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Identification of active leads in *Andrographis paniculata leaves* for controlling the growth of two opportunistic bacteria

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Abstract

Opportunistic pathogens like *E. faecalis* and *K. pneumoniae* exploit weakened immune systems, causing diverse infections, often resistant to antibiotics. This study explored Andrographis paniculata's potential against these pathogens. Extracts from its leaves, notably acetone, showed significant inhibitory effects on both bacteria. GC-MS analysis identified eighteen compounds; three showed promising drug-like properties. Molecular docking studies revealed Andrographolide's efficacy against essential bacterial enzymes. It inhibited triphosphohydrolase, lactate dehydrogenase, and lipoyl synthase in *E. faecalis*, and dihydrofolate reductase and SHV-11 beta-lactamase in *K. pneumoniae*. Andrographolide holds promise for developing novel antibacterial therapeutics. This research underscores the importance of exploring plant-based remedies in combating antibiotic-resistant pathogens, offering potential alternatives for pharmaceutical intervention. Further investigation into the mechanisms of action and *in vivo* efficacy of Andrographis paniculata extracts and their active compounds is warranted to advance their therapeutic potential in clinical settings.

Keywords: *Andrographis paniculata, E. faecalis, K. pneumoniae, docking, beta lactamase*

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Introduction

Opportunistic pathogens are like cunning tricksters hiding in plain sight. They peacefully coexist with us, often in our gut or skin, but wait for the right opportunity to strike [1-6]. When our immune system weakens due to illness, age, or medical treatment, these "friendly neighbors" can turn into troublemakers, causing infections like pneumonia, UTIs, and even serious blood infections [7-8]. These clever bugs are notorious for developing resistance to antibiotics, making them even harder to combat.

Enterococcus bacteria are Gram-positive cocci, typically occurring in pairs or short chains under a microscope and are considered opportunistic bacteria. They possess a thick cell wall composed of peptidoglycan, which provides structural support and contributes to their resistance to environmental stressors and antibiotics. *Enterococcus* is a complex genus of bacteria with a dual nature [9-11]. While some species reside harmlessly in our intestines, others can cause serious infections, especially in healthcare settings. Most commonly, *Enterococcus faecalis* and *Enterococcus faecium* live peacefully in our gut, aiding digestion and potentially boosting immunity. Some strains even find use in probiotics, helping restore gut health after antibiotic use. In weakened individuals or when they escape the gut, these bacteria can activate infections such as urinary tract infections (UTIs), bloodstream infections (sepsis), wound infections, and endocarditis (heart valve infection). Enterococci are infamous for acquiring resistance to various antibiotics, making their infections tougher to treat. The emergence of multidrug-resistant strains of *Enterococcus* is a significant threat to public health in recent years [12-15]. Common antibiotics used for controlling the growth of this pathogen include ampicillin, penicillin, vancomycin, daptomycin, linezolid, and nitrofurantoin (for UTIs).

Klebsiella pneumoniae is an opportunistic pathogen, often affecting individuals with compromised immune systems or underlying health conditions. It is a Gram-negative, rod-shaped bacterium commonly associated with hospital-acquired infections, particularly pneumonia. It is also known to cause urinary tract infections (UTIs), bloodstream infections (sepsis), wound infections, and infections in other parts of the body [16-19].

Controlling the growth of *Klebsiella pneumoniae* involves a combination of infection prevention measures and antibiotic therapy. Due to its propensity to develop resistance, careful antibiotic selection based on susceptibility testing is essential. To prevent the transmission of Klebsiella pneumoniae in healthcare settings, it is crucial to strictly follow infection control protocols, such as practising proper hand hygiene, ensuring thorough environmental cleaning, and sterilising medical equipment. Additionally, surveillance efforts to monitor for outbreaks and antimicrobial resistance trends play a vital role in controlling the spread of this pathogen.

The careful selection of antimicrobial agents for Klebsiella pneumoniae infections depends on various factors, such as the infection's severity, the anatomical site involved, and the susceptibility profile of the organism. Commonly used classes of antibiotics for treating *K. pneumoniae* infections include carbapenems, cephalosporins, fluoroquinolones, aminoglycosides, and tigecycline. However, due to increased antibacterial resistance [20-21], we may be entering an era where there are 'a lot of antibiotics with less efficacy.' Pharmaceutical scientists are continuously searching for novel antibacterial agents with higher potency. Many synthetic and natural compounds are expected to exhibit antibacterial activity.

Plants can hold valuable resources for exploring potential solutions to microbial challenges [22-24]. Some medicinal herbs, like echinacea, garlic, and oregano, have been studied for their antimicrobial properties. Another herb, *Andrographis paniculata* (APA), native to Southeast Asia, has shown promise in research. Studies have investigated its potential impact on various conditions, including microbial activity. *Andrographis paniculata*, commonly known as the king of bitters, is a medicinal herb native to Southeast Asia, particularly found in countries like India, Sri Lanka, and parts of China (Fig.1). The vernacular name of this plant In Kerala (India) is Kiriyath [25-27].

Fig. 1 *Andrographis paniculata*

It has a long history of use in traditional medicine systems, including Ayurveda and traditional Chinese medicine, for its diverse health benefits. The plant is characterised by its distinctive bitter taste and slender leaves. Some research suggests that *Andrographis paniculata* extract exhibits potent antimicrobial, anti-inflammatory, and immunomodulatory properties [28-29]. Due to its therapeutic potential, *Andrographis paniculata* has gained attention in modern scientific research for its possible applications in treating various health conditions. Despite these early findings, further research is necessary to comprehensively understand its effectiveness and safety, particularly regarding specific types of microbes and potential applications.

In the present study, we screened the APA leaf extract against two main opportunistic bacteria, *Enterococcus faecalis* and *Klebsiella pneumoniae*. We identified the components using GC-MS analysis and attempted to determine the active component responsible for the antibacterial efficacy by *in silico* investigations.

Materials And Methods

Preparation of plant extract

The *Andrographis paniculata* plant was sourced from Mannuthy, Thrissur, Kerala, India, and its leaves were meticulously washed before being air-dried in a shaded environment for seven days. Following drying, the leaves were finely powdered and subjected to extraction using a Soxhlet apparatus, utilising 200 g of the powdered

material. Initially, hexane extraction was employed to eliminate chlorophyll and other hydrophobic components, after which the sample underwent drying at 50°C in a hot air oven. Subsequently, ethyl acetate, acetone and methanol extraction were carried out using the Soxhlet extractor, followed by removal of excess solvent via a rotary evaporator under reduced pressure. The resulting dried solid mixture was then dissolved in 5% DMSO, yielding a stock solution concentration of 1 mg/l. The antibacterial screening was conducted using this stock solution, with application volumes ranging from 50 to 80 microliters.

Antibacterial Studies

Aseptic cultures of *K. Pneumoniae* (ATCC No: 13883) and *E. faecalis* (ATCC No: 700802) were acquired from the Microbial Type Culture Collection and Gene Bank located in Chandigarh, India. To sustain viable cultures for antibacterial experimentation, the nutrient broth was prepared with specific nutrients and adjusted to a neutral pH. The assessment of the antibacterial properties of various plant extracts was conducted using the well-diffusion method, adapted from the protocol outlined by Collins and Lyner in 1987. Bacterial cultures were propagated in a nutrient broth medium and evenly spread onto nutrient agar plates using sterile swabs. Wells, each measuring 3 mm in diameter, were meticulously formed on the solidified agar surface with a well borer, maintaining a 2 cm separation between each well. A stock solution was prepared by dissolving the solid extract (hexane, ethyl acetate, acetone and methanol extracts) in a 2% DMSO solution at a concentration of 1 mg/l. Different concentrations of plant extracts (50, 60, 70, and 80 µl) were then dispensed into the wells on the nutrient agar plates using a micropipette. After an incubation period of 24 hours at 37°C, the diameters of any clear zones surrounding the wells, indicative of inhibited bacterial growth, were precisely measured to determine the efficacy of each extract concentration against the resilient pathogens.

GC-MS analysis

Since the acetone extract of the APA plant only displayed antibacterial activity, the GC-MS analysis of the same was performed for the identification of phytochemicals. The chromatograph utilises a fused

silica-packed capillary column measuring 30 meters in length, with a diameter of 0.25 millimetres and a film thickness of 0.25 micrometres. The temperature profile for analysis was programmed as follows: initially, the temperature was maintained at 110°C for 2 minutes, followed by a linear increase to 150°C at a constant rate of 15°C per minute. Subsequently, the temperature was further ramped up to 250°C at a rate of 10°C per minute. Helium gas was employed as the carrier gas, with a constant flow rate of 1 millilitre per minute, and chromatographic separation was achieved within a duration of 42 minutes. Identification of phytochemical constituents in the extract was performed by correlating retention time indices obtained from the NIST library.

Computational studies

Leveraging the capabilities of computational tools, this research delves deep into the leaf constituents of *Andrographis paniculata*, aiming to identify potent compounds against bacterial species. Gas chromatography-mass spectrometry (GC-MS) methodically reveals the concealed compounds present in the acetone extract of APA leaves. The molecular structures of these identified compounds were optimised and sourced from the extensive Pubmed library in SDF format. Evaluation of drug-like properties was conducted using the Swiss ADME web server [30], focusing solely on compounds demonstrating pharmaceutical potential. Crystal structures of structural receptors for various pathogens were obtained from the Protein Data Bank (PDB) [31]. Tables 1 and 2 detail the characterisation methods, functions, and sequence lengths of target proteins of *Klebsiella Pneumoniae* and *Enterococcus faecalis*. These crystal structures were prepared for docking studies utilising Biovia Discovery Studio 2024 software [32]. Compounds exhibiting drug-like properties were subjected to docking with pathogen receptors using the CB-dock2 web server, assisted by Autodock docking software [33]. Each docking simulation unveiled potential interactions, meticulously analysed using Discovery Studio 2024.

Table 1 Structural and functional details of proteins in *E. faecalis*

Sl. No.	PDB ID	Sequence length	Resolution (Å)	Function
1	7M3H	423	1.27 (XRD)	Reductase: crucial role in mevalonate pathway; synthesis of cholesterol and other essential cellular components in the bacterium.
2	6QXS	315	2.88 (XRD)	Synthase: It plays a crucial role in DNA synthesis by catalyzing the conversion of deoxyuridylate to thymidylate
3	6EP5	207	1.93 (XRD)	FIC protein: the family of proteins called Fic, known for their involvement in protein post-translational modifications (PTMs)
4	4M7U	176	2.10 (XRD)	reductase (DHFR): critical for the bacterium's survival. DHFR catalyzes a crucial step in folate biosynthesis.
5	2O6I	480	2.55 (XRD)	Triphosphohydrolase: crucial role in nucleotide metabolism; breaking down deoxynucleotide triphosphates, which are essential precursors for DNA synthesis.
6	6BSQ	648	1.80 (XRD)	Penicillin-binding protein 4: It plays a crucial role in cell wall biosynthesis.
7	4LRL	480	2.35 (XRD)	Lactate dehydrogenase: an enzyme that catalyses the conversion of L-lactate to pyruvate with the concomitant oxidation of NAD+ to NADH
8	6ORI	411	1.40 (XRD)	Lipoyl synthase: It plays a crucial role in various metabolic pathways, including fatty acid and branched-chain amino acid synthesis
9	6GED	315	1.79 (XRD)	Adhesin domain of PrgB: facilitating adhesion of Enterococcus faecalis bacteria to host cells

Table 2 Structural and functional details of proteins in *K. Pneumoniae*

Sl. No.	PDB ID	Sequence length	Resolution (Å)	Function
1	5ECX	157	1.95 (XRD)	Dihydrofolate reductase A1: It plays a crucial role in folate metabolism, which is essential for the bacterium's survival and growth.
2	5EIX	741	3.35 (XRD)	DNA topoisomerase IV: plays a crucial role in DNA replication and repair.
3	8SKO	230	1.30 (XRD)	Beta-lactamase NDM-4: inactivate beta-lactam antibiotics, rendering them ineffective.
4	6NFD	268	1.17 (XRD)	beta-lactamase SHV-11: It breaks down beta-lactam antibiotics, a widely used class including penicillin and cephalosporins.
5	5UL8	290	1.15 (XRD)	Carbapenemase (KPC-2), belonging to the class A β-lactamase family. Its primary function is to break down carbapenem antibiotics, rendering them ineffective against the bacteria.
6	6MGY	243	1.60 (XRD)	New Delhi metallo-beta-lactamase (NDM-5): known for their ability to break down various antibiotics, including cephalosporins, carbapenems, and penicillins.
7	5NBK	251	2.60 (XRD)	NDM-1 metallo-beta-lactamase: it uses a metal ion to break down the beta-lactam ring, a key component of many antibiotics.

Results And Discussion

Antibacterial screening

The acetone extract derived from *Andrographis paniculata* leaves only demonstrated notable *in vitro* antibacterial efficacy against *E. faecalis* and *K. pneumoniae*. Other extracts, such as hexane, ethyl acetate and methanol extrac,t did not show any activity against these pathogens. Particularly, the acetone extract exhibited robust activity against *E. faecalis*, evidenced by a substantial zone of inhibition measuring 22 mm at an 80 µl concentration. Conversely, its activity against *K. pneumoniae* was comparatively moderate, with a maximum inhibition zone of 11 mm observed. This antibacterial effect is likely attributable to the binding of active constituents within the extract to crucial enzymes involved in bacterial cell synthesis pathways. The APA acetone extract did not exhibit significant growth inhibition for *K. pneumoniae* and *E. faecalis* at concentrations below 15 µl and 7 µl, respectively. These values can be considered as Minimum Inhibitory Concentrations (MIC). It's worth noting that the control (5% DMSO) did not exhibit any antibacterial activity. Further details regarding the antibacterial activity of the APA leaf extract on *K. pneumoniae* and *E. faecalis* in a 5% DMSO medium can be found in Table 3. Fig. 2 represents well-diffusion antibacterial inhibition zones by the APA plant extract.

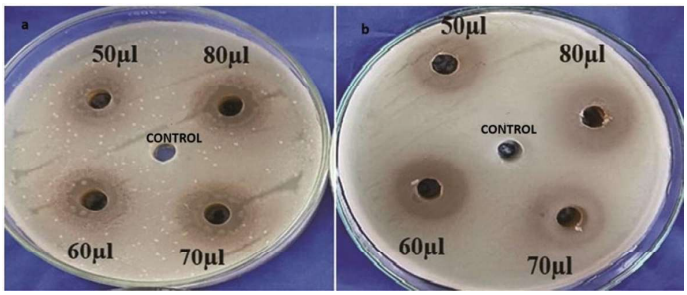


Fig. 2 Inhibition zones of a) *K. Pneumoniae* and b) *E. faecalis* by APA plant extract

Table 3 Antibacterial activity of acetone extract of APA leaves

	zone of inhibition (mm) of extract			
Species	**50µl**	**60µl**	**70µl**	**80µl**
Klebsiella Pneumoniae	**8**	**9**	**11**	**11**
Enterococcus faecalis	**16**	**19**	**20**	**22**

GC-MS analysis

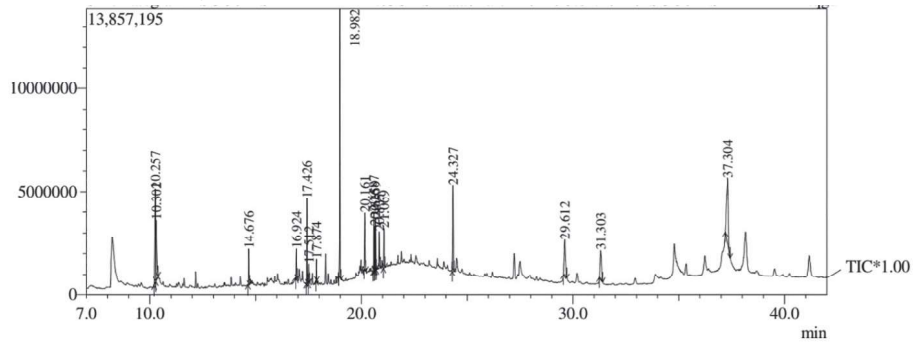


Fig. 3 Gas chromatogram of the acetone extract of APA leaves

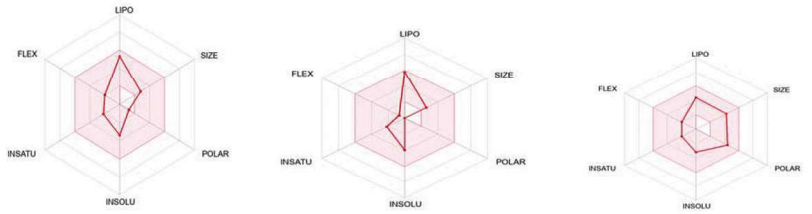
The gas chromatogram presented in Figure 3 illustrates the acetone extract of APA leaves. The mass spectral analysis identified 18 compounds within the extract, including 1,2,3-propanetriol-1-acetate (10.257)*, d-mannitol,1,4-anhydro (10.302), 1-hexadecene (14.676), E-15-heptadecenal (16.924), neophytadiene (17.426), hexahydrofarnesylacetone (17.512), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (17.874), ethyl palmitate (18.982), phytol (20.161), ethyl (9Z,12Z)-9,12-octadecadienoate (20.587), 9-octadecenoic acid (20.626), 9,12,15-octadecatrienoic acid (20.659), ethyl stearate (20.835), phytol acetate (21.069), 1,2-benzenedicarboxylic acid (24.327), cis-Z-. alpha.-bisabolene epoxide (29.612), alpha.-bulnesene (31.303) and andrographolide (37.304). (*Retention time is given in the bracket).

Out of the 18 compounds identified eight compounds were carboxylic acids or esters of fatty acids. Two components belonging to the alcoholic family such as 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol) and anhydro mannitol were observed. Other compounds such as E-15-heptadecenal, neophytadiene, and hexahydro farnesyl acetone were terpenoids. A notable drug-like component cis-Z-. alpha.-bisabolene epoxide, alpha-bulnesene and andrographolide were also detected in the GC of the extract.

ADME studies

Following the drug-likeness screening and ADME predictions, we opted for three molecules (bisabolene epoxide, bulnesene, and andrographolide) identified within the acetone extract. ADME screening was done by the SwissADME webserver. Table 4 showcases the structures of the notable phytochemicals in the APA

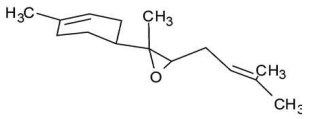
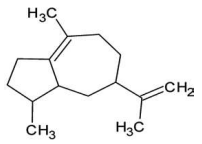
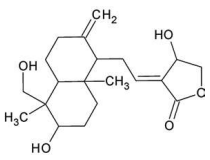
leaves extract, alongside their names and retention times in the chromatogram. ADME predictions for these compounds unveiled molecular descriptors and parameters aligning with drug-likeness criteria. Notably, factors such as lipophilicity, insaturation, molecular size, flexibility, insolubility, and polarity fell within the predicted limits of the webserver. Additionally, Fig. 4 depicts the bioavailability radar of these compounds, offering a graphical depiction of their bioavailability characteristics.



Bisabolene epoxide Bulnesene Andrographolide

Fig. 4 Bioavailability profiles of pharmacologically active compounds found within the acetone extract of APA leaves

Table 4 Structures of drug-like compounds present in the acetone extract of APA leaves

Sl. No.	Name	Structure of Phytochemical	Retention time (min) & Area %
1	cis-Z-.alpha.-bisabolene epoxide		29.612; 6.49%
2	alpha.-bulnesene		31.303; 5.56%
3	andrographolide		37.304; 14.08%

In silico investigations

To gain insight into the active component(s) responsible for the antibacterial activity in the APA leaf acetone extract, molecular

docking studies were conducted on selective structural receptors of opportunistic pathogens. Three molecules, namely bisabolene epoxide, alpha-bulnesene, and andrographolide, exhibiting drug-like characteristics, were chosen for virtual screening. By assessing the binding efficiency of these molecules to the microbial receptors, we aim to elucidate the bioactive ingredient present in the extract.

Screening of compounds on the receptors of E. faecalis

Nine structural receptors of *E. faecalis* were selected for the virtual screening of the molecules. Table 5 presents the binding scores of protein-ligand complexes. Alpha-bulnesene and bisabolene epoxide demonstrated binding energies ranging from low to good on various receptors of this pathogen. Bulnesene exhibited a maximum binding energy of -7.7 kcal/mol on receptors with PDB IDs 4M7U and 2O6I. Bisabolene epoxide showed binding scores ranging from -5.7 to -7.3 kcal/mol on different receptors. The binding energies of andrographolide-receptor complexes ranged from -7.4 to -9.6 kcal/mol. From the virtual screening, it can be inferred that andrographolide acted as the main antagonist molecule for the growth of *E. faecalis*. Receptors 2O6I (tri-phosphohydrolase), 4LRL (lactate dehydrogenase), and 6ORI (lipoyl synthase) exhibited very high binding energy scores with andrographolide (-9.6, -9.0, and -8.7 kcal/mol, respectively).

The 2O6I-andrographolide complex displayed four conventional hydrogen bonds between the molecule and amino acid residues of the receptor at distances ranging from 2.04 to 3.58 Å (Tyr187; 2.04 Å, Arg63; 3.05 Å, Thr51; 3.58 Å, Ser52; 2.37 Å). Additionally, the Thr120 residue formed a non-conventional hydrogen bond with the five-membered ring of andrographolide at 3.54 Å. One strong pi-sigma bond was observed between the Tyr243 amino acid residue of the receptor and one of the methyl groups of the molecule (3.55 Å). The complex was further stabilised by three pi-alkyl interactions with the amino acid residues Tyr239, Tyr243, and Tyr187. Figure 5a illustrates the 2D interaction plot of the 2O6I-andrographolide complex and the possible conformation of the molecule in the binding cavity of the triphosphohydrolase receptor.

Figure 5b depicts the 2D and 3D interaction plots of andrographolide with the lactate dehydrogenase enzyme, which plays a crucial role in the conversion of lactate to pyruvate. Andrographolide inhibited the enzyme through two strong conventional hydrogen bonds with the amino acid residues Tyr243 (2.55 Å) and Arg63 (2.44 Å). Additionally, three carbon-hydrogen bonds formed between the receptor cavity and andrographolide (His129; 3.27 Å, His114; 2.89 Å, Ser248; 3.14 Å), contributing to the formation of a robust complex with lactate dehydrogenase, exhibiting a binding score of -9 kcal/mol.

The lipoyl synthase enzyme 6ORI, which is involved in the synthesis of fatty acids and amino acids in bacterial cells, was significantly inhibited by andrographolide, displaying a binding score of -8.7 kcal/mol. Analysis of the 2D interaction plot of the 6ORI-andrographolide complex (Fig. 5c) revealed six classical hydrogen bonds between the amino acids present in the binding site and the molecule, rendering it a potent complex. Amino acid residues such as Asn75 (1.78 Å), Val341 (2.02 Å), Ala342 (2.58 Å), Asn (2.2 Å), and Asp290 (2.77 Å, 2.33 Å) participated in the formation of the protein-ligand complex.

In conclusion, from in silico investigations, it can be inferred that the primary reason for the growth inhibition of E. faecalis by the acetone extract of APA leaf is attributed to the phytochemical andrographolide, which acts strongly as an inhibitor against the enzymes triphosphohydrolase, lactate dehydrogenase, and lipoyl synthase.

Table 5 Docking score (-kcal/mol) of drug-like molecules with the selected receptors of *E. faecalis*

PDB	Andrographolide	Alpha bulnesene	Bisabolene epoxide
7M3H	7.5	6.8	6.4
6QXS	7.9	6.1	5.7
6EP5	7.4	6.8	6.4
4M7U	7.8	7.7	6.4
2O6I	9.6	7.7	6.9
6BSQ	7.9	6.4	7.2
4LRL	9.0	7.1	7.3
6ORI	8.7	6.8	7.2
6GED	7.5	6.9	6.9

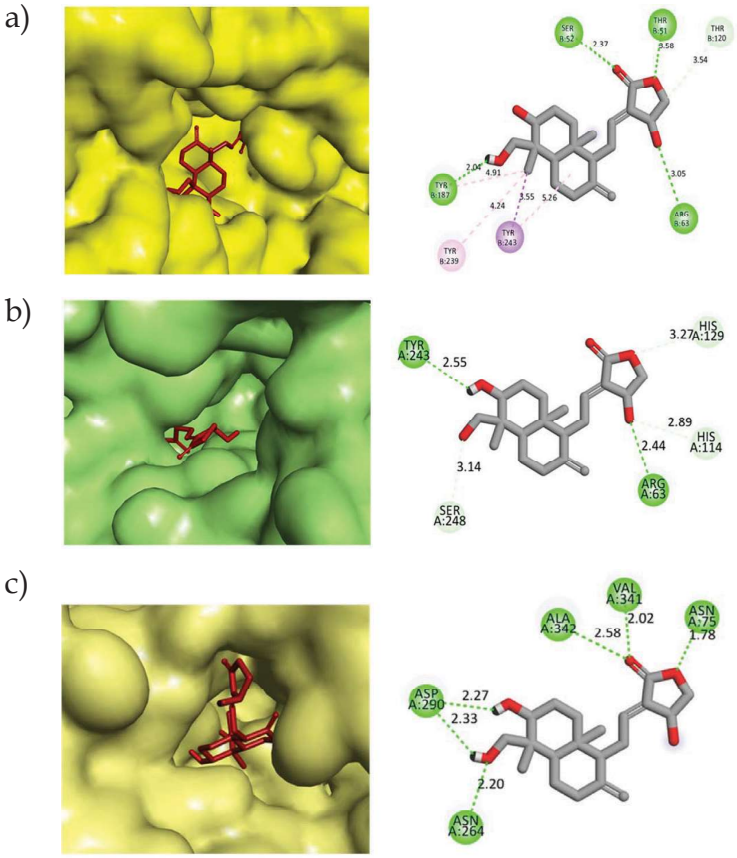


Fig. 5 3D and 2D interaction plots of andrographolide with receptors a) 2O6I b) 4LRL and c) 6ORI

Screening of compounds on the receptors of K. pneumoniae

Table 6 presents the binding scores of andrographolide, bulnesene, and bisabolene epoxide on selective receptors of *K. pneumoniae*. Bisabolene epoxide exhibited only low to moderate values of binding scores on various enzymes of this pathogen. Among the seven receptors selected for *in silico* studies, the 5ECX-ligand complex of alpha-bulnesene displayed a binding energy of -7.9 kcal/mol. Other receptors showed a low affinity to bulnesene, similar to bisabolene epoxide. Conversely, the andrographolide molecule demonstrated superior binding scores compared to the other two molecules on the enzymes of the pathogen.

Andrographolide exhibited strong binding within the cavity of the receptor 5ECX, which represents dihydrofolate reductase, an enzyme

crucial for bacterial survival and growth. The 5ECX-andrographolide complex displayed a binding score of -9.2 kcal/mol, stabilised by three conventional hydrogen bonds between the molecule and amino acid residues Met5 (2.28 Å), Ser49 (2.27 Å), and Ile20 (2.12 Å). Additionally, two non-conventional bonds formed between the alkyl parts of the molecule and the amino acids (Pro18; 3.32 Å, Ser96; 3 Å), further enhancing complex stability. The amino acid residue phenylalanine 31 engaged in three pi-alkyl interactions with the molecule, contributing to the stability of the 5ECX-andrographolide complex. The most probable conformation of the andrographolide molecule within the enzyme's binding cavity and the 2D interaction plot of the 5ECX-ligand complex are depicted in Fig. 6a.

The beta-lactamase SHV-11 enzyme (PDB ID: 6NFD) also exhibited good binding affinity towards andrographolide (-8.5 kcal/mol). By inhibiting beta-lactamase, andrographolide presents a promising avenue to prevent the antibacterial resistance behaviour of the pathogen *K. pneumoniae*. In other words, beta-lactam antibiotics will exhibit enhanced effectiveness in the presence of andrographolide. The beta-lactamase-andrographolide complex was stabilised by four classical hydrogen bonds. Amino acid residues Asn128 (2.32 Å), Ser126 (2.0 Å), Arg (4.77 Å), and Asn270 (2.74 Å) within the binding pocket of the beta-lactamase were involved in hydrogen bond formation. Additionally, a pi-sigma bond was observed between Tyr101 (3.66 Å) and the molecule. Furthermore, three pi-alkyl interactions (Val212, Ala233, and Tyr101) contributed to the stability of this complex. Figure 6b illustrates the 2D and 3D interaction plots of the 6NFD-andrographolide complex.

Table 6 Docking score (-kcal/mol) of drug-like molecules with the selected receptors of *K. pneumoniae*

PDB	Andrographolide	Alpha bulnesene	Bisabolene epoxide
5ECX	9.2	7.9	7.1
5EIX	7.7	6.4	6.8
8SKO	7.8	6.1	6.0
6NFD	8.5	7.0	6.4
5UL8	7.7	6.2	6.3
6MGY	7.2	6.2	6.5
5NBK	7.6	6.2	6.6

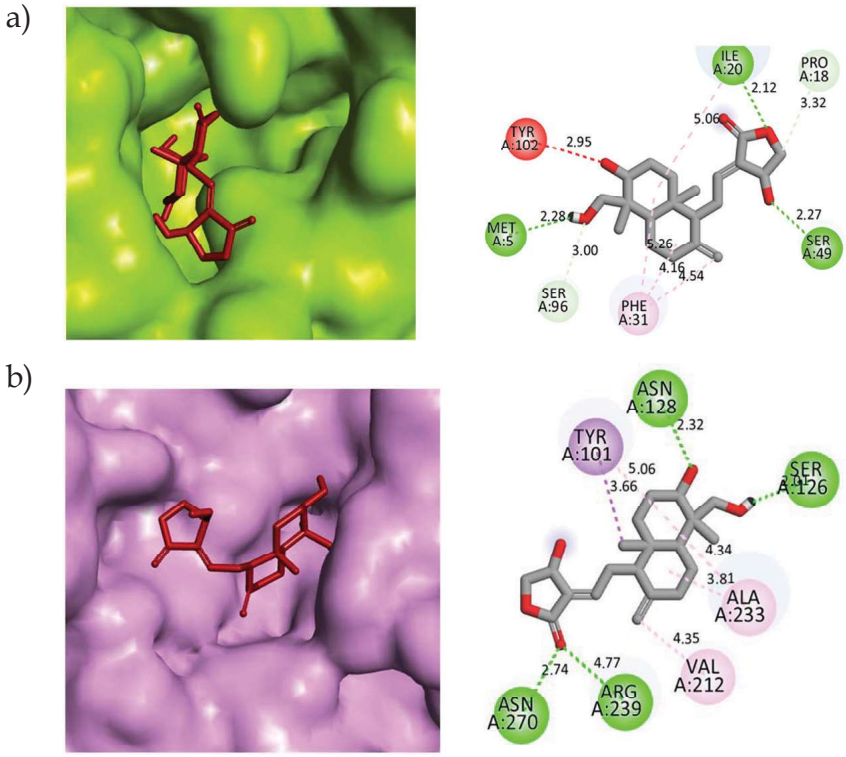


Fig. 6 3D and 2D interaction plots of andrographolide with receptors of *K. pneumoniae* a) 5ECX b) 6NFD

Conclusions

In this work, we prepared hexane, ethyl acetate, acetone, and methanol extracts of *Andrographis paniculata* leaves and performed antibacterial screening against two opportunistic pathogens, *Klebsiella pneumoniae* and *Enterococcus faecalis*. Only the acetone extract inhibited the growth of *K. pneumoniae* and *E. faecalis*, as determined by the well diffusion method in in vitro studies. Analysis of the extracts using GC-MS identified 18 compounds. Subsequent ADME studies revealed three drug-like molecules: alpha-bulnesene, bisabolene epoxide, and andrographolide.

To identify the bioactive component, we performed *in silico* investigations on selected receptors of these pathogens. Notably, andrographolide emerged as the key player in inhibiting the growth of both *E. faecalis* and *K. pneumoniae*. This molecule demonstrated

remarkable inhibition efficiency against triphosphohydrolase, lactate dehydrogenase, and lipoyl synthase enzymes in *E. faecalis*. Additionally, andrographolide exhibited significant binding affinity towards dihydrofolate reductase and beta-lactamase SHV-11 enzymes of *Klebsiella pneumoniae*.

Both the *in vitro* and *in silico* studies revealed a trend: the extract displayed greater activity against E. faecalis compared to *K. pneumoniae*. This finding mirrored the observation of higher binding scores on the structural proteins of *E. faecalis* compared to *K. pneumoniae*. In conclusion, the presence of andrographolide in the acetone extract of *Andrographis paniculata* leaves appears to be the driving force behind its antibacterial activity against the two tested bacteria, with *E. faecalis* being potentially more susceptible than *K. pneumoniae*.

Conflict of interest

There is no conflict of interest between the authors.

Author Contribution

Bindu T K: Collection of plant and Solvent extraction, Vinod P Raphael: Computational studies, Shaju K S: Computational studies, Sunil Jose T: Anti-microbial studies

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References

[1] Rossi, F., Santonicola, S., Amadoro C., Marino, L., Colavita, G. (2023) Recent Records on Bacterial Opportunistic Infections via the Dietary Route. *Microorganisms* 29;12(1):69. doi: 10.3390/microorganisms12010069. PMID: 38257896

[2] Katharine, M., Jimstan N., Periselneris Jeremy S. (2023) Opportunistic bacterial, viral and fungal infections of the lung, *Medicine*, 51(11) 777-783, https://doi.org/10.1016/j.mpmed.2023.08.002.

[3] Wu Y., Wang Y., Yang H., Li Q., Gong X., Zhang G., et al. (2021) Resident bacteria contribute to opportunistic infections of the respiratory tract. *PLoS Pathog* 17(3): e1009436. https://doi.org/10.1371/journal.ppat.1009436

[4] Ogawa, T., Ikebe, K., Enoki, K., Murai, S., & Maeda, Y. (2012). Investigation of oral opportunistic pathogens in independent living elderly Japanese. *Gerodontology*, *29*(2). https://doi.org/10.1111/j.1741-2358.2010.00449.x

[5] MartíNez, J. L. (2014). Short-sighted evolution of bacterial opportunistic pathogens with an environmental origin. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00239

[6] Riccardi, N., Rotulo, G. A., & Castagnola, E. (2019). Definition of opportunistic infections in immunocompromised children on the basis of etiologies and clinical features: A summary for practical purposes. *Current Pediatric Reviews*, *15*(4), 197–206. https://doi.org/10.2174/1573396315666190617151745

[7] National Institutes of Health (US); Biological Sciences Curriculum Study. NIH Curriculum Supplement Series. Bethesda (MD): National Institutes of Health (US); 2007. Understanding Emerging and Re-emerging Infectious Diseases. Available from: https://www.ncbi.nlm.nih.gov/books/NBK20370/

[8] Abdoli, A., Falahi, S., & Kenarkoohi, A. (2021). COVID-19-associated opportunistic infections: a snapshot on the current reports. *Clinical and Experimental Medicine*, *22*(3), 327–346. https://doi.org/10.1007/s10238-021-00751-7

[9] Said MS, Tirthani E, Lesho E. Enterococcus Infections.. In: StatPearls Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK567759/

[10] Gök ŞM, Türk Dağı H, Kara F, Arslan U et al (2020) Investigation of Antibiotic Resistance and Virulence Factors of Enterococcus faecium and Enterococcus faecalis Strains Isolated from Clinical Samples. *Mikrobiyol Bul*, 54(1): 26-39. Turkish. doi: 10.5578/mb.68810. PMID: 32050876.

[11] Moon, B., Ali, M., Choi, J., Heo, Y., Lee, Y., Kang, H., Kim, T., Yoon, S., Moon, D. C., & Lim, S. (2023). Antimicrobial Resistance Profiles of Enterococcus faecium and Enterococcus faecalis Isolated from Healthy Dogs and Cats in South Korea. *Microorganisms*, *11*(12), 2991. https://doi.org/10.3390/microorganisms11122991

[12] Mikalsen, T., Pedersen, T., Willems, R. J. L., Coque, T. M.,

Werner, G., Sadowy, E., Van Schaik, W., Jensen, L., Sundsfjord, A., & Hegstad, K. (2015). Investigating the mobilome in clinically important lineages of Enterococcus faecium and Enterococcus faecalis. *BMC Genomics*, *16*(1). https://doi.org/10.1186/s12864-015-1407-6

[13] Moshokoa, M. F., Daramola, M. O., Adeleke, R., Ndaba, B., & Roopnarain, A. (2023). Influence of environmental factors on Enterococcus faecium for production of succinic acid in a batch reactor: A preliminary investigation. *Materials Today: Proceedings*. https://doi.org/10.1016/j.matpr.2023.08.078

[14] Zowalaty, M. E. E., Lamichhane, B., Falgenhauer, L., Mowlaboccus, S., Zishiri, O. T., Forsythe, S., & Hemly, Y. A. (2023). Antimicrobial resistance and whole genome sequencing of novel sequence types of Enterococcus faecalis, Enterococcus faecium, and Enterococcus durans isolated from livestock. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-42838-z

[15] Aung, M. S., Urushibara, N., Kawaguchiya, M., Ohashi, N., Hirose, M., Kudo, K., Tsukamoto, N., Ito, M., & Kobayashi, N. (2023). Antimicrobial Resistance, Virulence Factors, and Genotypes of Enterococcus faecalis and Enterococcus faecium Clinical Isolates in Northern Japan: Identification of optrA in ST480 E. faecalis. *Antibiotics*, *12*(1), 108. https://doi.org/10.3390/antibiotics12010 108

[16] Ashurst JV, Dawson A. *Klebsiella Pneumonia*.. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK519004/

[17] Kuş H, Arslan U, Türk Dağı H, Fındık D.(2017) Investigation of various virulence factors of Klebsiella pneumoniae strains isolated from nosocomial infections, *Mikrobiyol Bul,* 51(4) 329-339. Turkish. doi: 10.5578/mb.59716. PMID: 29153063.

[18] Karimi, K., Zarei, O., Sedighi, P., Taheri, M., Doosti-Irani, A., & Shokoohizadeh, L. (2021). Investigation of Antibiotic Resistance and Biofilm Formation in Clinical Isolates of Klebsiella pneumoniae. *International Journal of Microbiology*, *2021*, 1–6. https://doi.org/10.1155/2021/5573388

[19] Sharma A, Thakur A, Thakur N, Kumar V, Chauhan A, Bhardwaj N. Changing (2023) Trend in the Antibiotic Resistance Pattern of *Klebsiella Pneumonia* Isolated From Endotracheal

Aspirate Samples of ICU Patients of a Tertiary Care Hospital in North India. *Cureus. Mar,* 17;15(3) (2023) e36317. doi: 10.7759/cureus.36317. PMID: 37077586; PMCID: PMC10106535.

[20] Li Y, Kumar S, Zhang L, Wu H. (2022) *Klebsiella pneumonia* and Its Antibiotic Resistance: A Bibliometric Analysis. *Biomed Res Int,* 6 1668789. doi: 10.1155/2022/1668789. PMID: 35707374; PMCID: PMC9192197.

[21] Arato, V., Raso, M. M., Gasperini, G., Scorza, F. B., & Micoli, F. (2021). Prophylaxis and Treatment against Klebsiella pneumoniae: Current Insights on This Emerging Anti-Microbial Resistant Global Threat. *International Journal of Molecular Sciences*, *22*(8), 4042. https://doi.org/10.3390/ijms22084042

[22] Nyakudya, T.T., Tshabalala, T., Dangarembizi, R., Erlwanger, K.H., Ndhlala, A.R. (2020) The Potential Therapeutic Value of Medicinal Plants in the Management of Metabolic Disorders. Molecules. 25(11):2669. doi: 10.3390/molecules25112669.

[23] Cheng, J.T, Liu, I.M., Shen, S.C (2022) Plant Therapeutics. Plants (Basel). 11(20):2720. doi: 10.3390/plants11202720. PMID: 36297743; PMCID: PMC9611926.

[24] Shikha, J., Klaudi, K. V., Amit A., (2023) Plant-based therapeutics: current status and future perspectives, Phytopharmaceuticals and Herbal Drugs, Academic Press, 2023, Pages 3-11, ISBN 9780323991254, https://doi.org/10.1016/B978-0-323-99125-4.00003-2.

[25] Okhuarobo, A., Falodun, J. E., Erharuyi, O., Imieje, V., Falodun, A., & Langer, P. (2014). Harnessing the medicinal properties of Andrographis paniculata for diseases and beyond: a review of its phytochemistry and pharmacology. *Asian Pacific Journal of Tropical Disease*, *4*(3), 213–222. https://doi.org/10.1016/s2222-1808(14)60509-0

[26] Hossain MS, Urbi Z, Sule A, Hafizur Rahman KM. (2014) Andrographis paniculata (Burm. f.) Wall. ex Nees: a review of ethnobotany, phytochemistry, and pharmacology. *Scientific World Journal* 274905. doi: 10.1155/2014/274905. Epub 2014 Dec 24. PMID: 25950015; PMCID: PMC4408759.

[27] Kaewdech, A., Nawalerspanya, S., Assawasuwannakit, S., Chamroonkul, N., Jandee, S., & Sripongpun, P. (2022). The use of Andrographis paniculata and its effects on liver biochemistry of

patients with gastrointestinal problems in Thailand during the COVID-19 pandemic: a cross sectional study. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-23189-7

[28] Gonde, D. P., Bhole, B. K., & Kakad, K. S. (2024). Andrographolide, diterpenoid constituent of Andrographis paniculata: Review on botany, phytochemistry, molecular docking analysis, and pharmacology. *Annales Pharmaceutiques Françaises*, *82*(1), 15–43. https://doi.org/10.1016/j.pharma.2023.10.001

[29] Subramanian, R., Asmawi, M. Z., & Sadikun, A. (2011). A bitter plant with a sweet future? A comprehensive review of an oriental medicinal plant: Andrographis paniculata. *Phytochemistry Reviews*, *11*(1), 39–75. https://doi.org/10.1007/s11101-011-9219-z

[30] Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, *7*(1). https://doi.org/10.1038/srep42717

[31] Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. (2000) The Protein Data Bank. Nucleic Acids Res. 28(1) 235-42. doi: 10.1093/nar/28.1.235. PMID: 10592235; PMCID: PMC102472.

[32] BIOVIA, Dassault Systèmes, San Diego: Dassault Systèmes, [2024].

[33] Yang, L., Yang, X., Gan, J., Chen, S., Xiao, Z. J., & Cao, Y. (2022). CB-Dock2: improved protein–ligand blind docking by integrating cavity detection, docking and homologous template fitting. *Nucleic Acids Research*, *50*(W1), W159–W164. https://doi.org/10.1093/nar/gkac394