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Original Research Article

Trans-Cinnamaldehyde Inhibitory Activity Against *mrkA*, *treC*, and *luxS* Genes in Biofilm-forming *Klebsiella pneumoniae*: An *In Silico* Study

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ABSTRACT

Biofilm-forming *Klebsiella pneumoniae* is a multidrug resistant organism that causes severe infections in humans. The *luxS, treC* and *mrkA* genes play a significant role in the formation of *K. pneumoniae* biofilms. *Cinnamaldehyde* has been shown to exhibit antimicrobial and antibiofilm activities against pathogenic bacteria. The present study aims to investigate the antibiofilm activity of cinnamaldehyde *in silico*.

In silico study was done using Autodock Vina software and pharmacokinetics prediction using the pkCSM strategy. The ability of cinnamaldehyde to inhibit the *mrkA, treC*, and *luxS* genes of *K. pneumonia* was accessed by docking the 3D structure of *cinnamaldehyde* with the *luxS, mrkA*, and *treC* gene receptors. Post-docking analysis such as binding affinities, hydrophobic interactions, and pharmacokinetic predictions were carried out. Cinnamaldehyde showed low binding affinities for the three genes; *luxS* (-5.6 kcal/mol), *mrkA* (-5.0 kcal/mol), and *treC* (-6.0 kcal/mol). The root mean square deviation (RMSD) values were found to be 1.461, 1.210, and 1.426 for *luxS*, mrkA, and *treC* gene receptors, respectively. Cinnamaldehyde had a number of hydrophobic interactions as seen in the ligand-receptor interactions for *luxS* (Lys 13; Asn 15; His 11; Pro 43; Leu 159; and Val 9), *mrkA* (Phe 157; Ala 162; and Lys 129). Cinnamaldehyde had high bond-free energy similar to that of ciprofloxacin docked with the same gene receptor. From the pharmacokinetics predictions, cinnamaldehyde had a good pharmacokinetics profile. In conclusion, cinnamaldehyde has a high docking score comparable to ciprofloxacin and therefore has a potential for use as antibacterial and antibiofilm agent against *Klebsiella pneumoniae*.

***Keywords*:** *Antimicrobial, Trans-Cinnamaldehyde, Klebsiella pneumoniae, Molecular Docking, pkCSM.*

Introduction

Klebsiella pneumoniae is a bacterium belonging to the *Enterobacteriaceae* family. It is an opportunistic pathogen that causes a wide range of infections and frequently develops drug resistance.[1] *K. pneumoniae* has lately become known as an infectious agent due to an increase in the incidence of serious infections and a decline in the availability of effective therapies.[2] The rise of *K. pneumoniae* strains with extra genetic traits has become worrisome. These strains are either antibiotic resistance or hypervirulent (HV).[2] Previous studies have shown that *K. pneumoniae* strains with broad antimicrobial resistance spread quickly, especially when the bacteria can form biofilm. These bacteria have the capacity to generate a substantial extracellular biofilm layer, which facilitates their ability to adhere to both biotic and abiotic surfaces.[3]

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Infections caused by *K. pneumoniae* strains that form biofilms are more challenging to cure than infections caused by other strains.[3] Bacteria that developed biofilms had a much higher prevalence of antimicrobial resistance than those that do not, leading to chronic infection. The molecular distribution of biofilm generating genes show that *luxS* gene is present in 98% of the isolates, while 96% of the isolates has the *treC* gene, and 34% of the isolates has the *wza* gene.[4]

Exopolysaccharides, extracellular DNA, and proteins form a protective and adhesion-promoting extracellular matrix that surrounds cells adhering to a biotic or abiotic surface to form biofilms.[5] The *luxS, treC*, and *mrkA* genes are necessary for the formation of biofilms in *K. pneumonia*.[6] *Quorum Sensing* (QS), a gene that generates *Autoinducer-2 (AI-2)* increased biofilm formation and enhanced the gene expression from the *luxS* gene for *K. pneumoniae* (*AI-2*).[7,8] Previous investigations asserted that *AI-2* molecules are involved in the biofilm formation of *K. pneumoniae*.[9] The *treC* gene was also discovered to be essential for the creation of capsules and the development of biofilms by the use of trehalose and CPS modulation.[10] In addition, *type 3 fimbriae* expression; a crucial element in fostering the development of biofilms is significantly influenced by *mrkA* genes as only the *type 3 fimbriae*-coding *mrkA* gene was noticeably up-regulated in *K. pneumoniae mrkH+* (high biofilm production).[11, 12]

For centuries, the discovery and development of antimicrobial medicines have mostly been based on natural products.[13] Antimicrobials are largely developed using planktonic microorganisms to counteract host immunity and harmful bacteria that are multi-resistant to antibiotics.[14] The goal of the current study is to identify substances and strategies that can alter the bacterial phenotype without causing genetic

alteration. It has been speculated that genetic alteration will stop the selection pressure that leads to the development of resistance.[15] Various new strategies have been identified for inhibiting biofilm formation.[16-19] Plants, animals (including humans with the host defense peptide LL-37), bacteria, algae, and fungi are some of the sources of natural antimicrobials. These organic molecules emerged as a result of evolutionary pressure, and many of them served as the foundation for traditional remedies in the past.[20] Numerous research have shown how certain natural products essential oils and antibiotics can be used in combination to potentially combat the problem of antibiotic resistance in clinical setting.[21, 22] Despite the fact that very few studies have been carried out to investigate the mechanism of action of various essential oils against bacteria, the majority of these studies have hypothesized that essential oils exert their antimicrobial activity by disrupting bacterial cell membrane and/or their efflux systems.[23] Cinnamon (*Cinnamomum burmannii*) is one of the herbs that is widely used for medical treatment, it contains mainly essential oils and important compounds such as cinnamaldehyde, eugenol, cinnamic acid and cinnamate.[24,25] Cinnamon has an antibacterial effect on a variety of pathogenic microorganisms, including *Candida albicans*, *Staphylococcus aureus*, *Enterobacter spp., and Pseudomonas aeruginosa*.[26] Trans-cinnamaldehyde (trans-CNMA), a naturally occurring antibacterial and antibiofilm substance has been demonstrated to exhibit efficacy against commensal, enterohemorrhagic, and uropathogenic *Escherichia coli* (UPEC) strains in addition to Methicillin-resistant *Staphylococcus aureus* strains. Numerous CNMA analogs have recently been found to have antibiofilm actions on strains of *Streptococcus pyogenes*, *Vibrio species*, and *Candida albicans*.[27] Therefore, the present study conducted the inhibitory interactions of trans-cinnamaldehyde against *Klebsiella pneumoniae* biofilm genes by *in silico* model.

Materials and Methods

Materials

The tools, programs and applications used in this study were HP Intel CORE i3 laptop, Chemdraw (v16.0), Python (v3.10.0), YASARA (v21.6.2), Open Babel (v3.1.1.1), MGL Tools or AutoDock Tools (v1.5.7), Biovia Discovery Studio Visualizer 2021 (v21.1), AutodockVina, Command Window, pkCSM web server (http://biosig.unimelb.edu.au/pkcsm/). The three-dimensional structure of trans-Cinnamaldehyde, as test compound, and Ciprofloxacin, as standard compound were downloaded from https://pubchem.ncbi.nlm.nih.gov/. Target gene structures of *luxS* (PDB ID: 1j6w), *mrkA* (PDB ID: 5kgo), and *treC* (PDB ID: 5brp) were downloaded from www.rcsb.org.

Methods

In Silico Molecular Docking

Ligand and Protein Preparation

The proteins (*luxS*, *mrkA*, and *treC*), and ligand were prepared using the Biovia DS Visualizer application. The preparation of *luxS*, *mrkA*, and *treC* proteins was done by separating the proteins from their native ligands and the residues in their receptors. Then, all files were saved in .pdb format. Meanwhile, the ligand preparation was done by optimizing the 3D cinnamaldehyde structure, then the file was saved in .pdb and converted to .pdbqt by Autodock tools (v1.5.7).

Docking Parameter Validation

Docking method validation was performed using AutoDock software (v1.5.7), by redocking native ligand of *luxS* (PDB ID: 1j6w), *mrkA* (PDB ID: 5kgo), and *treC* (PDB ID: 5brp) proteins. The results of this process were obtained in the form of grid box parameters and Root-Mean-Square Deviation (RMSD). The RMSD was correlated with the area of the docking zone. Hence, to produce a RMSD value of <2 Å, it was necessary to adjust the docking area several times. The validated protein files were saved with the names *luxS*.pdbqt, *mrkA*.pdbqt, and *treC*.pdbqt, while the ligand files were saved with the name ligand.pdbqt.

The Grid submenu and Grid box were then used to set the protein and ligand docking region. The area of the docking zone and the Root-Mean-Square Deviation (RMSD) were associated in each conformation. Thus, it was required to change the docking area numerous times in order to get an RMSD value of < 2Å.

Molecular Docking

Molecular docking of cinnamaldehyde with each of the *luxS*, *mrkA*, and *treC* proteins was done using Autodock Vina software (v1.5.7). The grid box parameters were determined. Then, the coordinates of the grid box were written in conf.txt file and the tethering process was carried out using Autodock Vina. The docking score and RMSD were extracted from the docking process by typing vina.exe -config conf.txt -log log.txt into the Windows Command Prompt. All the files to be used in the docking process were contained in the same folder. The proteins *luxS*.pdbqt, *mrkA*.pdbqt, and *treC*.pdbq were then written into a new document called conf.txt, while the ligands were represented as ligand.pdbqt, and center x,y,z and size x,y,z were represented by the values provided in the grid box. The RMSD value was retrieved from the docking process by typing vina.exe -config conf.txt -log log.txt into the Windows Command Prompt or by typing "cmd" in the folder address section and pressing the 'Enter' key. Numerous conformations and docking scores were displayed by this code. The RMSD values for each conformation were also displayed. The conformation with RMSD value <2 Å was chosen. To make the visualization process easier, the output.pdbqt file was divided into numerous files and saved in the same folder according to their respective conformations.

Visualization of Molecular Docking Data

The visualization of molecular docking data was carried out using the Biovia DS Visualizer 2021 (v21.1) application. The visualization process aims to determine the position and image of the bond between the protein and the ligand in 3 and 2 dimensions. The receptor file and the best molecular docking conformation with the .pdbqt format were opened and then the conformation file with the receptor was linked. Then the results of the visualization were analyzed to determine the interactions of the compounds as well as the positions and descriptions of the protein bonds with each of the tested ligands.

Pharmacokinetics and Toxicity Analysis

Prediction of the pharmacokinetics and toxicity profile of cinnamaldehyde was carried out using the pkCSM web server. The results obtained were in the form of ADMET properties represented as absorption, distribution, metabolism, excretion, and toxicity properties as well as Lipinski's rule of five.

Results and Discussion

An *in silico* study was carried out to determine the bonding interactions between cinnamaldehyde and *luxS*, *mrkA*, and *treC* gene receptors. These genes are implicated in biofilm formation in *Klebsiella pneumoniae*. The *in silico* study was carried out by molecular docking and using the pkCSM model for pharmacokinetics prediction of cinnamaldehyde. Molecular docking with the applications mentioned above function to assess the activity of a compound against certain receptors which serve as a useful tool in rational drug design. Additionally, information on the activity of the drug candidate and its affinity for the functional protein is learned using molecular docking application and the pkCSM model for pharmacokinetics prediction.[19] The results of the molecular docking are presented as binding affinity (docking score), RMSD, and ligand interactions with amino acid residues in the receptor proteins (Table 1).

The major outcome of the molecular docking procedure is the binding affinity also referred to as the docking score. The stability of the association between the ligand and the receptor is impacted by the binding affinity value. The highest unfavorable binding affinity was selected. Negative values represent the minimum energy required for the receptor to connect with the ligand, allowing for spontaneous interaction.[20] The binding affinity value decreases as the interaction between the ligand and receptor becomes more stable. From the results of the molecular docking, each tested ligand has a unique binding affinity value for each of the receptor.

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The binding affinities of cinnamaldehyde when docked with the *luxS*, *mrkA* and t*reC* gene receptors were -5.6 kcal/mol, -5.0 kcal/mol, and -6.0 kcal/mol, respectively. These low values indicate that cinnamaldehyde has a high potential to interact with the gene receptors *luxS*, *mrkA* and t*reC*. On the other hand, the binding affinities of ciprofloxacin when docked with the *luxS*, *mrkA* and *treC* gene receptors were -6.0 kcal/mol, -7.5 kcal/mol, and -7.0 kcal/mol, respectively. This shows that cinnamaldehyde exhibits binding affinities to the *luxS*, *mrkA* and *treC* genes similar to the binding affinities of the standard compound ciprofloxacin. Cinnamaldehyde is predicted to have the ability to interact with the target receptor with the same binding affinities as that of the standard drug, Ciprofloxacin (Table 1).

The Root-Mean-Square Deviation (RMSD) value of the docked compound is determined from the docking results as the lowest bond energy of the compound when it attaches to the target protein. Since the test substance and target protein can be docked in the same grid box area, the approach is deemed to be valid if the RMSD value achieved is ≤ 2Å.[21] In this study, the RMSD values for *luxS*, *mrkA*, and *treC* as gene receptors were 1.461 Å, 1.210 Å, and 1.426 Å, respectively. The variation from the error that happens during docking is described by the RMSD value. Furthermore, the molecular docking of ciprofloxacin as the standard drug compound showed that the RMSD value was ≤ 2Å for each of the interaction with the *luxS*, *mrkA*, and *treC* genes. The values were 1.805 Å, 1.502 Å, and 1.942 Å for *luxS*, *mrkA*, and *treC*, respectively. Smaller RMSD value indicates little differences in the structure conformations during the docking.

The bond affinity is a measure of how well the test ligand interacts with the receptor. The ligand-receptor bond will be more stable if the free bond energy is lower.[22] The interaction of amino acid residues is one of the parameters to be considered in the molecular docking method (Table 2).

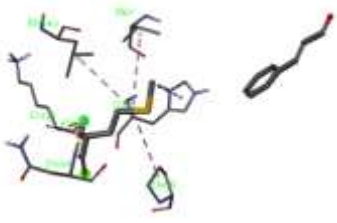
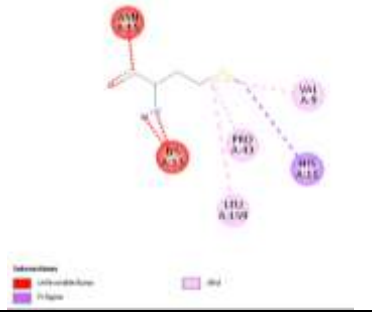
To determine the interactions between the ligand and the receptor, amino acid residue interactions are studied. Hydrogen bonds, hydrophobic interactions, and electrostatic interactions are the interactions that take place. The interaction types involving hydrogen bonds, hydrophobic interactions (alkyl, aryl), unfavorable bumps, and *van der Waals* forces were studied by two-dimensional complex structures. The binding energy of the ligand-protein complex is influenced by the types and amount of interactions.[23] The hydrogen bonds between the reference or natural ligands and the test ligands with the same amino acid residues display a comparable kind of interaction, in this case describing the similarity of their activity.[27] Hydrophobic and electrostatic interactions can increase conformational stability.[28]

Besides having anti-biofilm and antibacterial activity, cinnamaldehyde has been shown to have anticancer effect.[29] Cinnamon bioactive compounds have shown significant *Histone deacetylases 8 (HDAC8)* inhibitory activity suggesting their potential therapeutic applications in cancer therapy.[30] Inhibitors of biofilm formation with strong antihelminthic effects include 4-bromo and 4-chloro cinnamaldehydes, methyl and trans-4-methyl cinnamaldehydes.[29] Cinnamaldehyde showed fungicidal activity through a mechanism of action likely related to ergosterol complexation.[31] Major components of essential oils of cinnamon have been identified by molecular docking study as potential inhibitors of hemolysin, phospholipase, and biofilm formation in *Candida* spp.[32] This suggests many potential benefits of cinnamaldehyde as an antibiofilm agent against several microorganisms.

Table 1: Result of molecular docking between ligands (cinnamaldehyde and ciprofloxacin) and bacterial proteins

Ligand	Genom	Protein	Docking Score	*RMSD* (<2.00 Å)	Conformation of Amino Acid
Cinnamaldehyde	*K. pneumoniae*	*luxS*	-5.6	1.461	2
		mrkA	-5.0	1.210	2
		treC	-6.5	1.426	2
Ciprofloxacin	*K. pneumoniae*	*luxS*	-6.0	1.805	6
		mrkH	-7.5	1.502	2
		treC	-7.0	1.942	7

Table 2: 2D and 3D visualization of molecular docking between ligands (cinnamaldehyde and ciprofloxacin) and receptor (bacterial proteins)

Protein	Conformation	3D Visualization	2D Visualization
Cinnamaldehyde			
luxS	Conformation 2		

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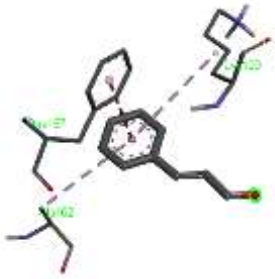
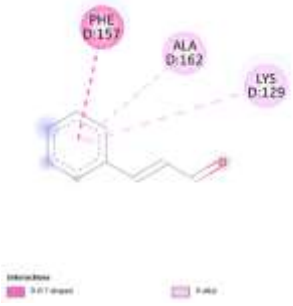
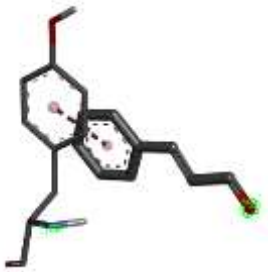
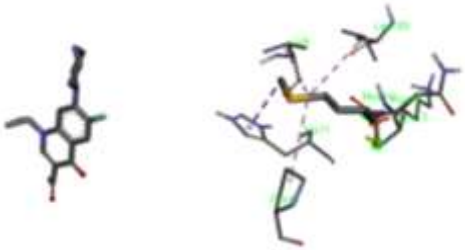
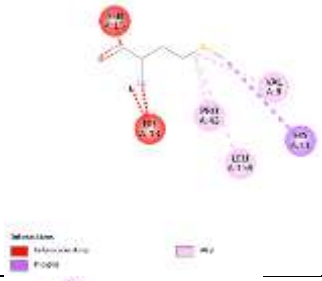
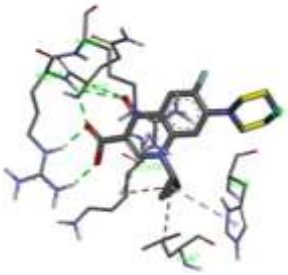
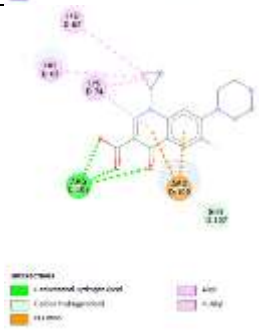
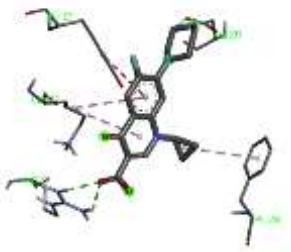
mrkA	Conformation 2		
treC	Conformation 2		
Ciprofloxacin			
luxS	Conformation 6		
mrkA	Conformation 2		
treC	Conformation 7		

On the basis of the binding affinity and the RMSD value of ≤ 2 Å for *luxS, mrkA,* and *treC* genes, cinnamaldehyde could be said to have potential anti-biofilm effect against *K. pneumoniae* and it is comparable to the standard drug ciprofloxacin.

The ADME and toxicity profile of cinnamaldehyde were predicted using the pkCSM strategy. According to the World Drugs Index database, molecules or compounds are more likely to have poor absorption or permeability if their molecular weight exceeds 500, their calculated octanol/water partition coefficient (log P) exceeds +5, they have more than five H-bond donors (expressed as the sum of O-H and N-H groups), and they have more than ten H-bond acceptors (expressed as the sum of N and O atoms). This is known as the Lipinski Rules of Five because all values are multiples of five.[33] In terms of absorption parameters, cinnamaldehyde has a molecular weight of less than 500 Da (132.162 Da), therefore it is predicted to have the ability to be easily absorbed by the body (Table 3).

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Table 3: Molecular properties of *Cinnamaldehyde* from pkCSM method

Molecule Properties (*Cinnamaldehyde*)	
Molecular Weight	132.162 (normal: <500)
LogP	1.8987
#Rotatable Bonds	2
#Acceptors	1
#Donors	0
Surface Area	59.998

Cinnamaldehyde has a high ability to be absorb with a predicted intestinal absorption rate of >80% (95.015%) (Table 4). The distribution parameter as measured by the volume of distribution (Vd) for cinnamaldehyde predicted that this compound can be distributed evenly in the body achieving similar concentration as in the plasma (Table 4). In calculating Vd in humans, the total dose of the drug is divided by the plasma concentration in a steady state. This condition occurs when all drug concentrations in the body are constant and the system is continuously infused with drug. The plasma protein binding of drugs play significant role in the pharmacological effect of the drug. The fraction of drug not bound to plasma protein is that which is available for diffusion through cell membrane and bring about pharmacological effect. The higher the fraction of unbound drug, the less protein-bound the drug is and *vice versa*.[34] A compound can be interpreted as having a good distribution profile, if the log Vd value is more than -0.15, and in this case, cinnamaldehyde is predicted to have a good distribution profile with log Vd value of 0.266 L/kg.

For drug metabolism, the most common reaction is oxidation and *cytochrome P450* (CYP450) is the enzyme responsible for the oxidative metabolism of drugs thereby facilitating the excretion of drug metabolites and other unwanted organic compounds. Results obtained from this study showed that the enzymes belonging to the *cytochrome P450* subtype, such as the *CYP2D6* were not inhibited by cinnamaldehyde. Therefore, it could be interpreted that cinnamaldehyde would be metabolized by *Cytochrome P450*.

The excretion ability of a compound is normally predicted from the ClTot value, which comprises the total cleaning ability during the excretion process and the constant of *Organic Cation Transporter 2 (OCT 2)/*kidney organic cation transport substrate. The higher the ClTot value, the better the excretion ability, and in this study, cinnamaldehyde did not affect the *OCT2* substrate (Table 4). In terms of the toxicity profile, cinnamaldehyde was predicted to have low toxicity such as not being hepatotoxic, and do not inhibit *hERG I* and *II* when administered *in vivo*, even though it is sensitive to the skin (Table 4). The pkCSM approach was used to predict the ADME and toxicity properties of cinnamaldehyde, the results shows that cinnamaldehyde exhibits good absorption, good distribution, would be metabolized by *Cytochrome P450,* and has low toxicity when administered *in vivo*. These findings demonstrates that cinnamaldehyde satisfies the criteria for a drug substance and could be considered as an antibiofilm agent against *K. pneumoniae*.

Table 4: Prediction of pharmacokinetics characteristic (Absorption, Distribution, Metabolism, Excretion, Toxicity) of *Cinnamaldehyde*

Cinnamaldehyde	
Absorption	
Water Solubility	-2.175 log mol/L
$CaCO_2$ Permeability	1.634 log Papp in 10^{-6} cm/s
Intestinal Absorption (human)	95.015% (normal: >80%)
Skin Permeability	-2.355 log Kp
P-glycoprotein Substrate	No
P-glycoprotein I Inhibitor	No
P-glycoprotein II Inhibitor	No
Distribution	
VDss (human)	0.266 log L/kg
Fraction Unbound (human)	0.3 Fu
BBB Permeability	0.436 log BB
CNS Permeability	-1.582 log PS
Metabolism	
CYP2D6 substrate	No
CYP3A4 substrate	No
CYP1A2 inhibitor	Yes
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Excretion	
Total Clearance	0.203 log ml/min/kg
Renal OCT2 Substrate	No
Toxicity	

AMES Toxicity	No
Max. tolerated dose (human)	0.876 log mg/kg/day
hERG I Inhibitor	No
hERG II Inhibitor	No
Oral Rat Acute Toxicity (LD50)	1.88 mol/kg
Oral Rat Acute Toxicity (LOAEL)	1.944 mg/kg_bw/day
Hepatoxicity	No
Skin Sensitisation	Yes
T.Pyriformis Toxicity	0.665 log µg/L
Minnow Toxicity	1.655 log mM

Conclusion

Cinnamaldehyde, a major constituent of *Cinnamommum burmanii bark oil* has a high docking score comparable to that of ciprofloxacin. Molecular docking of cinnamaldehyde with genes of biofilm-forming *Klebsiella pneumoniae* produced data that predicted substantial binding to *luxS*, *mrkA* and *treC* genes with docking scores of -5.6, -5.0, and -6.5 kcal/mol, respectively. The docking score of ciprofloxacin against the *Klebsiella pneumoniae* genes *luxS*, *mrkA* and *treC* were -6.0, -7.5, -7.0 kcal/mol, respectively. These values indicate that the activity of cinnamaldehyde against *luxS*, *mrkA*, and *treC* genes of *K. pneumoniae* is similar to that of the standard drug ciprofloxacin. Pharmacokinetics prediction using the pkCSM approach has also shown cinnamaldehyde as an active compound with good pharmacokinetics profile. Therefore, cinnamaldehyde has the potential to be developed as an antibacterial and antibiofilm agent against *Klebsiella pneumoniae*.

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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