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In-Vitro and In-Silico Analysis of Antimicrobial Efficacy in Annona Muricata Leaf Extracts.

Shivali U Shetty[1], Beena Antony[2], Animik Ray[3], Shruthi B[4], Jane Grace Dsouza[5], Sanjeev Rai B[6]

[1,4,5]Research Assistant, Father Muller Research Center, Kankanady, Mangalore, India
[2]Professor, Department of Microbiology, Father Muller Research Centre, Kankanady, Mangalore, India
[3]Scientist C, Father Muller Research Center, Kankanady, Mangalore, India
[6]Chief of Research, Father Muller Research Center, Kankanady, Mangalore, India

Abstract

Annona muricata and *Annona reticulata*, known for their traditional medicinal uses and antimicrobial properties, were investigated for their potential antibacterial effects. Extracts from fresh leaves were tested for antimicrobial activity, revealing that Annona muricata exhibited superior antibacterial properties compared to Annona reticulata, especially against *E. coli* strains. In silico analysis focused on 46 phytochemicals extracted from *Annona muricata*, which were docked with key bacterial proteins, including Gyrase, the 30S and 50S ribosomal subunits, Topoisomerase IV, and penicillin-binding protein. Notably, phytochemicals such as Quercetin 3–O-rutinoside (Rutin), Quercetin 3-O-neohesperidoside, Quercetin 3-O-glucoside (Isoquercetin), Luteolin 3´7-di-O-glucoside, and Homoorientin demonstrated strong docking scores and confidence score, indicating potential interactions that could lead to bacterial destruction. These findings suggest that certain phytochemicals of Annona muricata is a promising candidate for antibiotic development, opening avenues for further exploration in combating bacterial infections.

Keywords: Antibacterial Effects, Phytochemicals, *Anonna muricata*, Molecular Docking

Introduction

The Annonaceae family includes tropical plants like *Annona muricata* (Soursop) and *Annona reticulata* (Bulocks heart), sometimes used in traditional treatments. Annona muricata is one of these plants used in many cultures to treat various conditions. The leaves can be applied externally to treat rashes, edema, wound and eczema to promote rapid healing and prevent infection[1, 2]. *Annona muricata* is claimed by herbalists to be able to treat cancer (if detected in earlier stage). It can be a good substitute for chemotherapy since it has the advantage of destroying cancer cells without harming healthy cells[3]. Alkaloids, acetogenin, polyphenols, flavonoids, lectins and terpenes found in soursop leaves which gives them these properties[4]. While *Annona reticulata* has been conventionally utilized to manage various conditions such as epilepsy, dysentery, heart issues, parasite infections, constipation, bleeding, bacterial infections, fever, and ulcers. Its leaves have also been employed to treat helminthiasis [5].

A significant issue in the healthcare industry is infections brought on by pathogenic bacteria. Opportunistic Pathogens causes 90 percent of invasive infections, these pathogens have become resistant

to Antibiotic drugs. Using more broad-spectrum antibiotics, immuno-suppressants, and cancer treatments has weakened the host's immune system, making it a good place for the opportunistic pathogens to grow[6]. Bacteria are the human pathogens that are most capable of causing serious infections, it can trigger an exaggerated immune response which can also lead to tissue damage, organ failure sometimes death. Bacterial infections can range in intensity from less serious to more serious[7].

The evolution and spread of bacterial and fungal infections had forced the researches to create an efficacious, safer, and more potent novel treatment agents, as well as broader spectrum antibiotics. Plants, on the other hand, have been used to treat and prevent disease since antiquity. For thousands of years, medicinal plants have been used as natural sources of treatment for a variety of diseases. Identifying the antimicrobial capability of many traditional plants is becoming more common, owing to the fact that many synthetic drugs are potentially toxic and have side effects in Humans[3].

The majority of antibiotics, such as fluoroquinolones, Penicillin, Tetracyclines target crucial bacterial proteins such as gyrase, the 30S ribosomal protein, the 50S ribosomal subunit, penicillin-binding proteins, and topoisomerase. These proteins play essential roles in the smooth functioning of bacteria, and any damage to them, risk the overall survival of the bacterial cell. Given the significance of these proteins, they serve as common targets for a wide range of antibiotics[8, 9]

According to numerous research, *Annona muricata* and *Annona reticulata* plant extracts contain antibacterial as well as antifungal characteristics that are effective against a variety of microorganisms. The photochemical found in *Annona muricata's* tissues, which can be utilised as a medicine to kill microbes which give this plant its antimicrobial properties [1, 5]

As a part of rational drug design, Molecular docking studies utilizes in-silico techniques for drug discovery. This approach identifies potential therapeutic lead compounds by evaluating efficacy, predicting molecular interactions and toxicity, repurposing drugs, and adapting to artificial intelligence for target profiling and fishing [10, 11].

Current research is predominantly focuses on investigating the antimicrobial capability of leaf extracts obtained from Annona muricata and Annona reticulata against specific opportunistic pathogens. Furthermore, this study seeks to identify the phytochemical compounds present in Annona muricata leaf extracts that may able to bind to bacterial proteins like DNA gyrase, 30s and 50s ribosomal proteins, penicillin-binding proteins, and topoisomerase IV. The ultimate goal is to study the inhibition of these proteins by the phytochemicals present in Annona muricata through in-silico protein-protein molecular docking studies, leading to bacterial eradication.

Methods

Collection of Plant Leaves

Fresh leaves of Annona reticulata and Annona muricata had been randomly gathered from fully grown plants in Mangalore, Dakshina Kannada, Karnataka. Both sets of leaves, weighing approximately 250g each, were washed with tap water twice and distilled water once individually.

Preparation of extract

The washed leaves went through drying in hot air oven by setting the temperature to 70 degrees until it attains a crisper texture. Once the leaves had dried completely and became crisper, these were ground into a fine powder in a blender and sieved separately by maintaining the sterility. About 100gs of leaf powder was mixed with 70% ethyl alcohol in a sterile glass bottle with 1:5 ratio. To completely dissolve,

the mixture was maintained at room temperature on a mechanical shaker for 24 hours. The mixture was filtered, and the filtrate was evaporated to get a crude residue. The residue was diluted with DMSO at a ratio of 1:10 each [4].

Cultures

Twenty stock cultures each of E. coli, Pseudomonas species (including Pseudomonas aeruginosa, Pseudomonas stutzeri, Pseudomonas putida, Pseudomonas mosselli, Pseudomonas Monteilli), and Staphylococcus species (including S. aureus, S. capitis) were obtained. Additionally, ten stock cultures each of Acinetobacter species (including Acinetobacter baumannii, A. variabilis, A. towneri) and Enterococcus species (including Enterococcus faecalis, Enterococcus faecium) were obtained. Furthermore, three cultures of Klebsiella pneumoniae were obtained from the Department of Microbiology at Father Muller Research Centre in Mangalore, Karnataka, India.

The organisms, namely E. coli, Pseudomonas, Acinetobacter, and Klebsiella, were subcultured onto MacConkey media, while Staphylococcus and Enterococcus were subcultured onto Blood agar. Incubation was carried out at a temperature of 37 degrees Celsius for overnight.

Once the growth has come in the plates the organisms were transferred into the nutrient broth and incubated for 3 to 4 hours to match 0.5 McFarland turbidity. When the required turbidity had attained the inoculum was swabbed into the AST plates

Antimicrobial Sensitivity Test

Antimicrobial test is performed using agar-based well diffusion approach. The Muller-Hinton agar plates were utilized to examine the antimicrobial property. After equally swabbing the inoculum into the plates evenly, wells of 6 mm diameter were punched into the agar medium. 100µl of each the plant extract was added separately to the wells and allowed to diffuse at ambient temperature for a period of 1 h. The plates have been subsequently left to incubate for a 24-hour period in an upright position at 37°C. Following incubation, the widths of the growth inhibition zones were determined in millimeters[12].

Experiments were conducted in duplicates for both the extract to minimize error, and the mean values were tabulated [13].

Selection and Preparation of Ligands and receptor for Molecular Docking

After an extensive literature search, 212 phytochemicals were identified in *A. muricata*, with the leaves alone containing 111 bioactive chemicals[14]. A detailed list of these were compiled, and their molecular structures were extracted from the PubChem library. Subsequently 46 three-dimensional structures of these substances available in the database were obtained in SDF format from PubChem these structures were then translated into PDB format using UCSF Chimera [15] aiming to prepare them for further analysis particularly for docking studies

The proteins which act as receptors in this study include Gyrase, the 30S and 50S ribosomal subunit, Topoisomerase IV and penicillin-binding protein which are commonly targeted by antibiotics. These proteins play essential roles in bacterial replication, protein synthesis, cell wall synthesis, and cell division. Inhibition of these proteins can cause DNA damage, impaired replication, weakened cell walls, and bacterial cell death.

The structures of these proteins were sourced from the RCSB PDB database [16] using the respective PDB identifiers: 4PRV, 7OE1, 6QUL, 5CRF, and 3FV5. The retrieved structures contained additional

elements such as ligands, water molecules, and ions. To use the proteins for docking analysis, all nonstandard atoms were removed using UCSF Chimera software.

Molecular docking

Using the H-DOCK online tool, molecular docking was performed to evaluate docking scores, interactions, and binding poses. Hdock produces 10 distinct docked complexes or models with unique binding poses and docking scores for all the runs. Among these, model 1 stand as the optimal choice, as indicated by the software's assessment. Docking scores were calculated using knowledge-based iterative scoring functions (ITScorePP or ITScorePR), where a more negative score indicates a more favorable binding model[17]. The blind docking was carried out with the gyrase enzyme docking with 46 selected bioactive compounds in PDB format. Subsequently, the top five structures with the best docking scores were further subjected to docking with the remaining proteins, namely the 30S ribosomal subunit, 50S ribosomal subunit, penicillin-binding protein, and Topoisomerase IV.

Results

The results of the antimicrobial sensitivity assay clearly indicate that Annona muricata possesses superior antibacterial properties in comparison to Annona reticulata, as outlined in Table 1. The leaf extract from the soursop plant demonstrates notable effectiveness, showcasing a zone of inhibition ranging from 18mm to 27mm against various E. coli strains. Out of the 20 E. coli isolates tested, 9 exhibited sensitivities to A. muricata. Additionally, two species of Staphylococcus aureus showed inhibition zones of 21mm and 17mm, respectively, and one isolate of Enterococcus faecium displayed a zone of inhibition of 18mm. In contrast, all selected organisms demonstrated resistance to Annona reticulata.

Table 1: Sensitivity Pattern of The Extracts

Organism Sensitivity	Extracts	
	Annona muricata	**Annona Reticulata**
Pseudomonas sps N=20	-	-
Ecoli N=20	9	-
Staphylococcus Sps N=10	2	-
Acinitobacter sps N=10	-	-
Enterococcus Sps N=10	1	-
Klebsella Sps N=3	-	-

Table represents the sensitivity of various organisms to the leaf extracts of *Annona muricata* and *Annona reticulata.*

Molecular docking

Among the 46 phytochemicals from *Annona muricata*, phenols like Quercetin 3–O-rutinoside, Quercetin 3-O-neohesperidoside, Quercetin 3-O-glucoside, Luteolin 3´7-di-O-glucoside, and Homoorientin demonstrated superior docking scores and confidence score compared to others when docked to gyrase using Hdock. Notably, Quercetin 3–O-rutinoside achieved the highest docking score of -199.83 and a confidence score of 0.7304.

These phytochemicals were then subjected to docking analyses against 30s and 50s ribosomal subunit, topoisomerase IV, and the penicillin-binding protein, docking scores and confidence scores are depicted in Table 2

In the 30S ribosomal subunit, homoorientin ligand exhibited a notable docking score of -258.52 with a confidence value of 0.8976, signifying a robust interaction. Similarly, quercetin 3-O-glucoside demonstrated the highest docking score of -263.13 with a confidence value of 0.9057 when docked to the 50S ribosomal subunit. Quercetin 3-O-rutinoside displayed the highest docking score of -264.92 with a confidence value of 0.9088 in the interaction with topoisomerase IV, indicating a strong binding affinity. Additionally, luteolin 3'7-di-O-glucoside achieved the highest docking score of -221.2 with a confidence value of 0.806 when docking to the penicillin-binding protein (PBP).

Table 2: Docking Simulation Scores of The Protein-Ligand Complexes

Protein	Phytochemicals	Docking Score	Confidence score	Ligand RMSD (Å)
DNA Gyrase	Quercetin 3–O-rutinoside	-199.83	0.7304	50.87
	Quercetin 3-O-neohesperidoside	-194.43	0.7086	44.56
	Quercetin 3-O-glucoside	-192.8	0.7018	25.69
	Luteolin 3´7-di-O-glucoside	-190.26	0.6911	43.45
	Homoorientin	-184.81	0.6673	25.88
30s Ribosomal Subunit	Homoorientin	-258.52	0.8976	343.67
	Luteolin 3´7-di-O-glucoside	-254.98	0.8909	311.88
	Quercetin 3-O-rutinoside	-251.27	0.8834	311.45
	Quercetin 3-O-neohesperidoside	-251.27	0.8834	311.6
	Quercetin 3-O-glucoside	-251.11	0.8831	311.98
50s Ribosomal Subunit	Quercetin 3-O-glucoside	-263.13	0.9057	211.72
	Homoorientin	-263.13	0.9057	211.73
	Quercetin 3-O-neohesperidoside	-260.8	0.9017	211.93
	Luteolin 3´7-di-O-glucoside	-258.72	0.8979	211.93

	Quercetin 3-O-rutinoside	-241.48	0.8617	250.52
Topoisomerase IV	Quercetin 3-O-rutinoside	-264.94	0.9088	76.7
	Homoorientin	-261.68	0.9032	76.13
	Quercetin 3-O-glucoside	-261.52	0.9029	76.18
	Quercetin 3-O-neohesperidoside	-260.62	0.9014	76.52
	Luteolin 3´7-di-O-glucoside	-260.08	0.9004	76.21
Penicillin-binding Protein	Luteolin 3´7-di-O-glucoside	-221.2	0.806	239.33
	Quercetin 3-O-neohesperidoside	-215.18	0.7864	239.44
	Homoorientin	-210.27	0.7695	239.66
	Quercetin 3-O-rutinoside	-208.57	0.7634	239.23
	Quercetin 3-O-glucoside	-202.62	0.7412	239.84

The table shows docking score, confidence score and ligand RMSD of the selected phytochemicals with 5 different bacterial proteins

The docking results affirm robust interactions between the proteins and the five phytochemicals. Notably, interactions with the 30S and 50S ribosomal subunit, and topoisomerase IV outweigh those with gyrase and penicillin-binding protein. Typically, protein-protein complexes in the PDB have a Hdock docking Score of about -200 or better. A confidence scores greater than 0.7 indicates a high binding probability between the two molecules.

Discussion

The rise of drug resistance among pathogens is a major health concern, necessitating continuous efforts in new drug discovery. Researchers globally are exploring alternative therapeutic strategies, including drug repurposing, with a focus on phytochemicals. These natural compounds, found in Annona muricata extracts, exhibit broad-spectrum antibacterial activities. In vitro screening of phytochemicals is time-consuming, leading to an increased reliance on in silico computational approaches like cheminformatics, molecular docking, and artificial intelligence in drug design [18]

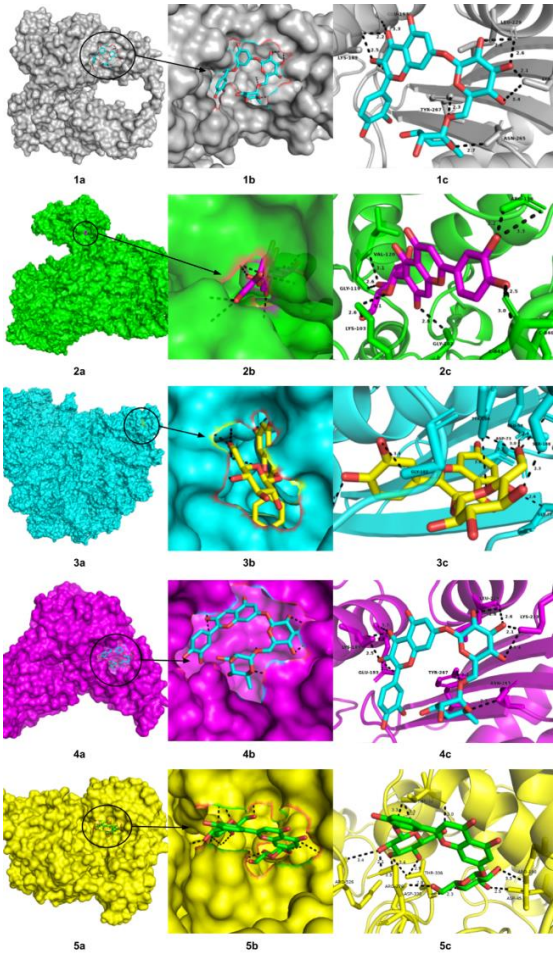
Researchers analyzing *Annona muricata* extracts identified diverse secondary metabolites, including alkaloids, phenolics, flavonoids, acerogenins, vitamins, and tannins. These compounds exhibit potential anticancer, antimicrobial, and antioxidant properties.

The effective use of in-silico molecular docking has enabled the prediction of ligand-target interactions,

enhancing our understanding of the molecular basis of natural product biological activity. Among the numerous phytochemicals present in Annona muricata, the leaf extracts contain approximately 111 compounds, including alkaloids, acerogenins, phenols, and vitamins[14]. Out of these, 46 compounds with available structures from a public database were selected for docking against bacterial proteins, including Gyrase, the 30S and 50S ribosomal subunits, Topoisomerase IV, and penicillin-binding protein. The resulting docked structures and their interactions with the top-scoring ligands are depicted in Figures 1-5.

Quercetin 3–O-rutinoside interacts with amino acid residues including Glutamine, Lysine, Tyrosine, Leucine, Asparagine, on gyrase, as illustrated in Figure 1. Homoorientin or Isoorientin, a flavonoid-like compound, binds to specific amino acid residues, including Arginine, Valine, Glycine, Lysine, etc., on the 30s ribosomal protein, as depicted in Figure 2. Quercetin 3-O-glucoside, also known as isoquercetin, a flavonoid, which binds to forms hydrogen bonds with amino acid residues such as Alanine, Glycine, Tyrosine, Aspartic acid, Serine, Arginine, etc., as illustrated in Figure 3. Quercetin 3-O-rutinoside, also known as Rutin, is a rutinoside, that is quercetin with the hydroxy group at position C-3 substituted with glucose and rhamnose sugar groups interacts with amino acid residues including Glutamine, Lysine, Tyrosine, Leucine, Asparagine, on topoisomerase IV, as illustrated in Figure 4. Luteolin 3´7-di-O-glucoside, a flavonoid glycoside, interacts with specific amino acid residues such as Arginine, Aspartic acid, Threonine of PBP as shown in Figure 5.

Figure 1: Protein-Ligand Docked Structures



The figure illustrates the interaction between the grey-colored DNA Gyrase enzyme structure and the blue-colored Quercetin 3–O-rutinoside, showcasing the binding interaction, bond lengths in angstroms, and the specific amino acid residue to which the ligand binds. In Figure 2, the green structure represents the 30s ribosomal protein, while the pink structure represents homoorientin, highlighting their binding interaction with details of the involved amino acid residues. Figure 3 displays the binding interaction between the blue-colored 50s ribosomal protein structure and the yellow-colored Quercetin 3-O-glucoside, providing insights into their interaction. In Figure 4, the interaction between the pink-colored Topoisomerase IV structure and the blue-colored Quercetin 3-O-rutinoside is depicted, along with bond lengths and information about the amino acid residue to which the ligand binds. Lastly, Figure 5 showcases the docked structure of the yellow-colored Penicillin binding protein and the green-colored Luteolin 3´7-di-O-glucoside, elucidating the binding interactions and relevant details.

Conclusion

The in vitro study results indicated that *Annona muricata* extract exhibited effective antibacterial properties against *Escherichia coli* and certain *Staphylococcus* and *Enterococcus* species, as evidenced by antimicrobial susceptibility testing. This highlights the superior antimicrobial capabilities of *Annona muricata* compared to *Annona reticulata*, which showed no zone of inhibition in the antimicrobial test. The in-silico analysis using molecular docking revealed promising interactions between the phytochemicals and the bacterial proteins, as indicated by favourable docking scores and confidence scores. These interactions suggest a potential mechanism for bacterial destruction and demonstrate the potential of *Annona muricata* phytochemicals in developing antibiotics. However, further research is necessary to determine the safety and efficacy of these phytochemicals, including comprehensive drug investigations that involve analysis of pharmacological properties and preclinical and clinical studies.

Conflict of Interest: No conflict of interest

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