RESEARCH PAPER

GC-MS Steered Insilico Discovery of Anti-Uropathogenic Phytocompounds from the Ethanolic Extract of Aegle marmelos targeting Uropathogenic Escherichia coli

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ABSTRACT

Urinary tract infections (UTIs) caused by uropathogenic Escherichia coli (UPEC) remain a significant clinical problem, largely due to bacterial adhesion mediated by fimbrial proteins such as Type I fimbriae (FimH) and P fimbriae (PapGII). In the present study, bioactive compounds from the ethanolic leaf extract of Aegle marmelos were identified using GC–MS analysis, and their anti-adhesive potential was evaluated through in silico molecular docking and pharmacokinetic profiling. Among eight compounds detected, four ligands—naphthalene, phytol, N-hexadecanoic acid, and oleic acid—were selected based on reported biological activities and docked against FimH (PDB ID: 6GTW) and PapGII (PDB ID: 3MEO). Docking results revealed that N-hexadecanoic acid exhibited binding affinity toward both FimH and PapGII, whereas phytol, oleic acid, and naphthalene showed strong interactions primarily with PapGII. Interaction analysis indicated that hydrogen bonding, hydrophobic interactions, and π–alkyl interactions contributed to the stability of the protein–ligand complexes. ADMET predictions suggested favorable pharmacokinetic properties for oleic acid, N-hexadecanoic acid, and phytol, while naphthalene exhibited potential toxicity concerns. Overall, the findings suggest that bioactive compounds from Aegle marmelos, particularly N-hexadecanoic acid, may serve as promising lead molecules for the development of anti-adhesive therapeutics targeting UPEC-mediated urinary tract infections. Further experimental validation is warranted to confirm their biological efficacy.

Keywords: Urinary tract infections, UPEC, Aegle marmelos, GC-MS, Molecular docking, FimH, PapGII, Anti-adhesive therapy, Phytocompounds.

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1.Introduction

Urinary Tract Infections (UTIs) are the common type of infection that affect the urinary tract system, which include kidney, bladder, ureters and urethra (1)(2).UTIs are one of the most common bacterial infections, affecting both male and female gender of all ages (3). The prevalence rate of UTIs depending on the population were studied, even though women tend to be more susceptible to UTIs as compared to men. It is estimated that about 40 to 60% of women will experience at least one UTI in their lifetime (4) with an incidence rate 50-fold higher among the 20–50 years age group (5), and it increases up to 60% in their postmenopausal time period.

Several epidemiological, serological and bacteriological studies revealed that Escherichia coli is the most frequently associated microorganism with UTIs (6). E.coli is a normal flora found in the healthy humans and animals occurring in to the gastrointestinal tract by participating in digestion and synthesis of certain vitamins (7).Even though a beneficial organism, it can also cause serious infections if it gets in to urinary tract.

Epidemiologically and phylogenetically UPEC strains are different from communsal like E.coli residing in the gastrointestinal tract (8). For a severe infection within the host, UPEC possess a vast array of virulence and fitness markers (9) that enable the bacteria to attach, invade, colonize and establish infection in the urinary tract. These virulence factors include surface structural components like lipopolysaccharide (LPS) and capsules, adhesion factors like pilli, fimbriae, curli and a fimbrial adhesins, outer membrane proteins, mechanisms for acquisition of nutrients (e.g. siderophores), toxins (e.g., cytotoxic necrotizing factor-1, hemolysin, serine autotransporters) (10). The attachment of Surface virulence factors on the

uroepithelial cells of host is a very crucial step in UPEC pathogenesis. This UPEC attachment on to the host cells is a complicated process which may be enabled with the help of some proteinaceous molecules called adhesions . The most important adhesion factors such as Type1 fimbriae, P fimbriae, F1C fimbriae, S fimbriae, and fimbrial adhesions(afa) and curli are highly associated with UPEC.

Currently, the empirical treatment of UTIs is an issue of concern due to the increasing rates of antibiotic resistance and the development of multidrug resistance. The antimicrobial susceptibility pattern of the urinary pathogens has been changing over the years and is influenced by such factors as the changing patient population, especially immune compramised patients (diabetic) patients and the extensive use and misuse of antimicrobial agents, which contribute to alterations in the microbial profile of urinary tract isolates. (11).

Common antibiotic resistance mechanisms among UPEC strains include inactivation of hydrolytic enzymes by β-lactamases; non-hydrolytic enzyme inactivation by amino glycoside acetyl transferase enzymes; permeability alteration through active efflux pumps; inactivation of the target site and resistance acquired by the horizontal transfer of genetic elements (12).

The multidrug resistance of uropathogenic bacteria in diabetic patients shows that hyperglycemic condition modify the bacterial epidemiology of urinary tract infections and leads to greater resistance to the antibiotics used to treat them (13). Multidrug resistance exhibited by the ESBL producing UPEC further urges the need for prompt diagnosis and effective treatment of UTI in order to avoid haematogenous spread of these pathogens leading to bacteremia associated morbidity and mortality among the diabetic patients. Currently, the clinically available treatment is not effective against the antibiotic resistance developed by some bacterial species, leads to the development of an alternative therapeutic system including medicinal plants.

Aegle marmelos is an important medicinal plant of India. Leaves, fruits, stem and roots of A. marmelos have been used in ethno medicine to exploit its' medicinal properties including astringent, antidiarrheal antidysenteric, demulcent, antipyretic and anti- inflammatory activities (14),(15) mentioned that A. *marmelos* leaves have potential anti-cancer and antioxidant effects and also, it is a valuable natural therapeutic agent for managing oral cancer and infections.

Phytochemicals present in the ethanolic extract of *Aegle marmelos* may be responsible for antioxidant and antibacterial activity. The crude extracts of A. marmelos revealed the presence of various biologically active phytochemicals with highest quantity of alkaloids, flavonoids, and phenols (16).Increasing antibiotic resistance and development of multidrug resistance in UPEC leads to search of an alternative for antimicrobial drugs. Leaf part of Aegle marmelos plant has been used for the treatment of various diseases and infections. In addition, they are known to possess very good antibacterial activity. Hence the current study was designed to evaluate the insilico analysis of anti-UPEC activity of active compounds present in the ethanolic leaf extract of Aegle marmelos.

2.Materials and Methods:

The phytocomponents in ***Aegle marmelos*** plant extract were identified using mass spectral patterns, retention time, peak area and peak height to those in MS library (National Institute of standards and Technology (NIST) library's spectrum database of genuine compounds, USA). The components were identified by comparing them to mass spectral fragmentation patterns in the MS library.

a.Gas Chromatography -Mass Spectrometry:

The GC-MS analysis was done on QP-2010, Shimadzu. The samples were loaded into a thin capillary column, which was 60 meters long and had a special coating inside. The carrier gas used was helium, which flowed at a rate of 1 milliliter, per minute.The temperature of the column was first set at 80^{o}C then it was slowly increased by 10^{0}C every minute until it reached 280^{o}Ce, where it was kept for 11 minutes. The whole process took 36 minutes. The temperature of the part where the sample was injected was 280^{o}C and the temperature of the part that connected the column to the mass spectroscopy was 290^{o} C Celsius. We injected a small amount of the sample just 1 microliter. The mass spectra were recorded using a method called Electron Ionization with an energy of 70 electron volts.To identify the compounds we compared the time it took for them to come out of the column with the times of known compounds. We also looked at how they broke apart and compared that to the mass spectra in a special library called NIST, which is stored in the computer software version 1.10 beta(17).

b.PASS prediction:

In this study, we utilised pass prediction investigation to identify the possible pharmacological effectiveness of the phytocompounds present in *Aegle marmelos* using pass online website. This web based program has the

capability to predict the bioactivity spectrum of a molecule by predicting the numerous probable pharmacological effects based on the molecule's structure. The bioactivity prediction for compounds was conducted using the Pass online web server, accessible at https://www.way2drug.com/passonline/index.php. This platform enables the prediction of the bioactivity spectrum at different threshold values, specifically denoted as Pa (Probable activity) and Pi (Probable inactivity). (18)

c.Preparation of Ligand:

The 3D structure of selected bioactive compounds Phytol, n-hexadecanoic acid, oleic acid and naphthalene were retrieved from Pubchem database at NCBI (http://pubchem.ncbi.nlm.nih.gov/)(19)

d.Protein Preparation:

The 3D structure of major adhesion proteins in UPEC such as *fimH* and *papGII* were selected as the target proteins and retrieved from Protein Data Bank(PDB),to find the best-fit orientation with ligand(20). The crystal structure were rebuilt and both water and small molecules were removed by using discovery studio software. To perform energy minimization and geometry optimization, polar hydrogens were added, and non-polar hydrogens were merged into molecules by AutoDock tools.

***e.Insilico* analysis of Pharmacokinetic analysis:**

The drug-likeness of the compounds found in *Aegle marmelos* was calculated using Swiss ADME (http://www.swissadme.ch/). The molecular structures of the compounds were converted into SMILES format prior to analysis. Only the ligands that compiled with Lipinski's five rule variations(calculated Log P should be less than five, polar surface area, the number of hydrogen bond donars should be less than ten and the molecular weight should be less than 500 with no more than one violation were selected for molecular docking experiments. (21)

f.Molecular docking:

Molecular docking was performed using Auto Dock Vina version 1.5.6. The retrieved proteins and ligands were prepared using Auto Dock Tools, and a grid box was generated to encompass all potential binding sites of the target protein. The grid parameters were defined and saved as a configuration file. Docking simulations were executed using command-line instructions in Auto Dock Vina. Among the various conformations generated for each ligand, the binding pose with the lowest binding energy (kcal/mol) was selected as the optimal ligand protein complex.

Hydrogen bonds and hydrophobic interactions play a crucial role in stabilizing protein–ligand complexes. To evaluate the stability of the best-docked conformations, hydrogen bonding interactions between the protein and ligands were analysed to identify key amino acid residues involved in binding. In addition to hydrogen bonds, other non-bonded interactions such as hydrophobic interactions were also examined(22). Furthermore, the final docking results, including protein–ligand interactions (bonded and non-bonded), pharmacokinetic properties (ADME), and pharmacodynamic characteristics, were systematically analysed.

3.Results and Discussion:

GCMS analysis revealed the presence of many bioactive compounds with their known biological activity.Among the 8 compounds identified from the GCMS analysis crude extract of the leaves of *Aegle marmelos* by GC-MS analysis(Figure 1)(Table 1) , 4 bioactive compounds were selected on the basis of their biological activities as documented in various literature(23). They are Naphthalene, phytol, N- hexadecanoic acid and oleic acid were listed in the Table 2. These compounds were further investigated for *insilico* molecular docking studies against Type I fimbrial protein (fimH) and P fimbriae coding protein (papGII)like curcumin against these bacterial Proteins.(24).

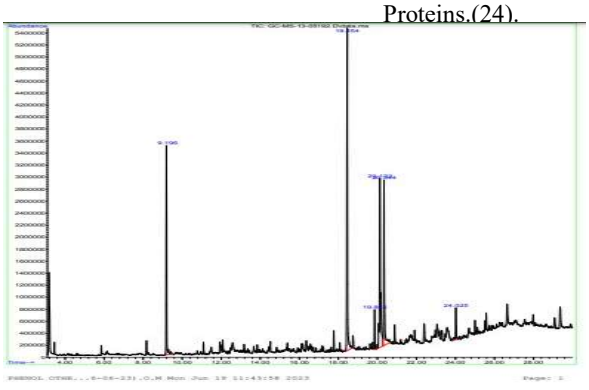
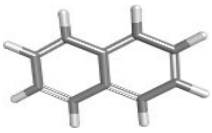
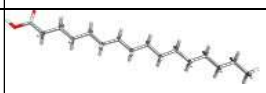
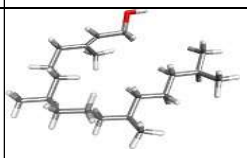
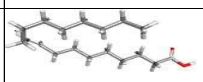
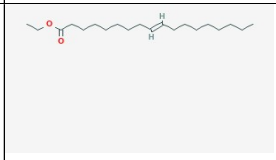
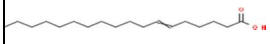


Figure 1: GCMS Chromatogram of *Aegle marmelos.*

Table 1: GC- MS analysis of ethanolic leaves extract of *Aegle marmelos*

S. No.	Compound	RT	Molecular formula	Molecular weight	Structure
1	Naphthalene	9.14	C10H8	128.1705 g/mol	
2	n-hexadecanoic acid	18.45	C16H32O2	256.43 g/mol	
3	phytol	19.86	C20H40O	296.53 g/mol	
4	Oleic aid	20.13	C18H34O2	282.46 g/mol	
5	9-octadecenoic acid	20.13	C18H36O2	282.5 g/mol	
6	6-octadecenoic acid	20.13	C18H34O2	282.4614 g/mol	

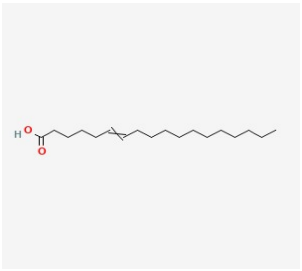
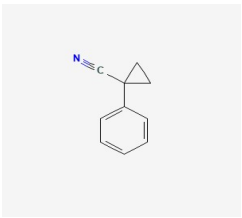
7	Octadecanoic acid	20.34	C18H36O2	284.48 g/mol	
8	1- phenyl-1 cyclopropanecarbon i trile	24.025	$C_{10}H_9N$	143.18 g/mol	

Table 3 : Selected ligands and their Pubchem ID

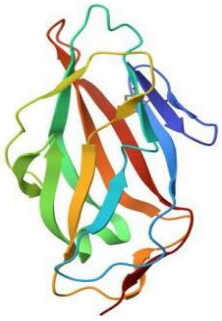
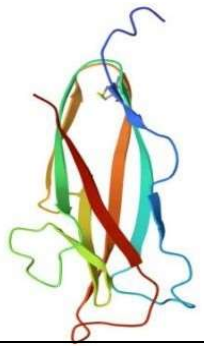
S No	Ligands	Pubchem ID
1	Naphthalene	931
2	Phytol	5280435
3	N-hexadecanoic acid	985
4	Oleic acid	445639

N-hexadecanoic acid and naphthalene activity may be due to the disruption of the integrity of microbial cell membranes and inhibition of microbial growth(25). Oleic acid induce antimicrobial activity by the disruption of cell membranes of microorganisms leading to their death or inhibition of growth(26). Apart from these, oleic acid exhibit insulin sensitivity and improve β-cell survival within human body. Phytol exhibit antioxidant, antiallergic, anti-inflammatory and antimicrobial activities(27).

Table 4: The proteins retrieved from the RCSB PDB database and their protein database ID was listed

Table 4:Proteins with pubmed ID		
1	*fimH*	6GTW
2	*papGII*	3MEO

Table 5: The 3D structure of Protein from PDB.

1	***fimH*-6GTW**	
2	***papGII*-3MEO**	

Among the four ligands were screened, only N-hexadecanoic acid showed the binding affinity against the protein *fimH* with a Libdock score of 81.579 (Table 6). Remaining three compounds did not exhibit an interaction with this protein in the study. In this study all the four ligands exhibited highest binding affinity towards the P fimbriae protein *papGII* . Among four ligands phytol showed in highest binding affinity with the the Libdock score of 111.823 followed by oleic acid (108.011), N-hexadecanoic acid (100.147) and naphthalene (57.7229). Compared to four ligands N-hexadecanoic acid showed strong affinity towards both the Proteins.

Docking result of the four ligands (oleic acid, n-hexadecanoic acid, phytol and naphthalene) with the two target protiens (Pdb id:6GTW-*fimH* and Pdb id: 3MEO-*papGII*) were illustrated in Table 6

Table 6:Libdock result of the ligands with target proteins

S.No	Ligands	Proteins	
		fimH	***papGII***
1	Phytol	Nil	111.823
2	Oleic acid	Nil	108.011
3	N-Hexadecanoic acid	81.579	100.147
4	Naphthalene	Nil	57.7229

The 3Dstructure of protein-ligand complexes revealed predictive binding affinity of ligands relative to the molecular orientation of protein molecules. 2D interaction analysis revealed the amino acid structures of the

ligands interaction with target proteins.

The ligand N-hexadecanoic acid being interacted the protein 6GTW with amino acid threonine at 5 and asparagine at 7th position by conventional hydrogen bond, valine at 20th position by alkyl bond and tyrosine at 21 by Pi-Alkyl bonds and glutamine at 41th position by conventional hydrogen bond.

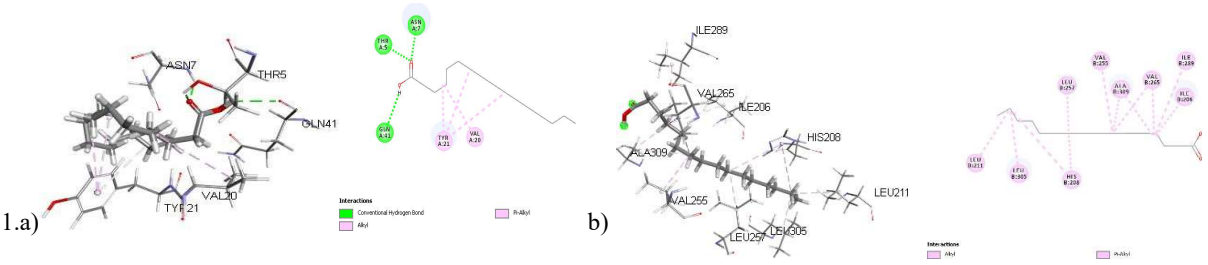


Figure 2: a) Molecular structure represents the 3D interaction of **n-Hexadecanoic acid** with ***fimH(6GTW***) b)Molecular structure represents the 3D interaction of **n-Hexadecanoic acid** with ***papGII*** **(3MEO)**

The ligand *N-hexadecanoic acid* interacted with the protein **6GTW** through conventional hydrogen bonds with threonine at position 5 and asparagine at position 7. An alkyl interaction was observed with valine at position 20, while a π–alkyl interaction occurred with tyrosine at position 21. Additionally, glutamine at position 41 formed a conventional hydrogen bond with the ligand.

The ligand *N-hexadecanoic acid* also interacted with the protein **3MEO** via an alkyl interaction with isoleucine at position 206 and a π–alkyl interaction with histidine at position 208. Further π–alkyl interactions were observed with leucine at positions 211, 257, and 305; valine at positions 255 and 265; isoleucine at position 289; and alanine at position 309.

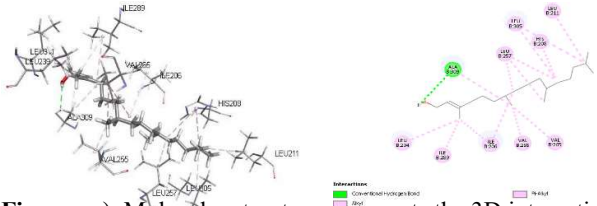


Figure : a) Molecular structure represents the 3D interaction of **Phytol** with ***papGII*** **(3MEO)**

The ligand phytol interacted with the protein 3MEO through alkyl interactions with leucine at position 204, isoleucine at position 206, leucine at positions 211, 257, and 305, valine at positions 255 and 265, and isoleucine at position 289. A π–alkyl interaction was observed with histidine at position 208, while a conventional hydrogen bond was formed with alanine at position 309.

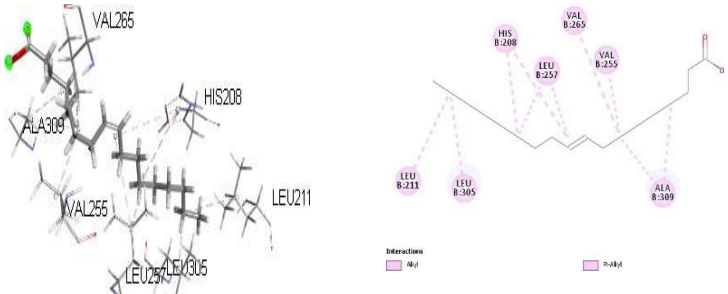


Figure 4: Molecular structure represents the 3D interaction of **Oleic acid** with ***papGII*** **(3MEO)**

The ligand **oleic acid** interacted with the protein **3MEO** through a π–alkyl interaction with histidine at position 208. Additional π–alkyl interactions were observed with leucine at positions 211, 257, and 305; valine at positions 255 and 265; and alanine at position 309.

Figure 5: Molecular structure represents the 3D interaction of **napthalene** with ***papGII*** **(3MEO)**

The ligand **naphthalene** interacted with the protein **3MEO** through π–sigma and amide π-stacking interactions with histidine at position 208. π–alkyl interactions, along with van der Waals forces, were observed with glycine at position 209. Additional π–alkyl interactions were identified with leucine residues at positions 211, 257, and 305 .

Molecular docking facilitate the binding geometry of two interacting molecules with known structures. It acknowledge the preferred orientation of receptor or protein and a ligand bioactive compounds to each other to form a stable complex (28).

FimH protein plays a key role to the binding of Type I fimbriae to the specific mannosilated receptor moieties present on the bladder epithelial cell lines. Likewise papGII is a member of pap gene clusters mediate binding of P fimbriae to kidney epithelial cell receptors. Thus inhibition of fimH and papGII molecules may offer therapeutic benefits in the treatment of UTI especially in diabetic patients.

Nasi *et al*., 2023 (29)studied the *insilico* interaction between chemically synthesized peptide-analogues to the FimH pilin domain and explore the use of antimicrobial peptide against urinary tract infection. Sudhir *et al*., 2018 reported that phytochemicals such as quercetin-3-glucoside, ethyl caffeate, liquiritoside, liquiritin and isoliquiritigenin as potential phytochemicals have antiadhesive activity to FimH protein. Badiger*et al*., 2023 (30) reported that the compounds garcinol and kaempferol from kokum and cranberry respectively could act as the potential FimH inhibitors (anti-adhesins).

In this study hydrogen bonds, hydrophobic interactions, and electrostatic interactions were found among the protein-ligand complexes. Interestingly, these interactions provide stability to the protein–ligand complexes have been reported (Umar *et al*., 2021). High binding scores of the phytocompounds represents good protein–ligand interaction and indicates that they may be biologically active as well as highly efficient compounds.

4.Pharmacokinetic analysis:

The absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies of isolated compounds oleic acid, n-hexadecanoic acid, phytol and naphthalene were predicted from Swiss ADME as illustrated in Table 7. Also the observed values of the ligands were checked with normal values Table 8. The ADMET profiling of oleic acid, *N*-hexadecanoic acid, phytol, and naphthalene revealed that all four compounds possessed a neutral total charge. The predicted aqueous solubility values indicated low solubility for all compounds, with naphthalene exhibiting comparatively higher solubility. Blood–brain barrier (BBB) permeability predictions varied among the ligands, with oleic acid and phytol showing higher BBB levels, while *N*-hexadecanoic acid and naphthalene exhibited lower permeability.

All compounds were predicted to be non-inhibitors of CYP2D6, supported by their applicability domain values and statistically significant p-values. Hepatotoxicity prediction indicated that oleic acid, *N*-hexadecanoic acid, and phytol were non-hepatotoxic, whereas naphthalene was predicted to be hepatotoxic. Absorption levels differed among the ligands, with phytol showing the highest absorption level.

Plasma protein binding (PPB) prediction suggested strong binding affinity for all compounds. Carcinogenicity assessment classified oleic acid and *N*-hexadecanoic acid as non-carcinogenic, while phytol and naphthalene were predicted to be carcinogenic. Ames mutagenicity prediction indicated that oleic acid, *N*-hexadecanoic acid, and phytol were non-mutagenic, whereas naphthalene showed mutagenic potential.

Table 7 :Pharmacokinetics properties of the ligands

	Oleic acid	**N-hexa decanoic acid**	**Phytol**	**Naphthalene**
Pubchem total charge	0	0	0	0
Solubility	-4.996	-4.68	-5.73	-3.695
Solubility level	2	2	2	3
BBB level	4	0	4	1
ExtCYP2D6	-1.05655	-1.54147	-1.06037	-0.548389
CYP2D6 Prediction	False	False	False	False
CYP2D6 applicability #MD	13.791	12.3553	11.2039	11.7263
CYP2D6applicability#MD p value	4.45E-05	0.00134852	1.50E-02	0.00526988
EXT Hepatotoxic	-20.2521	-23.3726	-21.5212	1.63286
Hepatotoxic Prediction	False	False	False	True
Hepato toxic applicability	7.24294	6.08249	10.849	10.4639
EXT Hepatotoxicity Applicability#MDpvalue	0.988225	0.99997	0.00990612	0.0295639
Absorption level	2	1	3	1
EXTPPB	3.5875	2.95007	9.96433	0.327123
PPB prediction	TRUE	TRUE	TRUE	TRUE
PPB applicability#MD	0.872909	0.964909	0.277519	0.497286
Carcinogenecity	Non-carcinogen	Non-carcinogen	Carcinogen	Carcinogen
Ames prediction	Non-mutagen	Non-mutagen	Non-mutagen	Mutagen

Table 8 :Normal values for Pharmacokinetics study					
Level	**Level Solubility**	**BBB Value**	**BBB description**	**Absorption level**	**Absorption description**
0	Extremely low	Very high	Brian-bloodratio greater than 5:1	ADMET_ Absorption_T 2_2D<6.1261 (inside 95%)	Good absorption
1	No,very low,but possible	High	Brain-Blood ratio between 1:1 and 5:1	6.1261 ≤ ADMET_Absorption_T2_2 D	Moderate absorption
2	Yes,low	Medium	Brian-blood ratio between 0.3:1 and1:1	9.6026 <ADMET_ Absorption_T2_2D (outside 99%)	low absorption
3	Yes, good	Low	Brian-blood ratio less than 0.3:1	ADMET_PSA_2D ≥ 150.0 or ADMET_AlogP98 ≤- 2.0 or ADMET_AlogP98 ≥7.0	Very low absorption
4	Yes, optimal	Undefined	Outside99% Confidence ellipse	-	-
5	No, too soluble	AlogP98	Warning; molecules with one or more unknownAlog98 Typ	-	-
6	Warning; molecules with 1 or more unknown Alog 98 types	-	-	-	-

Thus the current study indicates that the active compounds of the ethanolic leaves extract of *Aegle marmelos* may possess the most adhesive effect against *fimH* and *papGII* the potent virulence factors of urinary infection through a molecular docking technique.

5.CONCLUSION:

This study illustrates that bioactive compounds extracted from the ethanolic leaf extract of *Aegle marmelos* exhibit significant anti-adhesive properties against critical virulence factors of uropathogenic *Escherichia coli* (UPEC), specifically the Type I fimbrial adhesin FimH and the P fimbrial adhesin PapGII. Among the four ligands identified through GC–MS for *insilico* assessment—oleic acid, N-hexadecanoic acid, phytol, and naphthalene. N-hexadecanoic acid demonstrated a remarkable binding affinity for both target proteins, underscoring its dual inhibitory capability. While only N-hexadecanoic acid interacted with FimH, all four ligands exhibited significant binding affinities towards PapGII, with phytol and oleic acid achieving particularly high LibDock scores.

Comprehensive interaction analyses indicated that the stability of the protein–ligand complexes was predominantly influenced by hydrogen bonding, hydrophobic interactions, and π–alkyl interactions with crucial amino acid residues, thereby reinforcing the validity of the docking results. These interactions imply that the chosen phytocompounds may disrupt bacterial adhesion mechanisms that are vital for colonization and persistence within the urinary tract.

Moreover, ADMET profiling revealed that oleic acid, N-hexadecanoic acid, and phytol have favorable pharmacokinetic and toxicity profiles, while naphthalene raised concerns regarding potential hepatotoxicity and mutagenicity, which may restrict its therapeutic applicability. In summary, the results indicate that the bioactive components of *Aegle marmelos*, particularly N-hexadecanoic acid, phytol, and oleic acid, hold promise as lead compounds for the creation of anti-adhesive agents aimed at combating UPEC-related urinary tract infections. Nonetheless, these in silico findings necessitate further validation through *in vitro* and *in vivo* investigations to establish their biological efficacy and safety.

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