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GC-MS Analysis of Endophytic Bacteria Isolate *Acalypha indica* L. Compounds as Antibacterial

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Abstract : Pneumonia is an acute respiratory infection that primarily affects the lungs and is caused by various microorganisms, including viruses, fungi, and bacteria. *Klebsiella pneumoniae*, a multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacterium, is a leading cause of widespread pneumonia. This study aimed to identify endophytic bacteria from the leaves of *Acalypha indica* L. and evaluate their antibacterial properties through both *in vitro* and *in silico* approaches. The objectives included isolating endophytic bacteria from *Acalypha indica* L., testing their antibacterial activity against *Klebsiella pneumoniae*, identifying the selected bacterial isolates using molecular techniques, analyzing their secondary metabolites via gas chromatography-mass spectrometry (GC-MS), and performing *in silico* molecular docking studies. The study identified BE 4, an endophytic bacterial isolate of *Bacillus pumilus*, as exhibiting the most potent antibacterial activity against *Klebsiella pneumoniae*. GC-MS analysis of the ethyl acetate extract of this isolate revealed 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester as the primary metabolite component. Furthermore, molecular docking analysis identified two natural compound ligands, 1,2-benzenedicarboxylic acid, diethyl ester (-6.5 kcal/mol), and lilial (-6.2 kcal/mol), as having potential efficacy against drug-resistant bacteria responsible for pneumonia. These findings suggest that endophytic bacteria and their bioactive compounds could serve as promising candidates for the development of new treatments against drug-resistant pneumonia.

Keywords : Endophytic bacteria, *Acalypha indica* L, Antibacterial, Gas chromatography-mass spectrometry (GC-MS), Molecular docking

Introduction

Klebsiella pneumoniae causes many diseases, including pneumonia, urinary tract infections (UTI), and sepsis.[1] *Klebsiella pneumoniae* is an enterobacter significantly considered one of the pathogens often resistant to antibiotics. *Klebsiella pneumoniae* became resistant to carbapenem antibiotics through the spread of extended-spectrum β-lactamase (ESBL) and the plasmid encoding carbapenemase, causing the bacteria to become multidrug-resistant (MDR) and extremely drug-resistant (XDR).[2]

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Acalypha indica L. is a plant commonly found in humid, temperate, and tropical areas. It is often considered a weed. *Acalypha indica* L. is a weed recognized as a valuable source of medicine.[3] *Acalypha indica L.* has been found to contain several bioactive compounds with potential as anticancer, antioxidant, and antibacterial agents. The phytochemical screening of methanol and ethanol extracts revealed the presence of phenolic compounds, flavonoids, steroids, terpenoids, and alkaloids.[4,5]

The ethanol fractions derived from *Acalypha indica* L. and *Croton bonplandianus* Baill have demonstrated the ability to inhibit the growth of multidrug-resistant (MDR) bacteria. This antibacterial activity is supported by gas chromatography-mass spectrometry/mass spectrometry (GC-MS/MS) analysis, which identified bioactive compounds in the ethanol fractions of both plants. These compounds are noted for their antibacterial and anticancer properties, highlighting the therapeutic potential of these plant extracts.[6] Plants benefit from endophytic bacteria that live in the host plant's tissues without causing adverse effects, the synthesis of composite material has many ways, for example, endophytic bacteria help the host plant grow and increase its resistance to various pathogens by regulating the synthesis of secondary metabolites.[7]

Endophytic bacteria are essential in promoting the growth of their host by dissolving macronutrients such as phosphorus, potassium, and zinc and synthesizing phytohormones, siderophores, hydrogen cyanide, and ammonia.[8] Some bioactive compounds in endophytic bacteria include antibiotics, alkaloids, phenols, terpenoids, nanoparticles, phytohormones, and various enzymes.[9]

Endophytic bacteria live in the tissues of plants and provide protection against pathogenic bacteria; endophytic bacteria can induce biotic and abiotic stress tolerance in plants. Additionally, they regulate the synthesis of secondary metabolites with medicinal properties and induce various biological effects.[10,11]

In this study, the identification of a compound using GC-MS is accomplished by comparing the experimental mass spectrum with the mass spectra in a spectral library. The most diagnostic intensities in the mass spectrum are those with higher m/z values.[12] GC–MS analysis of the ethyl acetate extract of endophytic revealed several antibacterial compounds, including 1,2-benzenedicarboxylic acid, hentriacontane, and bis (2-methylpropyl) ester. The ethyl acetate-based endophytic extract showed antibacterial and antifungal activities against test microbes, with the highest inhibition observed against pathogens.[13]

Inspired by these works, it is possible to utilize endophytic bacteria found in *Acalypha indica L*. as antibacterial compounds.

Experimental

Isolation of endophytic bacteria from *Acalypha indica L.*

Fresh leaves of *Acalypha indica* were collected and washed with running water. The leaves were cut into small pieces. The leaf samples were sterilized in 70% ethanol for 1 min, 5.25% sodium hypochlorite (NaOCl) for 5 min, and 70% ethanol for 30 s. They were then rinsed three times for 1 min with sterile water. After placing the sample on the surface of nutrient agar (NA), the bacterial culture was incubated at 30ºC for 48 h.

Antibacterial activity test against *Klebsiella pneumoniae*

The study utilized the disc diffusion method to conduct the antibacterial activity test, with Lefoproxacin as a positive control. Using the streak method, one ose of *Klebsiella pneumoniae* pathogenic bacterial isolate was inoculated on a mueller-hinton agar (MHA). Further, a sterilized blank disc was soaked in endophytic bacteria isolates were inoculated into nutrient broth (NB) and incubated for 24 h and Lefoproxacin for 10 min. The sterile disc is positioned onto the surface of MHA that has solidified, followed by a 72 h incubation at 37ºC. Subsequently, the caliper is utilized to measure the diameter of the inhibition zone.

Molecular identification of selected bacterial isolates

Molecular identification was carried out on bacteria isolates with the highest inhibition zone. Endophytic isolates were identified using 16S-rRNA sequences using primers 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') conducted at the Laboratory of Science Research and Development (LPPS) Hasanuddin University, Makassar. Furthermore, the PCR results were thoroughly analyzed at 1stBASE through PT Genetika Science Indonesia. The sequencing results were searched for sequences similar to the BLAST (Basic Local Alignment Search Tool) program from NCBI (National Center for Biotechnology Information) online on the website (http//www.ncbi.nlm.nih.gov).

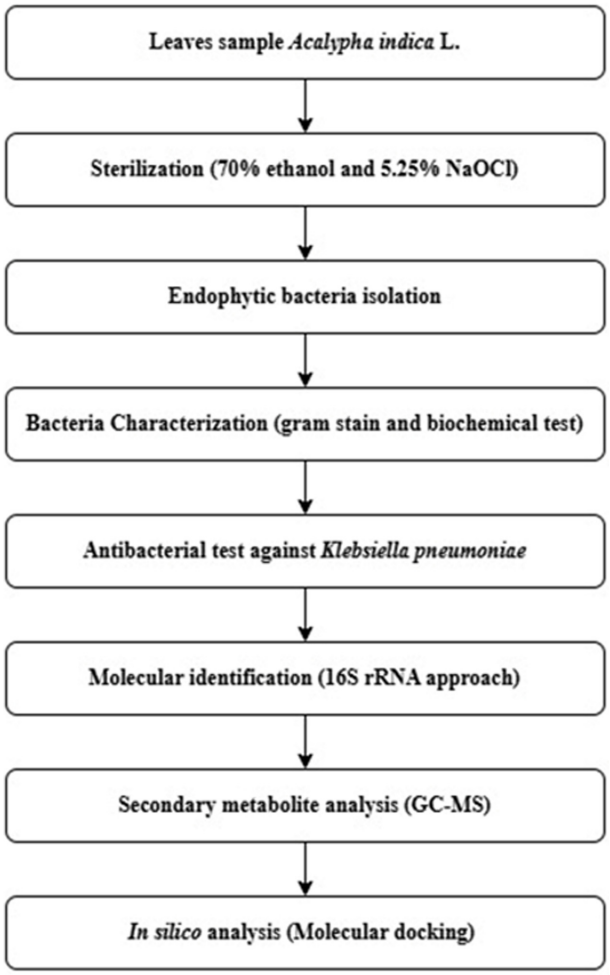


Figure 1. Schematic diagram of the method used to identify endophytic bacteria *Acalypha indica* L. compounds.

Secondary metabolite analysis of endophytic bacteria

Secondary metabolite analysis was performed using GC-MS. GC-MS is a technique frequently used in metabolomics analysis to study cometabolism. GC-MS experiments generate large amounts of data and require several analysis steps to decipher relevant biological information. The steps

involved in this analysis include spectrum deconvolution to convert raw data into a peak list, identification of metabolites associated with chromatographic peaks, measurement of metabolite abundance in different samples, association network analysis to find correlations between changes in the abundance of multiple metabolites, and pathway analysis to understand the biochemical relationships between several different metabolites in a coordinated or differential manner.[14]

The instrument was equipped with an SH-Rxi-5Sil MS column, measuring 30 meters in length with an internal diameter of 0.25 mm. The column temperature program started at 70°C, held for 2 min, followed by a ramp-up to 200°C at a rate of 10°C per min. The temperature was then further increased to a final temperature of 280°C at a rate of 5°C per min, where it was held for 9 min, resulting in a total analysis time of 36 min. The injector temperature was set to 250°C in, with a pressure of 76.9 kPa and a carrier gas flow rate of 14 mL/min at a split ratio of 1:10. The ion source and interface temperatures were maintained at 200°C and 280°C, respectively. A solvent cut time of 3 min was applied, and the mass spectrometer scanned in the range of 40-700 m/z.

In-silico analysis using molecular docking

Molecular docking is an essential part of the drug discovery process.[15] Docking is a molecular modeling technique used to predict a protein's interaction with ligands.[16] In performing molecular docking, the following preparations are required.

a) Ligand preparation, the ligand is taken from the 3D structure of the compound obtained from the ethyl acetate extract of endophytic bacteria *Acalypha indica* L. and levofloxacin from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The 3D structure was then downloaded in sdf format.

b) Target protein preparation, the target protein is taken through NCBI (https://www.ncbi.nlm.nih.gov/), and then the target protein is downloaded in FASTA format. Then, visualization is done using the Swiss Model (https://swissmodel.expasy.org/) and the FASTA format is entered. The website will then create a 3D structure of the target protein. After that, the target protein is then downloaded in pdb form. The 3D structure of the target protein obtained was then cleaned from water molecules using the Pymol application and saved in pdb format.

c) Molecular docking, target protein, and ligand are then docked using Pyrx software, and the ligand is minimized using an open babel contained in Pyrx and stored in pdb form. Then docking is done using the vina wizard by entering the ligand that has been minimized and the target protein, pressing the forward button, and automatically entering into the run vina. Then, the software will show the binding affinity value of the target protein and ligand interaction. The result of docking is then saved in pdb format.

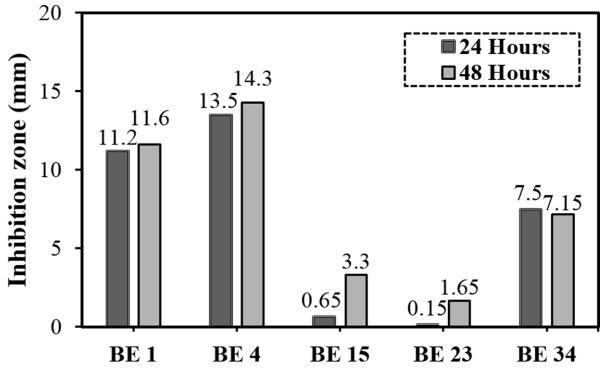
d) Visualization of docking results, docking results from target proteins and ligands are then visualized using discovery studio 2021. Previously, docking files and target proteins cleared of water molecules are opened using the pymol application and then saved in one pdb format. Furthermore, the pdb file that has been put together is then opened using discovery studio 2021 software. It visualizes the interaction in 3D and 2D and then saves it in PNG form.

Results and Discussion

Antibacterial activity test against *Klebsiella pneumoniae*

Endophytic bacterial isolates were inoculated into NB media and incubated for 24 h. Subsequently, the antibacterial activity of the isolates was tested using the disc diffusion method. This method involves diffusing antibacterial compounds from a blank disc into the media, which has been inoculated with test bacteria. Sterile blank discs immersed in NB media containing endophytic bacterial isolates and Lefoproxacin as a positive control are attached to MHA media inoculated with *Klebsiella pneumoniae* bacteria and then incubated for 24-48 h. The following are the results of the antimicrobial efficacy test for *Klebsiella pneumoniae*.

Endophytic bacterial isolates inoculated in NB media were tested for antibacterial activity inhibiting *Klebsiella pneumoniae* using the disc diffusion method. In the graph shown in Figure 2 about five endophytic bacterial isolates observed for 24 h were positive in inhibiting the growth of *Klebsiella pneumoniae*, with BE 4 having the highest inhibition zone activity. Furthermore, the isolates were observed for 48 h, the five endophytic bacterial isolates showed an increase in the diameter of the inhibition zone in inhibiting the growth of *Klebsiella pneumoniae*. This shows that the endophytic bacterial isolate *Acalypha indica* L., bactericidal, inhibits *Klebsiella pneumoniae*. On the comparison of the level of antibiotics in killing pathogenic bac-



*BE (Endophytic Bacteria)

Figure 2. Diameter chart of inhibition zone of endophytic bacteria isolate *Acalypha indica* L.

teria, there is a difference between bacteriostatic and bactericidal, There is an increase in bactericidal antibiotics rather than bacteriostatic, which remains stable without an increase or decrease.[17] This reinforces that the endophytic bacterial isolate *Acalypha indica* L. is bactericidal by obtaining an increase in the diameter of the inhibition zone.

Endophytic bacteria play a crucial role in protecting plants from harmful pathogens, largely through the production of secondary metabolites that mirror those of the plant host. This similarity in bioactive compounds positions endophytes as promising candidates for the development of new treatments against multidrug-resistant (MDR) pathogens.[18] For instance, bacterial isolates derived from *Phragmites australis* have demonstrated the ability to inhibit the growth of a wide range of MDR pathogens, including those found in food, clinical patients, and hospital environments. Moreover, certain endophytic bacteria share phylogenetic similarities with human opportunistic pathogens, further underscoring their potential as a source of novel antibiotics.[20]

Molecular identification of selected bacterial isolates

The sequence analysis results of endophytic bacteria isolates obtained from 1stBASE through PT Genetika Science Indonesia were then analyzed at gen bank using BLAST. The identification results showed that the endophytic bacterial isolate BE 4 species was *Bacillus pumilus* with a query cover value of 99%.

Bacillus pumilus bacteria are known to have antibacterial agents that can inhibit pathogens. Besides that, these bacteria are also helpful in spurring plant growth.[21,22] *Bacillus pumilus* bacteria have the potential antibacterial ability to inhibit three pathogens*: Escherichia coli, Staphylococcus,* and *Salmonella enteritidis.* Based on the results of high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) analysis, and mass spectrometry (MS) of the ethyl acetate extract from *Bacillus pumilus*, the following compounds were identified such as 3,4-dipentylhexane-2,5-diol, 1,1′-(4,5-dibutylcyclohexane-1,2-diyl)bis(ethan-1-ol), and 1,1′-(4,5-dibutyl-3,6-dimethylcyclohexane-1,2-diyl)bis(ethan-1-one). Additionally, four compounds methyl isobutyl ketone, ethanol, 5-methyl-2-heptanone, and S(-)2-methylbutylamine are recognized as biocontrol agents that inhibit the growth of food spoilage fungi during the storage of agricultural products.[21,24]

Analysis of secondary metabolites of endophytic bacteria

The ethyl acetate extract of endophytic bacteria isolate BE 4 was analyzed using gas chromatography-mass spectrometry (GC-MS) to identify and quantify the compounds present. The analysis, which was conducted under specific GC-MS conditions, revealed approximately 17 distinct compounds, as detailed in Table 1.

Chromatogram data obtained from the analysis were interpreted using the NIST and Wiley 9 libraries, which led to the identification of several key compounds. Among these, 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester was the most abundant, comprising 53.31% of the extract. This was followed by 1,2-benzenedicarboxylic acid, diethyl ester at 12.26%, bis (2-ethylhexyl) phthalate at 7.39%, and 1,4-benzenedicarboxylic acid, bis (2-ethylhexyl) ester at 5.15%, all of which are classified under

Table 1. Phytocomponents identified in the ethyl acetate extract of endophytic bacteria isolate BE 4 by GC-MS.

Peak	Compound name	Retention time (min)	Area (%)	Chemical formula
1	2-decanone	3.048	8.67	$C_{10}H_{20}O$
2	1,2,3-propanetriol, triacetate (CAS)	9.46	2.74	$C_9H_{14}O_6$
3	3,3-diethoxy-1-propanol, propyl ether	9.617	0.57	$C_{10}H_{22}O3$
4	2,4(1H,3H)-pyrimidinedione, dihydro-5-hydroxy-5-methyl-	9.708	0.38	$C_5H_8N_2O_3$
5	Dihydro-nor-dicyclo-pentadienyl acetate	10.61	2.55	$C_{12}H_{16}O_2$
6	Lilial	12.072	0.64	$C_{14}H_{20}O$
7	1,2-benzenedicarboxylic acid, diethyl ester	12.844	12.26	$C_{12}H_{14}O_4$
8	Octanal, 2-(phenylmethylene)-(CAS)	14.909	2.07	$C_{15}H_{20}O$
9	7-AC-6-ET-1144-ME4-tetralin	16.192	0.8	$C_{18}H_{26}O$
10	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	16.344	53.31	$C_{16}H_{22}O_4$
11	Cyclopropaneundecanal, 2-nonyl-	16.783	0.06	$C_{23}H_{44}O$
12	1,2-benzenedicarboxylic acid, dibutyl ester (CAS)	17.258	0.72	$C_{16}H_{22}O_4$
13	1,2-benzenedicarboxylic acid, dibutyl ester (CAS)	18.308	2.26	$C_{16}H_{22}O_5$
14	E-11-tetradecenol, trimethylsilyl ether	28.047	0.31	$C_{17}H_{36}OSi$
15	(1R,2S)-1-carboxy-2-(hydroxymethyl) cyclopropane	29.992	0.12	$C_5H_8O_3$
16	Bis (2-ethylhexyl) phthalate	30.178	7.39	$C_{24}H_{38}O_4$
17	1,4-benzenedicarboxylic acid, bis (2-ethylhexyl) ester	33.653	5.15	$C_{24}H_{38}O_4$

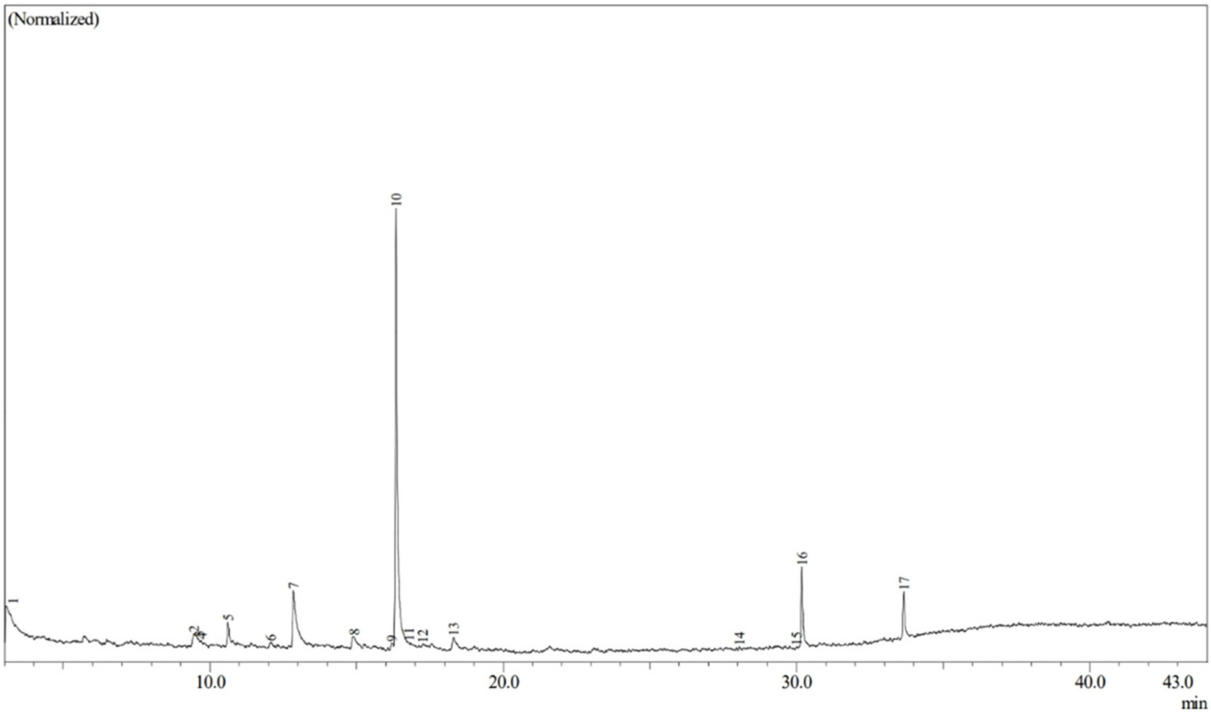


Figure 3. GC-MS chromatogram of ethyl acetate extract of endophytic bacteria isolate BE 4.

dicarboxylic acid-derived phthalic acid compounds. Additionally, the extract contained 2-decanone, which accounted for 8.67% and is categorized under butanone compounds. These results highlight the chemical diversity present in the ethyl acetate extract and underscore the predominance of phthalic acid derivatives alongside a significant presence of butanone compounds.

The compound 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester belongs to the group of dicarboxylic acid derivatives. GC-MS analysis of *Streptomyces cuspidosporus* bacterial extract identified this compound, representing 8.20% of the extract, and found it capable of inhibiting several pathogenic bacteria, including *Klebsiella pneumoniae*.[25]

1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester was also identified as a natural compound in endophytic bacteria isolates from the Bacillus group, specifically Bacillus atrophaeus and Bacillus mojavensis, both of which exhibit antifungal and antibacterial activity.[26] The dicarboxylic acid group, which includes compounds with antifungal potential, such as 1,2-benzenedicarboxylic butyl 2-ethylhexyl ester, is also known for its mucolytic properties. This compound, obtained from the endophytic bacteria isolate *Lysinibacillus sphaericus*, has been shown to inhibit *Rhizoctonia solani*, a fungal pathogen in rice plants..[27]

GC-MS analysis of bacterial extracts from *Bacillus pumilus* and *Bacillus thuringiensis* identified 2-decanone, a compound capable of inhibiting the growth of anthracnose pathogens in postharvest mango.[28] This supports the findings on the ethyl acetate extract of the endophytic bacteria BE 4, identified as *Bacillus pumilus*, which has been shown to inhibit the growth of the pathogenic bacterium *Klebsiella pneumoniae*.

In-silico analysis using molecular docking

In silico test using molecular docking to predict drug conformation by analyzing the ligand and target protein interaction. Furthermore, the docking results show the binding affinity value of the ligand to the target protein, which describes how strong their interaction.[29] The molecular docking results can be seen in the following table.

Targeting outer membrane protein A (OmpA) and conjugative pili protein (pilin) in *Klebsiella pneumoniae* presents a promising strategy for inhibiting bacterial growth and combating antibiotic resistance. OmpA plays a pivotal role in bacterial adherence to host tissues, immune evasion, and structural integrity, making it essential for virulence and infection establishment. Similarly, pilin facilitates bacterial conjugation, crucial for transferring antibiotic resistance genes, and contributes to bacterial adhesion and biofilm formation. Inhibiting these proteins could disrupt key processes such as adhesion, colonization, and biofilm development, thereby reducing *K. pneumoniae* virulence and enhancing the efficacy of existing antimicrobial therapies.[30,32]

Table 2 shows that two natural compound ligands, 1,2-

Table 2. Ligand binding affinity values of compounds of ethyl acetate extract of endophytic bacteria *Acalypha indica* L.

Sample	Compound ligand	Binding affinity (kcal/mol)	
		OmpA	pilin
BE 4 extracts	2- decanone	-4.6	-4.0
	1,2,3-propanetriol, triacetate (CAS)	-5.7	-4.6
	3,3-diethoxy-1-propanol, propyl ether	-3.4	-4.4
	2,4(1H,3H)-pyrimidinedione, dihydro-5-hydroxy-5-methyl-	-4.7	-4.9
	Lilial	-6.1	-6.2
	1,2-benzenedicarboxylic acid, diethyl ester	-6.5	-4.5
	Octanal, 2-(phenylmethylene)- (CAS)	-5.7	-4.0
	1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester	-5.7	-5.7
	1,2-benzenedicarboxylic acid, dibutyl ester (CAS)	-5.4	-5.7
Positive control	Levofloxacin	-6.3	-6.3

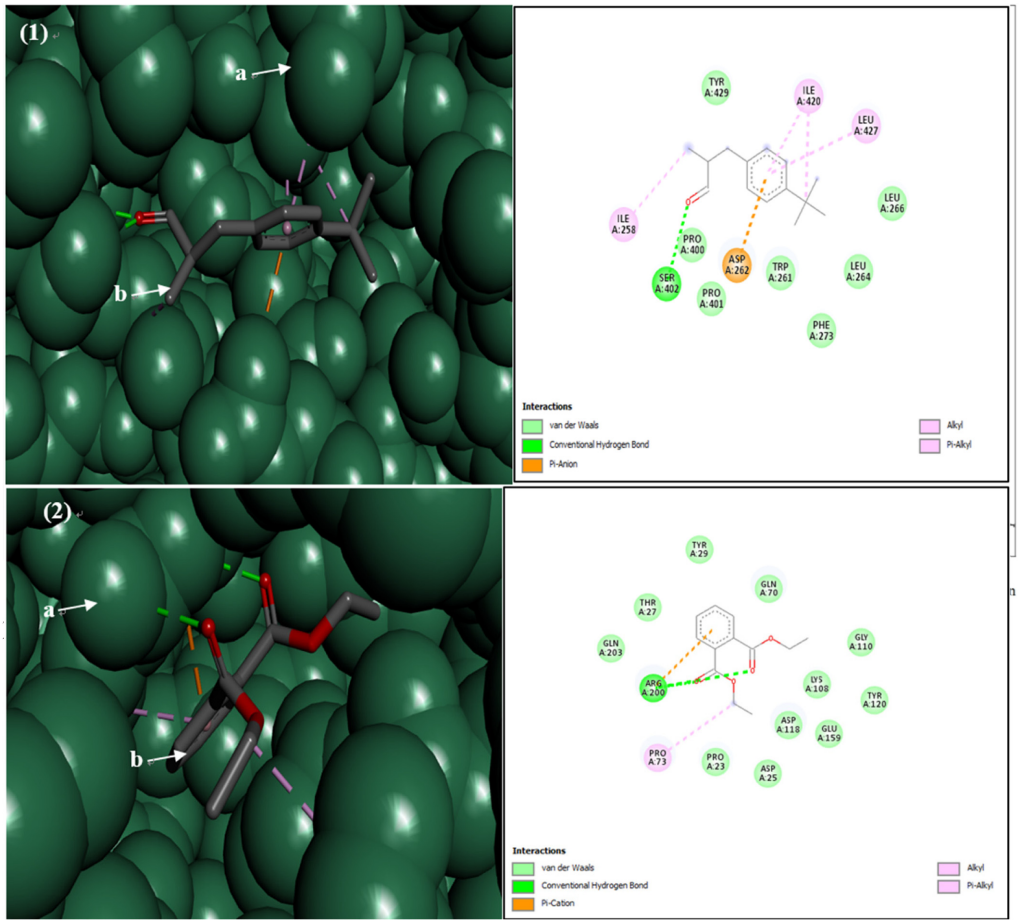


Figure 4. 3D and 2D visualization of ligand and target protein interaction. (a) Target protein; (b) Ligand compound. (1) Interaction of compound ethyl acetate extract of endophytic bacteria BE 4 1,2-benzenedicarboxylic acid, diethyl ester with outer membrane protein A (OmpA); (2) Interaction of of compound ethyl acetate extract of endophytic bacteria BE 4 lilial with conjugative pili protein (pilin).

benzenedicarboxylic acid diethyl ester (-6.5 kcal/mol) and lilial (-6.2 kcal/mol), derived from the ethyl acetate extract of endophytic bacteria BE 4, exhibited low binding affinity values. Binding affinity is a measure of a compound's ability to bind to a receptor, with lower binding affinity values indicating stronger interactions between the ligand and

receptor. In this case, the negative binding energy values suggest a relatively high affinity between the ligands and their target receptors, meaning the ligands are likely to bind more effectively.

The docking results were then visualized in 3D and 2D to analyze the interactions between the ligand and the target protein (Figure 4). The ligand forms various bonds with residues of the target protein such as van der waals bonds, hydrogen bonds, hydrogen carbon bonds, pi-cation, pi-anion, alkyl-alkyl and pi alkyl which are hydrogen bonds, hydrophobic and electrostatic bonds with seven amino acids (ARG A:200, PRO A:73, ILE A:258, SER A:402, ASP A:401, ILE A:420, LEU A:427). The type of chemical bond is a factor that affects a compound's affinity to the binding site.[33] The results indicate that the ligand forms a high-affinity bond with the amino acids of the target protein.

Conclusions

This study employed GC-MS analysis and molecular docking to explore the phytochemical profile and antibacterial potential of endophytic bacteria from *Acalypha indica* L. The analysis identified 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester as the most prominent and active compound. Molecular docking further revealed that 1,2-benzenedicarboxylic acid, diethyl ester and lilial demonstrated the lowest binding affinities, with values of -6.5 kcal/mol and -6.2 kcal/mol, respectively. These findings suggest that the primary compound exhibits significant bioactivity, highlighting its potential as an antibacterial agent against *Klebsiella pneumoniae*.

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