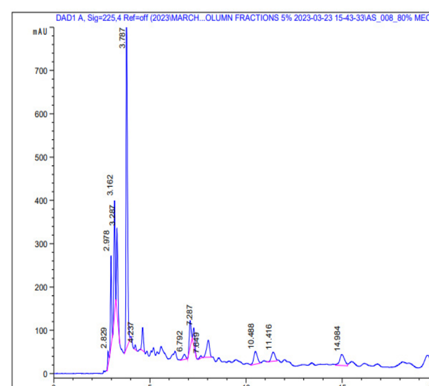
0-Normal: X-Not survived: Y-No any sign

Table 4: Details of Food intake and body weight

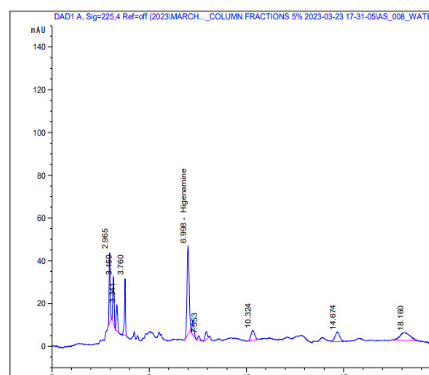
Food Intake			*Body weight (gm)*	
Animal ID	Day		Day	
	0	14	0	14
01	119 gm	114gm	185	208
02			204	225
03			184	204
04			187	212
05			194	207
06			187	210

standard Higenamine. Figure 4A represents the chromatogram for Batch AS_008_80% methanolic extract, 4B represents HGN peak at 6.938 minutes for 5% water fraction and 4C represents peak at 5.902 minutes for 10% water subfraction. The dried fraction showed 66% of enrichment of Higenamine. The developed HPLC method showed HGN peak at 6.21 minutes. The chromatograms for the same are attached with the report as given in Figures 3 and 4.

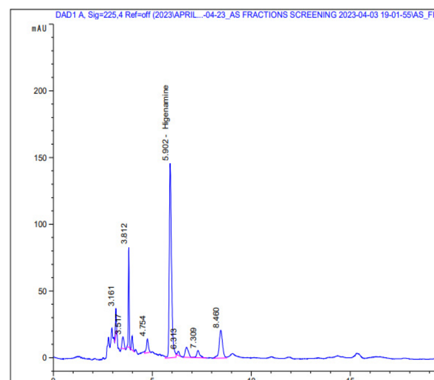
From 25 g water fraction, 20 g of the fraction was processed and 66% enrichment of Higenamine was achieved through column chromatography. The total 550mg Higenamine



(A) AS_008_80% Methanolic extract



(B) AS_008_Water fraction 5%



(C) AS _008_water subfraction 10%

Figure 4: HPLC chromatogram of A.80% Methanolic extract, B.5% water fraction, C.10% water subfraction.

Figure 5: Thin layer chromatography plate

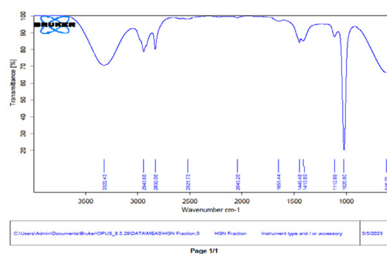


Figure 6: IR spectrum of the isolated fraction of Higenamine

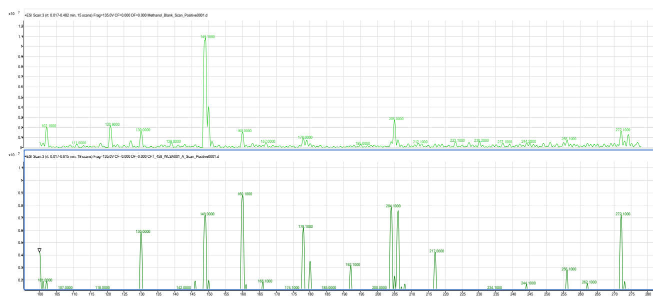


Figure 7: Mass spectrum of isolated fraction of Higenamine

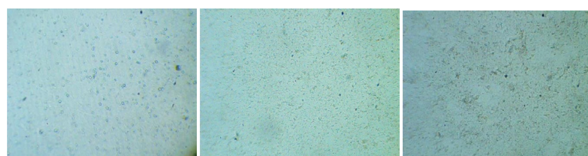
enriched fraction was generated. The extraction, analytical method and enrichment scheme for HGN from *A. squamosa L.* has been established successfully. The findings of the present study are limited to the HPLC analysis of *A. squamosa L* methanolic extract to estimate the presence of higenamine phytochemical from the chromatogram peaks.

Thin Layer Chromatography (TLC)

The compounds separated into distinct bands on the TLC plates, which were visible under white light. The developed TLC method showed a Higenamine band for 10% fraction at Rf 0.61. The TLC plate pictures are attached in Figure 5.

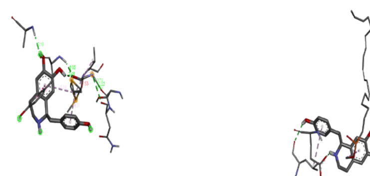
IR spectroscopy

Peaks: 3322, 2943, 2832, 2521, 1591, 1513, 1449, 1413, 1112, 1020, 616 cm^{-1} as shown in Figure 6. The isolated Higenamine



A. Control B. Standard(5- Fluorouracil) C. Higenamine(100 µg/ml)

Figure 8: Effects of anticancer activity of Higenamine compared with standard

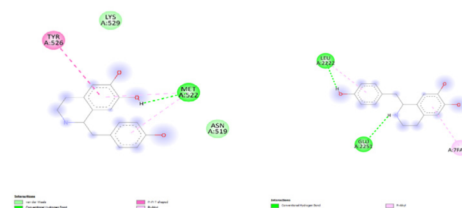


A.3D Higenamine with 3ERT B. 3D Higenamine with 3TJM

Figure 9: 3D images of Higenamine with PDBID: 3ERT, 3TJM

Table 5: Binding affinity of Higenamine and reference standard

Compound name	*Binding affinity (PDBID)*			
	2J6M	4OAR	3ERT	3TJM
Higenamine	-7.1	-6.6	-7.1	-8
Doxorubicin	-7.8	-9.9	-7.7	-8
5-Fluorouracil	-5.1	-5.7	-5.5	-5.3



A.2D Higenamine with 3ERT B. 2D Higenamine with 3TJM

Figure 10: 2D images of Higenamine with PDBID: 3ERT, 3TJM

showed all functional group values in a spectrum of Infrared spectroscopy as compared with standard.

Mass Spectrophotometry

EI-MS: m/z 272.1 (10%, M+) calculated for $C_{16}H_{17}NO_3$ as shown in Figure 7.

Cell Line

Previous studies have illustrated *A. squamosa L.* leaves extract anticancer activity on breast cancer cell lines. Therefore this study aimed to investigate anticancer activity on breast cancer for isolated Higenamine. At the different doses (10 µg to 100 µg) of different compounds carried out for antitumor

Table 6: ADME properties of Higenamine

GI absorption	*BBB permeant*	*Pgp substrate*	*CYP1A2 inhibitor*	*CYP2C9 inhibitor*	*CYP2C9 inhibitor*	*CYP2D6 inhibitor*	*CYP3A4 inhibitor*	*log Kp (cm/s)*
High	No	Yes	No	No	No	Yes	No	-6.36

Table 7: Predicted parameters in Swiss ADME

S. No.	*Consensus log Po/w*	*Solubility*	*Class*	*Bioavailability score*	*Synthetic accessibility*
Higenamine	-1.93	1.52e-01 mg/mL; 5.60e-04 mol/l	Moderately soluble	0.55	2.62

activity against MCF-7 cell line. The % inhibition for 10, 40, 100 µg/mL was reported as 49.21, 54.60 and 62.16%, respectively. The IC_{50} value for Higenamine was 42.39 µg/mL were observed in MCF-7 breast cancer which was better than 5-Fluorouracil having 39.22 µg/mL. The samples showed good activity as compared to the standard compound as shown in Figure 8.

Molecular Docking

Molecular docking studies were showed the binding interactions of phytochemical Higenamine of *A. squamosa L.* with PDBIDs: 2J6M, 3ERT, 3TJM and 4OAR along with 5-fluorouracil and Doxorubicin. Higenamine with PDBID: 3ERT showed amino acid residues MET522 conventional hydrogen bond, LYS529, ASN519 van der Waals forces, TYR526 π-π -T shaped interactions. Higenamine with PDBID: 3TJM showed amino acid residues LEU2222, GLU2251 conventional hydrogen bond, 7FA500 π-alkyl interactions. From the results, docking score was found to be significant for Higenamine for all PDBIDs (− 8 to − 6.6 Kcal/mol) as given in Table 5. This research has reported Molecular docking on breast cancer PDBIDs for higenamine first. It was found to be an effective anticancer molecule against breast cancer. The stable 3D and 2D confirmations of the ligands with breast cancer were represented in Figures 9 and 10.

ADME Studies

Furthermore, ADME properties of isolated compound was calculated using the SwissADME online tool in which Higenamine was found to have good pharmacokinetic profile as compared with 5-fluorouracil and doxorubicin as reported in Tables 6 and 7. Hence, Higenamine showed drug likeliness behavior.

CONCLUSION

In our study, Higenamine was isolated from leaves extract of *A. squamosa L.* and characterized by HPLC, IR, and LC-MS. Acute toxicity study confirmed the clinical safety of the phytochemical and *in-vitro* cell line studies indicated that Higenamine had anti-cancer activity comparable with the reference compound, and identified as a potential anticancer agent. The findings of our investigation suggest that Higenamine is a potential anticancer agent and can be further explored by its suitable pharmaceutical formulation.

ETHICAL APPROVAL

Protocol number: IAEC/Sangli/2022-23/04 at Biocyte Institute of Research and Development, Sangli.

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