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Original Research Article

GC-MS Profiling, Antimicrobial Activity of *Annona squamosa*: An *In-silico* and *In-vitro* Approach

Sasmita Dash[1], Nityananda Sahoo[1], Gurudutta Pattnaik[1], Chandan Das[2], Sovan Pattanaik[2], Kausik Bhar[3], Biswakanth Kar[2]*

[1]*School of Pharmacy and Life Sciences, Centurion University of Technology and Management, Jatni, India-751050*
[2]*School of Pharmaceutical Sciences, Siksha O Anusandhan Deemed to be University, Bhubaneswar, India-751030*
[3]*Bengal School of Technology, Sugandha, Hooghly, West Bengal - 712102*

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ABSTRACT

Annona squamosa Linn. (Annonaceae) is a perennial plant that possesses anti-diabetic, antioxidant, anti-tumor, analgesic, and antimicrobial activity. The current study was designed to explore the phytochemicals using GC-MS analysis and to evaluate the antimicrobial activity followed by *in-silico* molecular docking study. The leaf powder of *A. squamosa* was successively extracted with n-hexane and methanol. The n-hexane fraction was subjected to GC-MS analysis. The extract was evaluated for antimicrobial activity to determine the zone of inhibition by disc diffusion method at concentrations of 12.5, 25, and 50 mg/ml. The minimum inhibitory concentration was performed at 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml concentration, against gram-positive bacteria (*Staphylococcus aureus* ATCC-25923, *Staphylococcus aureus* ML-267, *Staphylococcus aureus* ATCC-29737, *Staphylococcus aureus* ATCC-29157, *Bacillus subtilis*-6633), and gram-negative organisms (*Escherichia coli* PBR-332, *Escherichia coli* JM-109, *Klebsiella pneumoniae* PB-12) using streptomycin (1 mg/ml) as standard. *In-silico* docking was carried out with AUTO-DOCK Tool 1.5.6 using Discovery Studio 3.5. The GC-MS study revealed 13 compounds, the major compounds were 16-hentriacontanone (41.95%); [(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylene-tricyclo-[4.4.0.02,7]-decane] (15.91%) and caryophyllene (12.07%). The better zone of inhibition was found at a concentration of 50 mg/mL against all organisms whereas the Minimum Inhibitory Concentration was detected at 6.25 mg/mL. The docking results revealed through Auto-Dock tool, a highest docking score of -5.2 each obtained from both the compounds caryophyllene and [(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo-[4.4.0.02,7]-decane], which was compared to the that of standard trimethoprim as reported previously. The antimicrobial potential of *A. squamosa* leaf might be attributed to the synergistic influence of bioactive components identified from GC-MS analysis.

Keywords: *Annona squamosa* L, GC-MS, *in vitro* antimicrobial, *in silico* molecular docking

Introduction

In many traditional systems of medication, plants as whole and their various parts are utilized as a remedy for various ailments by Vaidya, tribals and local healer; and are enriched time to time for showing better efficacy.[1] Different plant species contain many phytoconstituents which have broad range of medicinal properties without showing any adverse effects. Infectious diseases are still a major health concern accounting for 41% of the global disease. One of the main causes of this problem is the bacterial resistance to antibiotics. The present antibiotic therapies are commonly accompanied by a variety of toxic effects. [2] Antimicrobial drugs may produce adverse effects like an allergic reaction and hypersensitivity upon consumption. Additionally, a major concern is that some antimicrobial drugs have triggered multiple drug resistance in hosts.

*Corresponding author. E mail: biswa_kanth2005@yahoo.co.in
Tel: +91 9692769860

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Due to this problem of resistance against antibiotics, attention is now being shifted towards biologically active components isolated from plant species communing used as herbal medicine, as they may produce a new potent source of antibacterial and antifungal activities. [3] Therefore, it was vital to seek a natural drug that could overcome these disease conditions without causing any other adverse effects. [4]

Annona squamosa Linn is a shrub that belongs to the family Annonaceae. [5] It is a perennial tree with thin, oblong-lanceolate, alternate leaves. It is abundantly native to tropical America, the Caribbean, and Indomalaya. [5] The fruits, leaves, bark, and root possess a wide variety of medicinal and nutritional value as it contains vitamin C, amino acid, thiamine, riboflavin, potassium, calcium, and dietary fibers.[5] *A. squamosa* exhibits several biological activities like hypoglycemic,[6] antimicrobial,[7] anticancer,[8] analgesic,[9] and antioxidant.[10] The leaf extract of *A. squamosa* L significantly reduce the average testicular-index, that happened due to the testicular weight shrinkage in animals. [11] The phytoconstituents like aporphine and annonaine alkaloids, flavonoids, and glycosides were identified from the leaves and bark exhibited many biological activities. [5] The anti-microbial activity of the methanolic leaf extract of *A. squamosa* was performed against Gram-positive and Gram-negative species like *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *E. coli*, using the agar disc diffusion method. [12] However, the anti-microbial effect of n-hexane extract from the leaves of the plant is not performed yet. Hence the present study was designed to evaluate the antimicrobial activity and to investigate the compounds by GC-MS analysis and; further, an *in-silico* docking study to support its claim.

Materials and Methods

Collection and Identification

The leaves of *A. squamosa* plant were collected from the local area near Chinsurah, Hooghly, West Bengal, India, located at Latitude 22.90°N, Longitude 88.39°E (Source: https://en.wikipedia.org/wiki/Chinsurah_subdivision) during the month of October-November 2021 and authenticated by Dr. Pratap Chandra Panda, Taxonomist, Centre of Biotechnology, Bhubaneswar, Odisha bearing voucher specimen No. 2247. The leaves were shade dried for one week at room temperature and made into a coarse powder using a mechanical blender.

Extraction

About 600 gm of dried leaf powder was successively extracted with n-hexane (95% v/v) and methanol (99.00% v/v) using a Soxhlet apparatus. The solvent was subjected to evaporation using a rotary evaporator of model no. Sare-29G (Labex) R.S. Scientific, India under reduced pressure to collect the extract. The obtained extract was kept at a temperature of 2-8ºC for further use.[13]

Qualitative phytochemical tests

The qualitative tests were done to establish the phytochemical content of n-hexane leaf extract of *A. squamosa* L (LEAS) by Molisch's test,[14] Iodine test,[15] Fatty acid test,[16] Mayer's test,[17] Liebermann-Burchard test,[18] Shinoda test,[19] ferric chloride test,[20] and phenolic test,[21] for carbohydrates, starch, fatty acid, alkaloids, steroids, flavonoids, tannins and phenolic compounds, respectively.

Gas Chromatography Mass Spectrometry (GC-MS) analysis

The n-hexane of LEAS was subjected to GC-MS analysis using Agilent Technologies GC systems by Agilent 5977 MSD Module Display equipped with a column dimension of (30 meters length×250 micrometer diameter×0.25 micrometer thickness). The spectroscopic study by GCMS required some electronic energy utilizing high-energy electrons of 70 eV. A source of pure helium gas (99.995%) was taken as a carrier system having a 1.2 mL/min flow rate. The preliminary temp was fixed at 40°C with an increasing value of 2°C per min and the holding time was around 5 mins. Finally, the system temperature was raised to 325-350 °C with a pressure of about 9.1473 psi. Identification of compounds was confirmed with the help of a database found in the NIST (National Institute of Standards and Technology) library.[22]

In vitro antimicrobial study

Disc diffusion method: The antimicrobial study of n-hexane of LEAS was performed by disc diffusion method at 12.5, 25, and 50 mg/mL concentration.[23] About 100 µL of different bacterial strains were allowed to grow in 20 mL of freshly prepared agar media till they attained a desired count of nearly 108 cells per mL. An aliquot of 10 µL at different concentrations of the leaf extract was placed over a sterile Whatman paper disc (No. 1) of diameter 5.5 mm on the agar plates. The streptomycin (1 mg/mL) was used as the positive control, and filter paper discs soaked with 10 µL of DMSO solvent served as the negative control. The plates were incubated in a BOD incubator of model no. 109A (Dixell tecchnology) India for about 24 hrs at 37 °C, and the zone of inhibition was measured.

Minimum Inhibitory Concentration (MIC)

The MIC of the n-hexane LEAS was performed by agar well dilution method. It is the least concentration of the sample that inhibits the growth of microorganisms.[3] Serial dilution was done consequently with the concentrations of 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, and 1.56 mg /mL. 2 mL of each of the dilution of LEAS solution was added to 18 mL of Mueller-Hinton agar and transferred into Petri-plates and allowed to be solidified. The Petri plates containing agar were streaked with the isolates of bacterial overnight broth culture and incubated overnight.[24] 1 ml of the standard antibiotic streptomycin was mixed into the test tube as the positive control whereas the negative control contains the selected ATCC microbial strains. The result found after 36 hours of incubation was compared equally to both positive control and negative control. The MIC of the LEAS is the definite concentration of the extract at which the growth of the bacteria was inhibited.

In silico molecular docking study

Selection of receptor

In silico study was carried out by the selection of protein Dihydrofolate Reductase (DHFR) (PDB ID: 3SRW) Figure 1 was retrieved from protein data bank (PDB) database with the X-ray diffraction technique at a resolution of 1.70 Å showing the R-Value (Free and work) was 0.221 and 0.192 respectively as reported.[25] Further, the docking preparation of this targeted protein 3SRW was prepared by removing the attached ligand and water molecules.

Ligand selection

The 3-D structure of the selected nine phyto-components identified from GC-MS analysis was acquired from the PubChem database in structural data format (SDF). Furthermore, OPEN BABEL's GUI (graphical user interface) software was used to convert the SDF format to PDB format.

Molecular docking of receptor and ligand

The phyto-components detected in the GC-MS analysis were subjected to *In-silico* molecular docking by using AUTO-DOCK Tool 1.5.6. Then, the grid box was fixed to X = -0.038, Y = -28.906, Z = 9.337 with a spacing of 0.392 Å for 3SRW including all the required active moieties like ALA 8, GLN 20, LEU 21, GLY 95, PHE 99.[25] The Auto-Dock tool helps to quantify the stability of the interaction between two residues with the scoring function.[26] Individually, the interaction of the ligand and protein molecule was monitored using the Discovery Studio Visualizer (3.5), and the result was stored in the image format.[27]

Statistical analysis

All the findings were expressed as mean ± standard error of mean (S.E.M.) The values are considered to be mean± SEM and n=3. $p<0.05$was taken as significant. The values were calculated by (one-way ANOVA) following Dunnet-multiple tests.

Results and Discussion

The major phytoconstituents identified in the LEAS were fatty acids, steroids, terpenoids, tannins, cardiac glycosides, and alkaloids. On the other hand, the extract did not give a positive result for flavonoids, phenols, carbohydrate and starch which is provided in Table 1. The use of plants gradually increases day by day in the treatment of various diseases. This is owing to the safety concept of plants and cost-effectiveness and several adverse effects of conventional drugs.[39] Number of researchers experimentally documented the role of secondary metabolites formed by the plants to keep them safe from external invaders.[40] Dash *et al*. proposed in the literature that the secondary metabolites like flavonoids, phenols, alkaloids, tannins, steroids, etc., present in the plants are responsible for showing beneficial effects including antimicrobial properties.[5] Terpenes are the chief essential components of the plant extract that showed good antimicrobial activity.[41] Yamunadevi *et al*. suggested that tannins, and steroids are responsible to have antidiarrheal activity.[42] Therefore, the presence of tannins, alkaloids, fatty acids, terpenoids, and steroids could be attributed to the antimicrobial activity of the n-hexane LEAS. The terpenoids with the fatty acids identified in the leaf extract were showing good anti-bacterial function that is further confirmed in the database and the total ion chromatogram as provided in the GC-MS analysis.[20]

The GC-MS analysis of the n-hexane LEAS revealed 13 compounds. The detailed information on identified compounds were presented in Tables 2 and Figure 2. The major compounds were 16-hentriacontanone (41.95%); (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo-[4.4.0.02,7]-decane (15.91%); caryophyllene (12.07%); bicyclogermacrene (5.28%); caryophyllene oxide (3.20%); cyclohexene-4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl) (3R-trans) (2.14%); cyclohexane-1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]- (2.42%); humulene (2.04%); γ-muurolene (1.04%); (-)-spathulenol (1.96%); tau.-Cadinol (1.86%);

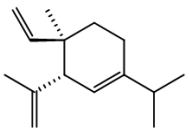
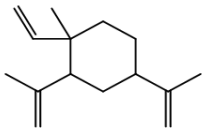
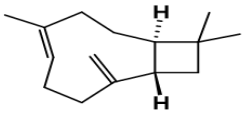
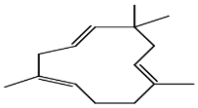
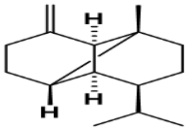
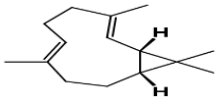
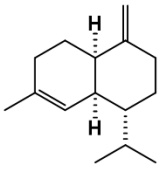
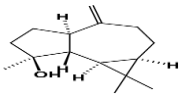
1,6,10,14-Hexadecatetraen-3-ol-3,7,11,15-tetramethyl-, (E, E)-(1.77%) and n-Hexadecanoic acid (1.67%). The compounds identified by the GC-MS study exhibited different therapeutic values. Some compounds were found to contain fatty acid which enhances the antimicrobial potential of the plant. Yoon *et al*. mentioned that fatty acids and triglycerides are most well-known for their antibacterial activities.[43] The antimicrobial effect of the LEAS which were supposed to be the existence of fatty acid n-Hexadecanoic acid derived from GC-MS analysis might destabilize the Gram +ve and Gram -ve bacterial cell wall.[44] The bactericidal activity mostly depends on the environmental factors, the species, concentration, and the mechanism involving its amphipathic effect.[45] They disturb the mechanism of the electron transport chain (ETC), oxidative phosphorylation, and membrane enzymes that regulate bacterial energy formation.[43] This leads to alteration in the membrane integrity resulting in the permeability of the cell, cell growth inhibition, cell lysis, and finally cell death.[43]

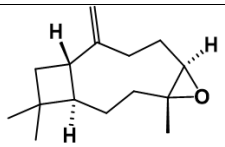
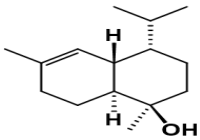
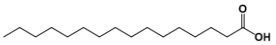
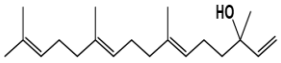
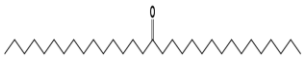
Table 1: Preliminary phytochemical study of the n-hexane leaf extract of *A. squamosa* L.

Extract	Phytochemical Tests									
	Carbohydrate	**Starch**	**Fatty acids**	**Steroids**	**Terpenoids**	**Flavonoids**	**Tannin**	**Phenols**	**Cardiac glycosides**	**Alkaloids**
n-hexane	-	-	+	+	+	-	+	-	+	+

'+' sign indicates presence of phytoconstituents and '-' sign indicates absence of phytoconstituents

Table 2: Phytoconstituents of *A. squamosa* leaf extract detected by GC-MS

Peak	RT	% Area	Molecular weight (g/mol)	Molecular formula	Compound name	Chemical Structure	Pharmacological activities
1	11.77	2.14	204.35	$C_{15}H_{24}$	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans		Antimicrobial[28]
2	13.11	2.42	204.35	$C_{15}H_{24}$	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]-		Antimicrobial[29]
3	13.80	12.07	204.35	$C_{15}H_{24}$	Caryophyllene		Antimicrobial[30]
4	14.62	2.04	204.35	$C_{15}H_{24}$	Humulene		Antimicrobial[31]
5	15.28	15.91	220.35	$C_{15}H_{24}O$	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane		Antibacterial[31]
6	15.65	5.28	204.35	$C_{15}H_{24}$	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene		Antimicrobial[32]
7	16.05	1.04	204.35	$C_{15}H_{24}$	γ-Muurolene		Antimicrobial, antioxidant and anti-inflammatory[33]
8	17.53	1.96	220.35	$C_{15}H_{24}O$	(-)-Spathulenol		Antimicrobial and antioxidant[34]

9	17.67	3.20	220.35	$C_{15}H_{24}O$	Caryophyllene oxide		Antimicrobial[30]
10	18.93	1.86	222.36	$C_{15}H_{26}O$	.tau.-Cadinol		Antibacterial[35]
11	26.67	1.67	256.42	$C_{16}H_{32}O_2$	n-Hexadecanoic acid		Antioxidant, antibacterial[36]
12	28.42	1.77	290.48	$C_{20}H_{34}O$	1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)-		Antimicrobial[37]
13	48.37	41.95	450.8	$C_{31}H_{62}O$	16-Hentriacontanone		Antimicrobial and antioxidant[38]

RT: Retention Time; g/mol: gram/mol

Table 3: Antimicrobial activity of n-Hexane extract of *A. squamosa* leaf against various gram-positive and gram-negative bacteria

Organism	Zone of Inhibition ± Standard (mm)				
	Concentration				
	Positive control (Streptomycin 1mg/ml)	**Negative control (DMSO)**	**12.5 mg/ml (LEAS)**	**25 mg/ml (LEAS)**	**50 mg/ml (LEAS)**
S. aureus ATCC-25923	35 ± 0.56	0	6 ± 0.47	8 ± 0.52	11 ± 0.58
S. aureus ML-267	34 ± 0.38	0	-	6 ± 0.42	12 ± 0.59
S. aureus ATCC-29737	21 ± 0.32	0	6 ± 0.59	7 ± 0.71	8 ± 0.70
S. aureus ATCC-29157	38 ± 0.35	0	6 ± 0.53	8 ± 0.64	10 ± 0.68
B. subtilis 6633	28 ± 0.25	0	6 ± 0.47	7 ± 0.62	10 ± 0.69
E. coli PBR-332	40 ± 0.58	0	5 ± 0.56	9 ± 0.56	14 ± 0.56
E. coli JM-109	35 ± 0.47	0	6 ± 0.56	7 ± 0.56	9 ± 0.56
K. pneumoniae PB-12	33 ± 0.52	0	-	-	11 ± 0.56

± value represents the standard error mean. The values are considered to be mean± SEM and n=3. * $p<0.05$was taken as significant

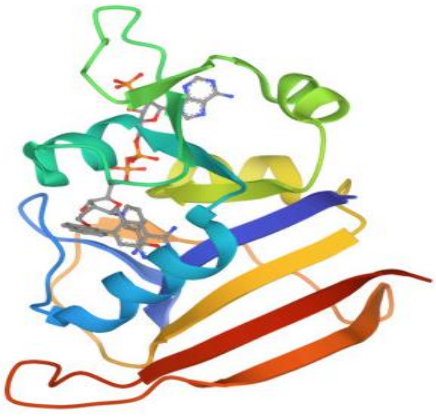


Figure 1: Structure of *Staphylococcus aureus* receptor (PDB ID:3SRW)[25]

The phyto-constituents found in the fraction of essential oil disrupt the metabolic pathway of the pathogenic microorganism.[47] Moreover, they reduce the membrane potential, affect the proton pump and ion channels, hinder the pathway of Cyt C, and inhibit protein metabolism. Herein, it was found that *A. squamosa* essential oil exhibited a remarkable antimicrobial effect which may be credited to the chemical components identified. Cyclohexane-1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]- is showing excellent effect against gram-negative organisms like *E. coli* and *E. cloacae;* and gram-positive bacteria *S. aureus.*[26] The richness of γ-Muurolene in essential oils (EOs) of *Ocimum* shows excellent anti-bacterial activity against *E. coli.*[46] The abundance of β-Caryophyllene and Caryophyllene oxide in the essential oil of *Ocimum* shows higher antimicrobial and antifungal effects.[47] Sesquiterpenes present in different plants are reported as having potent antimicrobial activity.[44,48] The identification of sesquiterpenes, caryophyllene, humulene, bicyclogermacrene, γ-muurolene, and caryophyllene oxide in GC-MS analysis of LEAS might be responsible for the antimicrobial potential.

The antimicrobial potential of LEAS on gram-positive and gram-negative bacteria was reported in Table 3 and compared with the standard streptomycin. The LEAS exhibited moderate to good zones of inhibition against the organisms at 12.5, 25, and 50 mg/mL concentrations. *The* sample showed better zones of inhibition at 50 mg/mL concentration against all organisms. However, the zones of inhibition of the extract were not found at a concentration of 12.5 mg/mL against *S. aureus* ML-267 and at a concentration of 12.5 and 25 mg/mL against *K. pneumoniae* PB-12 as shown in Figure 3. MIC values of the LEAS against several bacterial strains were specified in Table 4. As per the result, the LEAS showed significant inhibition of microbial growth of strains of *S. aureus*, *B. subtilis* 6633, and *E. coli* PBR-332

except for *S. aureus* ML-267 and *K. pneumoniae* PB-12 at 6.25 mg per mL concentration. Significantly, the LEAS exhibited a better potent antibacterial effect on Gram-ve bacteria than Gram+ve bacteria. This effect on Gram-ve bacteria is due to the presence of an external membrane comprising lipopolysaccharide and lipoprotein that is selectively permeable and thus controls the entry to the basic cell structures.[49] This depicts the less susceptibility of Gram+ve organism to various extracts of the plant than the Gram-ve organism.[50]

Based on the previous literature, the specified molecular docking study was executed by selecting the target molecule related to antimicrobial potential. The result of the docking analysis reveals the binding score and the bond of the interaction of the phytoconstituents of LEAS with DHFR (PDB ID: 3SRW) as displayed in Table 5 and Figure 4. Compounds caryophyllene (-5.2); (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylene-tricyclo-[4.4.0.02,7]-decane (-5.2); Bicyclogermacrene (-4.0); (-)-Spathulenol (-4.6) and tau-Cadinol (-4.3) revealed good docking scores among the identified compounds. DHFR is a vital enzyme used in the pathway of thymidine synthesis.[29] Trimethoprim, an antibiotic used as a standard drug that interacts with the enzyme DHFR, inhibits the thymidine synthesis that eventually disturbs the synthesis of DNA.[29] Moreover, Salvi *et al*. mentioned that the inhibition of the enzyme DHFR (PDB ID: 3SRW) is expected as the apparent pathway for microbial protection.[29] Furthermore, the *in-silico* study of phyto-compounds has been conducted by molecular docking. Antibiotics restrict microbial growth by targeting microbial metabolism with the deactivation of the key enzymes used in the biosynthesis and restoration of cell walls, nucleic acids, and proteins.[26] Thus, in molecular docking, it is speculated that the phytocompounds with less binding energy in comparison to the known antibiotics might be more effective for microbial inhibition.[26] Also, if not active these constituents can be employed as the new antimicrobial entities. The *in silico* research showed that the phytocomponents identified from the LEAS viz. Caryophyllene (-5.2 kcal/mol), (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo-[4.4.0.02,7]-decane (-5.2 kcal/mol), Bicyclogermacrene (-4.0 kcal/mol), (-)-Spathulenol (-4.6 kcal/mol) and tau.-Cadinol (-4.3 kcal/mol) revealed good binding affinity for DHFR and compared to the binding affinity of the standard drug trimethoprim (-7.5 kcal/mol).

Table 4: Results of Minimum Inhibitory Concentration of leaf extract of *A. squamosa*

Bacterial Strains	**Dilution in mg/mL of the LEAS**						
	1.56	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
S. aureus ATCC-25923	-	-	+	+	+	+	+
S. aureus ML-267	-	-	-	-	+	+	+
S. aureus ATCC-29737	-	-	+	+	+	+	+
S. aureus ATCC-29157	-	-	+	+	+	+	+
B. subtilis 6633	-	-	+	+	+	+	+
E. coli PBR-332	-	-	+	+	+	+	+
E. coli JM-109	-	-	-	+	+	+	+
K. pneumoniae PB-12	-	-	-	-	-	+	+

'+' indicates the inhibition of microbial growth and '-' indicates no inhibition. ATCC: American Type Culture Collection; ML-267: A bacterial strain of *S. aureus*; PBR-332: a plasmid and firstly used *E. coli* cloning vector; JM-109: JM-109: K strain of *E. coli* (for Recombinant cloning and subcloning)
S. aureus: Staphylococcus aureus; B. subtilis: Bacillus subtilis, E. coli: *Escherichia coli; K. pneumoniae: Klebsiella pneumoniae*

Table 5: Molecular Docking study of phytoconstituents of the LEAS with target Protein 3SRW

Sl. No.	**Compounds Name**	**AUTO DOCK MBE (kcal/mol)**	**Residues of interaction**	**(Type of interaction) Active Binding Site Residues**
		3SRW	**3SRW**	**3SRW**
1	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans	-1.8	No Amino Acid	No interaction
2	Caryophyllene	-5.2	GLN:20, LEU:21, LEU:29	Pi-Alkyl
3	Humulene	-1.0	GLN:20, TRP:23, HIS:24, LEU:29	Van der Walls
4	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane	-5.2	LEU:21, HIS:24	Pi-Alkyl
5	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene	-4.0	No Amino Acid	No Interaction
6	(-)-Spathulenol	-4.6	GLN:20, LEU:21	Conventional hydrogen bond
7	Caryophyllene oxide	1.0	LEU:21	Alkyl
8	.tau.-Cadinol	-4.3	No Amino Acid	No interaction

9	n-Hexadecanoic acid	-2.3	GLN:20, ILE:51	Conventional Alkyl	hydrogen	bond,		

GLN: Glutamine; LEU: Leucine; TRP: Tryptophan; HIS: Histidine; ILE: Isoleucine

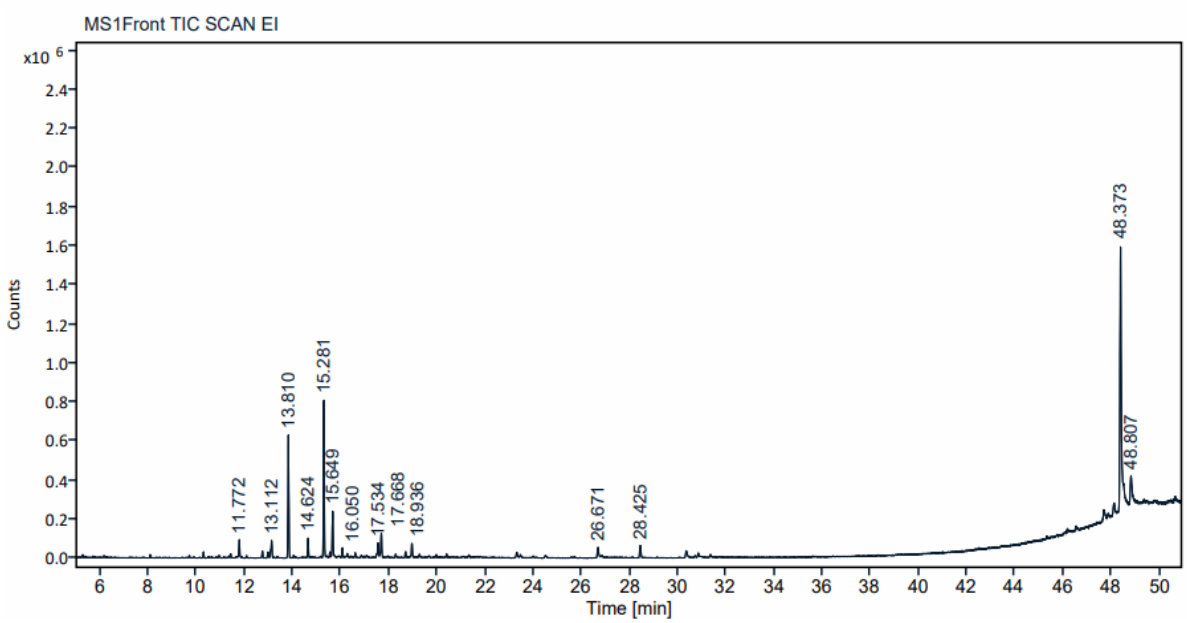
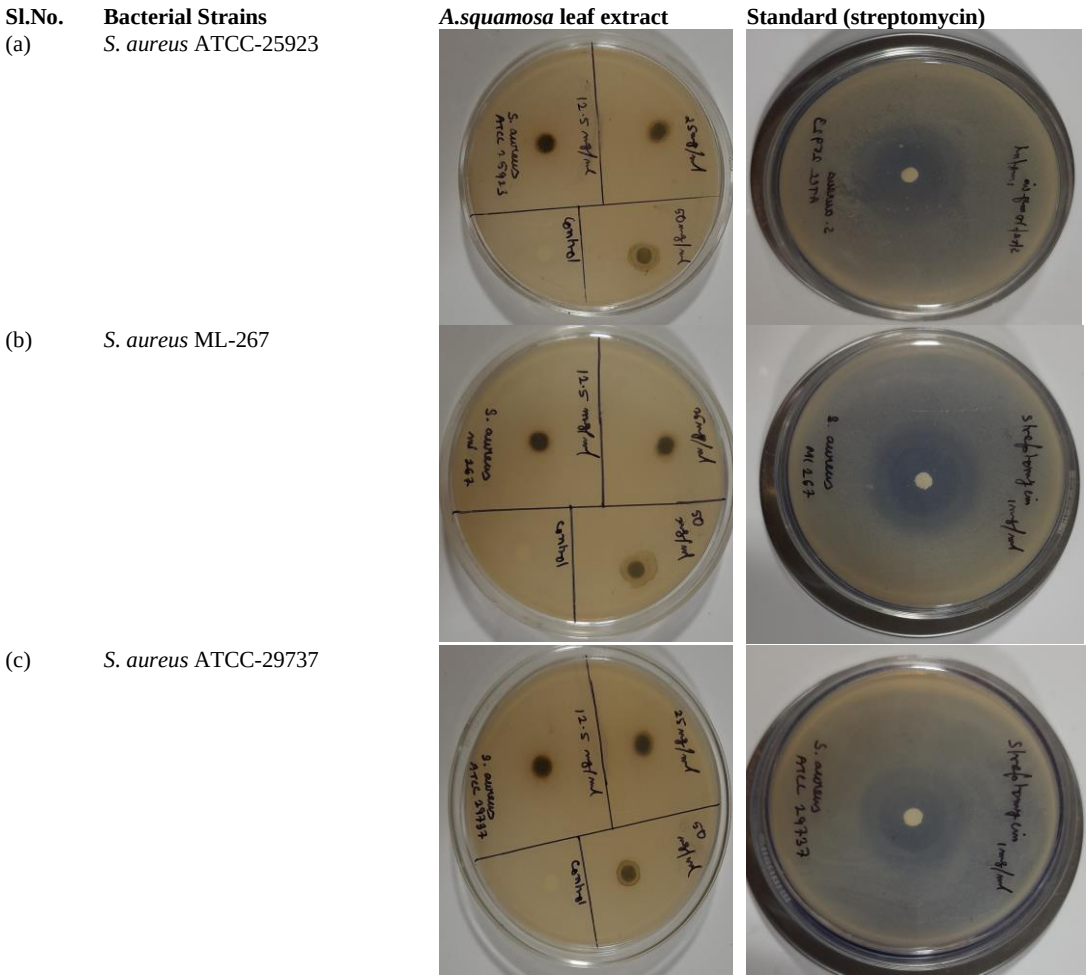


Figure 2: GC-MS Chromatogram of the n-hexane extract of *A. squamosal* leaf.



(d) *S. aureus* ATCC-29157

(e) *B. subtilis* 6633

(f) *E. coli* PBR-332

(g) *E. coli* JM-109

(h) *K. pneumoniae* PB-12

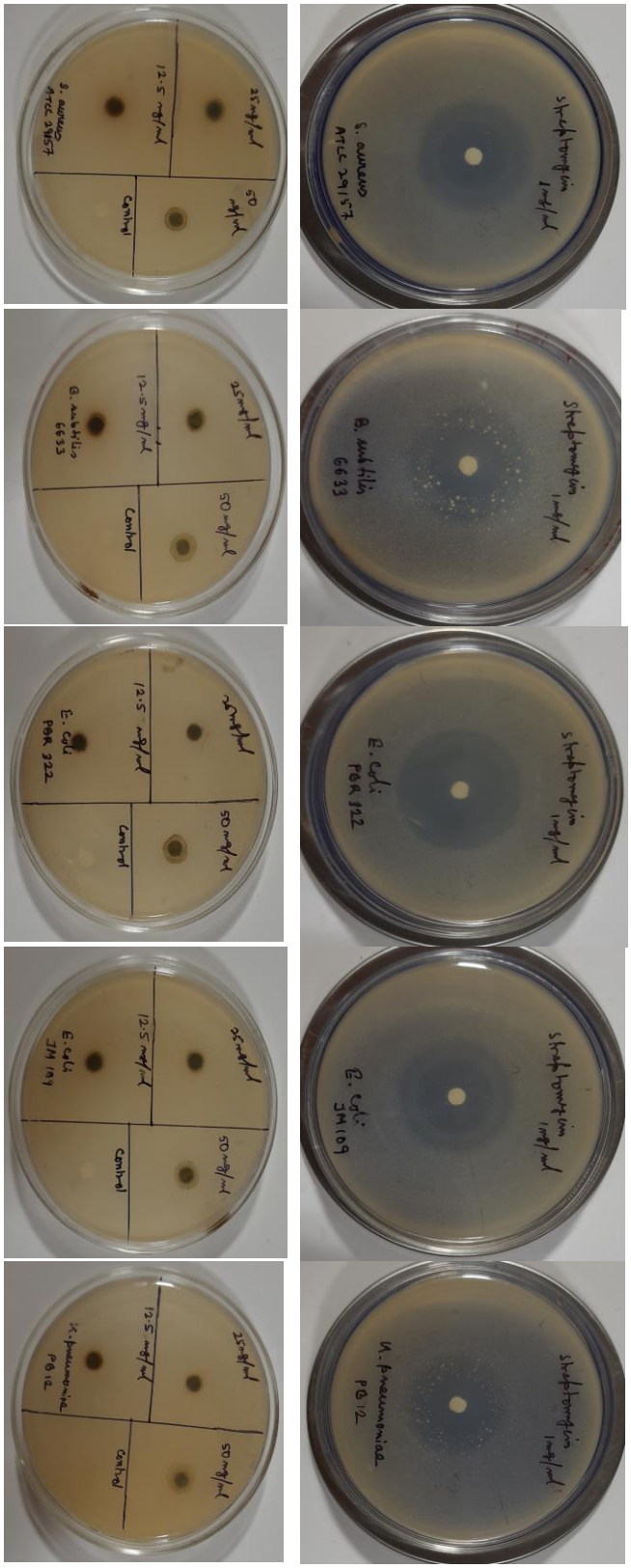


Figure 3: Zones of inhibition of *A. squamosa* leaf extract and standard drug Streptomycin against and (a) *S. aureus* ATCC-25923; (b) *S. aureus* ML-267; (c) *S. aureus* ATCC-29737; (d) *S. aureus* ATCC-29157; (e) *B. subtilis* 6633; (f) *E. coli* PBR-332; (g) *E. coli* JM-109 and (h) *K. pneumoniae* PB-12 at concentration of 12.5, 25, 50 mg/ml and 1mg/ml respectively.
S. aureus: Staphylococcus aureus; B. subtilis: Bacillus subtilis, E. coli: *Escherichia coli; K. pneumoniae: Klebsiella pneumoniae*

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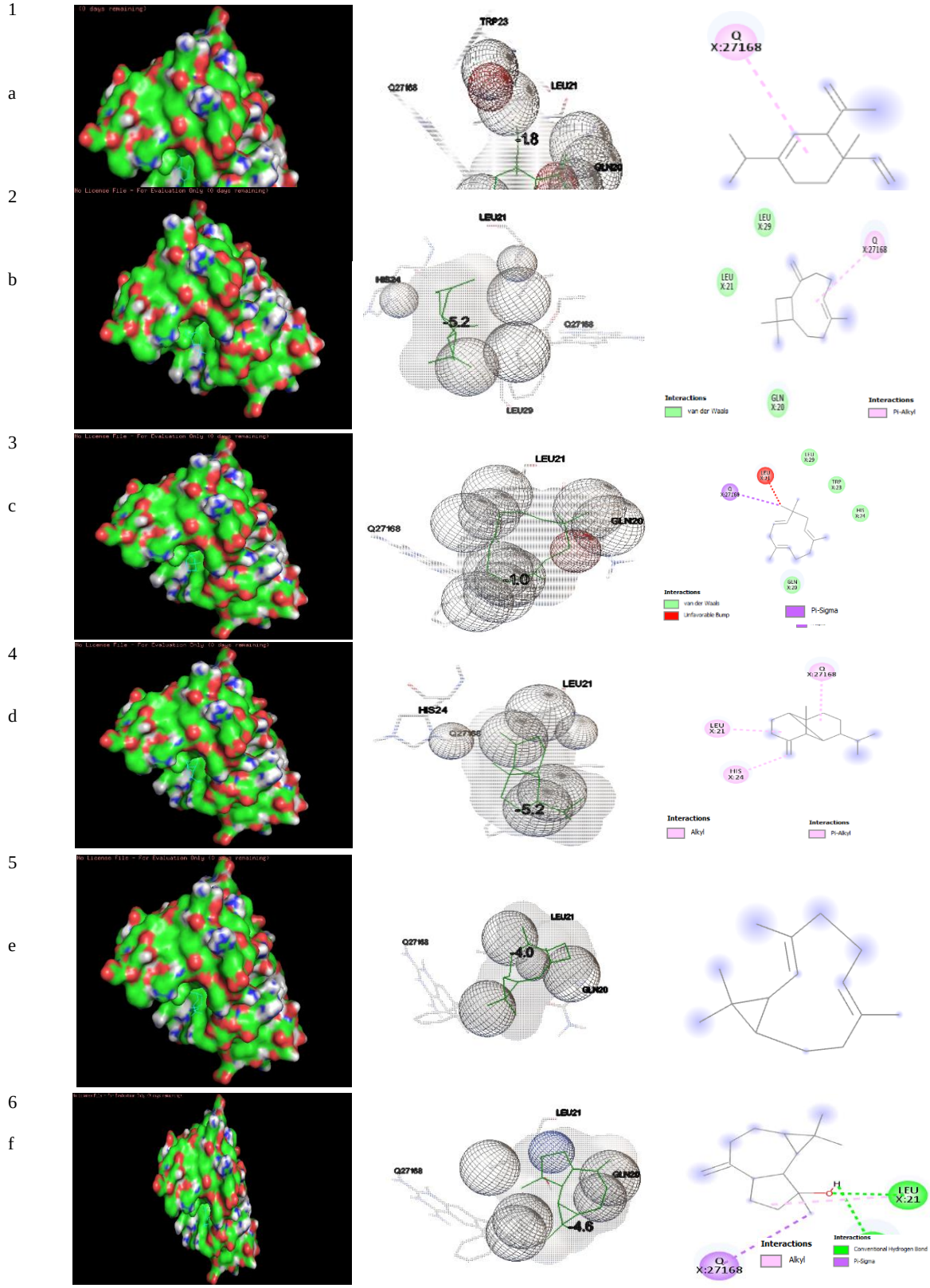

Figure 4: 3D visualisations illustrating the interacting patterns of compounds (a-i) with the effective site of DHFR with PDB ID/3SRW. (a) Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans with 3SRW; (b) Caryophyllene; (c) Humulene; (d) (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane; (e) (1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene; (f) (-)-Spathulenol; (g) Caryophyllene oxide; (h) tau.-Cadinol; (i) n-Hexadecanoic acid.
3D: 3-Dimensional; DHFR: Dihydrofolate Reductase; PDB ID: Protein Data Bank ID

Conclusion

The n-hexane extract of *A. squamosa* leaf revealed significant antimicrobial activity against gram-positive and gram-negative organisms by in vitro study at different concentrations. The phytoconstituents identified in GC-MS analysis were reported for the first time. The appreciable amount of caryophyllene, bicyclogermacrene, caryophyllene oxide, and n-hexadecanoic acid detected by GC-MS might be responsible for the anti-microbial effect. The anti-microbial effect of the plant was further supported by a docking study where compounds Caryophyllene, (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo-[4.4.0.02,7]-decane, (-)-Spathulenol, tau.-Cadinol, and Bicyclogermacrene exhibited highest score. Further investigation is rather needed to isolate and purify the important phytoconstituents that are responsible for antimicrobial activity. These findings of the current research might be essential in developing novel and effective antimicrobial treatments.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Abbreviation, TIC: Total Ion Chromatogram, MIC: Minimum Inhibitory Concentration, Eos: Essential, GC-MS: Gas Chromatography-Mass Spectrometry oils, Cyt C: Cytochrome C, ETC: Electron Transport Chain, SD: Standard Deviation, DHFR: Dihydrofolate Reductase, *A.squamosa: Annona squamosa*

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