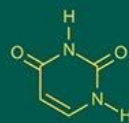


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Unravelling the antibacterial potential of hydroethanolic bark extract from *Melia azedarach*: *In vitro* and *in silico* investigation

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Abstract

In present study in-vitro and in silico analysis of hydroethanolic bark extract of *Melia azedarach* was carried out for development of new remediation against *E. coli* and *S. aureus*. The MIC of the bark extract against *E. coli* and *S. aureus* was found to be 8 mg/ml and 1 mg/ml, respectively. Furthermore, binding interactions were explored through molecular docking which showed good binding energy of Compounds viz. CID 535221 and 500095 analysed through GC-MS against target protein molecules of microbes *E. coli* (PDB ID 1IEM, 7NAE, 1HNJ) and *S. aureus* (PDB ID 2ZCO, 4CJN, 1JIJ). The current study suggests that bark extract from *M. azedarach* holds promising potential antimicrobial activity and bioactive compounds can be utilized for the development of an alternative agent to reduce antibiotic resistance.

Keywords: *Melia azedarach*, CID 535221, CID 500095, Antibiotic Resistance, *E. coli*, *S. aureus*

Introduction

Antimicrobial resistance is one of the predominant global concerns, leading to the emergence of innumerable resistant bacterial species (Prestinaci *et al.*, 2015) [23]. The main factor leading to the evolution of resistance bacteria is the indiscriminate use of antibiotics. According to Ramirez *et al.* (2020) [26], the careless use of antimicrobial medications causes adverse consequences such as allergic responses, a decrease in the gut microbiota, etc. Among the various bacterial species, *Staphylococcus aureus* and *Escherichia coli* are the most common pathogens that have been reported with high incidences of drug resistance against antibiotics. Previous studies regarding multi-drug resistance, *S. aureus*, and *E. coli* showed higher levels of resistance in ruminants as well as in non-ruminants (Haulisah *et al.*, 2021) [7]. Moreover, *S. aureus* and *E. coli* are also responsible for causing mastitis leading to tremendous economic loss to the farmers. Consequently, treating infectious infections in humans and animals has grown more difficult or even unfeasible, directly or indirectly responsible for huge morbidity and mortality. Despite the steadfast antimicrobial therapy's failure, no new class of antibiotics has been developed in the last two decades. Several cutting-edge substitute tactics for dealing with these (multi-drug-resistant) pathogenic microbes have been discovered (Murugaiyan *et al.*, 2022) [19].

A holistic approach along with a multidimensional, multisectoral, multidisciplinary 'one-health' approach has been advocated globally to combat (antimicrobial resistance) AMR across human-animal-environment interface (White and Hughes 2019) [37]. Addressing the growing threat of antimicrobial resistance (AMR) involves either minimizing unnecessary antibiotic usage or creating novel and potent antibiotics (McLinden *et al.*, 2014; Grant *et al.*, 2026) [16, 6]. Preventing infections is the most promising and long-term sustainable solution; for this purpose, several initiatives have been taken by the Government of India in the last decade to tackle the problem of AMR which helped to raise awareness about sanitation and hygiene in the community and hospitals, but the impact of these initiatives on reduction in antimicrobial use have not been succeeded (Rohr *et al.*, 2019; Jayalakshmi *et al.*, 2017) [28, 9]. Developing new antibiotics is a challenging task and reducing the use of antibiotics is again very challenging that will compromise the health of society and the chance to grow opportunistic microbes.

In this category of alternatives to antibiotics and sequential reduction in antibiotic use, new hope is generated from herbal medicine. Which has a very long history of treating many infectious and non-infectious diseases, a time when people have not heard the name of antibiotics. Hence, herbs or plants are hoped that provide a possible replacement for the antibiotics (Xie *et al.*, 218) [38].

Under the category of traditional medicinal plants, *Melia azedarach* well known and popularly named as Chinaberry tree or Mahanimba in India. This plant is known for its multiple pharmacological activities. It possesses various properties anthelmintic, antilithic, diuretic, deobstruent, antimalarial, and antifungal (Maciel *et al.*, 2006; Khan *et al.*, 2011) [14, 10]. Potential analgesic and antipyretic activities of *M. azedarach* L. leaf and *M.* root powder have been evaluated. Mild-to-moderate peripheral and central analgesic activity with antipyretic effect was reported (Vekariya *et al.*, 2016) [36]. Seed extracts of *M. azedarach* were found to be effective in controlling gram-positive and gram-negative human pathogenic infection agents under hospital conditions (Khan *et al.*, 2011) [10], while *M. azedarach* of flowers found effective against *S. aureus* and *E. coli* (Muhammad *et al.*, 2015) [18]. *M. azedarach* L. leaves contain huge quantities of limonoids of the nimbolinin type, which contribute to therapeutic effects, such as antimicrobial, antioxidant, and anticancer potential (Srivastava *et al.*, 2020) [35]. Further, ZnO NPs biosynthesized using *Melia azedarach* leaf extra have substantial efficient biological activities regarding antioxidant and antibacterial potential have been reported (Dhandapani *et al.*, 2020) [5]. Moreover, the broad-spectrum medicinal value of *A. indica* L. and *M. azedarach* L. leaf extracts have also been reported and recommended for the control of COVID-19 and other human microbes (Hemdan *et al.*, 2023) [8].

In the search for an alternative to the antimicrobials and looking towards the potential properties of *Melia azedarach*, it was decided to explore the more antimicrobial potential of the bark-ethanolic extract against the mastitis-causing pathogen. *In silico* analytical tools will provide information about different molecular interactions through possible ligand-receptor interaction studies (Rizvi *et al.*, 2013; Nabati *et al.*, 2020) [27, 20]. Hence, the advanced *in-silico* tool was used to explore the affinity of bioactive constituents of *Melia azedarach* against different targets of selected bacteria which establish the possible mechanism of action as an antibacterial agent.

Materials and Methods

Collection and processing of plant Materials

In the month of October 2022, the bark of *Melia azedarach* was collected from the Herbal Garden, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India.

Thereafter, the bark chapped into 5-6 cm sizes, washed with plenty of water, and allowed to dry for several days in an Oven Dryer at 45 °C. After completion of drying, the powder was prepared in a grinding mill.

Extraction of plant material

Powder of bark from *Melia azedarach* was soaked in 60% alcohol (aqua-ethanol) and kept for 72 hours in a rotatory shaker. After that, soaked content was filtered with a four-fold of muslin cloth followed by filtration using Whatman

No.1 paper. The aqua-ethanolic bark extract (AEBE) was dried in oven dryer at 45±1 °C. Prepared AEBE was stored in air tied container until used. The percent recovery was calculated by taking the difference in the weight of the empty petri dish and the weight after drying and multiplying by 100 and expressed as percent extractability.

Antibacterial Assays

Bacterial strain

Bacterial strains of *Staphylococcus aureus* (ATCC 25921) and *Escherichia coli* (clinical isolates) were obtained from the Department of Veterinary Microbiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Working bacterial cultures were prepared by transferring a colony to a fresh medium and then kept overnight at 37°C for further growth. Turbidity of the bacterial solution inside normal saline was matched with 0.5 Mc Farland standard and also measured in a spectrophotometer for the optical density at 625 nm and then was serially diluted to achieve 10⁷ CFU/mL with NSS.

Estimation of MIC

A minimum inhibitory concentration (MIC) of AEBE of *Melia azedarach*'s against *E. coli* and *S. aureus* was measured by broth microdilution method as recommended by CLSI protocol (CLSI, 2014). A 96-well plate was taken to measure MIC of extract against each bacterium (1 plate for each bacterium). Different dilution of plant extract ranging from 128 mg/ml to 0.25 mg/ml was prepared in 1% DMSO. 100 µl of Mueller Hinton broth (MHB), different concentrations of extract and 20 µl of bacteria were added simultaneously in each of the wells. Afterward it was incubated at 37 °C for 24 hrs. MHB + DMSO was used as a vehicle control. After completion of incubation period, 20 µl MTT was added and again incubated for 3 hrs. Change in colour from yellow to purple indicated as presence of bacteria. MIC was measured as the lowest concentration which do not change the colour.

GC-MS analysis

Gas Chromatography and Mass Spectrometer (GC-MS) analysis was carried out to find the phytochemical constituents present in the AEBE. GC-MS analysis data was obtained by proving the sample to Central Instrumentation Laboratory (CIL), Central University Punjab, Bhatinda (Shimadzu QP 2010 Ultra GC MS; Shimadzu Japan).

Molecular Docking Study

Phytochemical constituents identified by GC-MS analysis were selected as ligands for docking experiment. These constituents were first screened through PyRx (Dallakyan & Olson, 2015) [4] against protein of *S. aureus* (PDB ID-2ZCO, 4CJN, 1JIJ) and *E. coli* (PDB ID-1IEM, 7NAE, 1HNJ). Compounds with good binding energy were further selected for individual docking against both the proteins for validation by AutoDock 4.2. (Morris *et al.*, 2009) [17].

Protein preparation.

The crystal structures of receptor molecules of *S. aureus* (2ZCO, 4CJN, IJIJ) and *E. coli* (1IEM 7NAE, 1HNJ) were obtained from PDB database. Proteins were cleaned by removing the heteroatoms. Removal of water molecules and addition of hydrogen atoms were done by AutoDock tools 1.5.7. Afterwards, proteins were converted to PDBQT

format. On the basis of the literature review, the amino acids involved in the active sites were selected and the grid box was placed covering active site. The grid-center for all

receptor molecules is represented in tabulated form (Table 1).

Table 1: Important amino acid involved in binding pocket of target protein

Target Protein	Reference	Code	Amino acid involved in binding pocket
Carotenoid dehydrogenase of <i>S. aureus</i>	(Liu <i>et al.</i> , 2008) ^[13]	2ZCO	ASP 172, ASP 48, ASP 52, ASN 168
<i>S. aureus</i> tyrosyl - tRNA synthetase.	(as per plip-tool)	1IJJ	LEU A 70, GLN A 174, TYR A 36, GLY A 38, ASP A 40, ASP A 80, TYR A 170, GLN A 174, ASP A 177, GLY A 193, GLN A 196, HIS A 50, ASP A 195.
PBP2a (<i>S. aureus</i>)	(as per plip-tool)	4CJN	VAL 256B, VAL 277B, LYS 148B, ARG 151B, ASN 164B, ARG 241B, GLU 150B, HIS 293B
AmpC beta-lactamase of <i>E. coli</i>	(as per plip-tool)	1IEM	SER 64A, GLN 120A, TYR 150A, ASN 152A, ALA 318A, GLY 320A, ASN 343A, THR 316A, THR 319A, GLY 321A, TYR 221A
Beta-Ketoacyl-ACP Synthase III + Malonyl-COA	(as per plip-tool)	1HNJ	THR 37A, ILE 156A, ILE 250A, THR 28A, ARG 151A, GLY 209A, ASN 247A, ARG 249A, TRP 32A, ARG 36 A
<i>Escherichia coli</i> dihydrofolate reductase.	(Krucinska <i>et al.</i> , 2022) ^[12]	7NAE	MET 20, ASN 23, TRP 22, ASP 27, LEU 28, PHE 31, ARG 57, ARG 52, LEU 54, ASN 18, SER 49, ILE 50

Ligand preparation

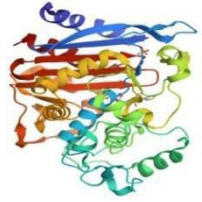
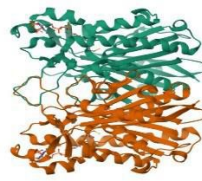
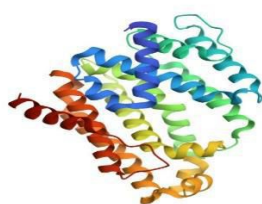
All phytochemical constituents identified by GC-MS were retrieved in SDF format from the PubChem database. Discover studio visualizer was used to prepare a library of retrieved phytochemical constituents. PDB file of all constituents were prepared by entering the smile of all phytochemical constituents in CORINA CLASSIC.

Docking study

On the basis of the literature review, the amino acids involved in the active sites were selected (Table 2) and the

grid box was placed to covering active site. The gridcenter and coordinates for XYZ of all receptor molecules are represented in tabulated form (Table 3). Before performing the docking, grid map file was generated for individual protein through AutoGrid. After that, docking parameter file was generated. Docking was performed through AutoDock 4.2. software. Analysis of docking was carried out on the basis of free binding energy and inhibition constant K_i . Visualization of protein-ligand interaction was carried out by Discovery studio visualizer and Pymol, software.

Table 2: 3D structure of bacterial protein.

Protein	Code	Structure
AmpC beta-lactamase of <i>E. coli</i>	1IEM	
Beta-Ketoacyl-ACP Synthase III + Malonyl-COA	1HNJ	
<i>Escherichia coli</i> dihydrofolate reductase	7NAE	
Carotenoid dehydrogenase synthase	2ZCO	

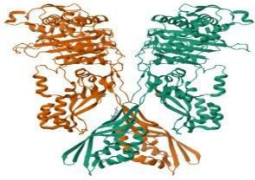
Penicillin binding protein 2a	4CJN	
<i>S. aureus</i> tyrosyl-tRNA synthetase in complex with SB-239629	1JJJ	

Table 3: Gridcenter for all targeted molecules

Protein	Gridcenter			Coordinates for XYZ		
	x	Y	z	x	y	z
1IEM	24.814	6.214	16.67	62	56	60
7NAE	-13.271	24.208	-7.263	58	78	70
1HNJ	27.872	10.174	29.899	66	60	62
2ZC0	50.964	6.187	53.635	80	66	78
4CJN	9.658	-1.662	-70.269	52	52	52
1JJJ	-13.291	11.253	85.522	68	76	68

Drug-likeness studies: This prediction studies was carried out by using PreADMET (<http://preadmet.bmdrc.org/>).

Results and discussion

Effect on percent extractability and MIC

AEBE from *M. azedarach* showed the percent extractability under standard laboratory conditions was 6.45±0.613 (g %). MIC of AEBE from *M. azedarach* against *S. aureus* and *E. coli* were found to be 1mg/ml and 8 mg/ml, respectively. The AEBE showed very good antibacterial activity against both strains of bacteria (Fig. 1 and 2).

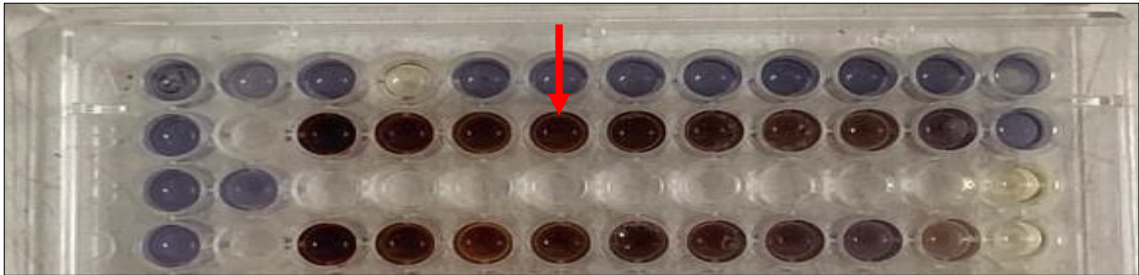


Fig 1: MIC of 8 mg/ml against *E. coli*

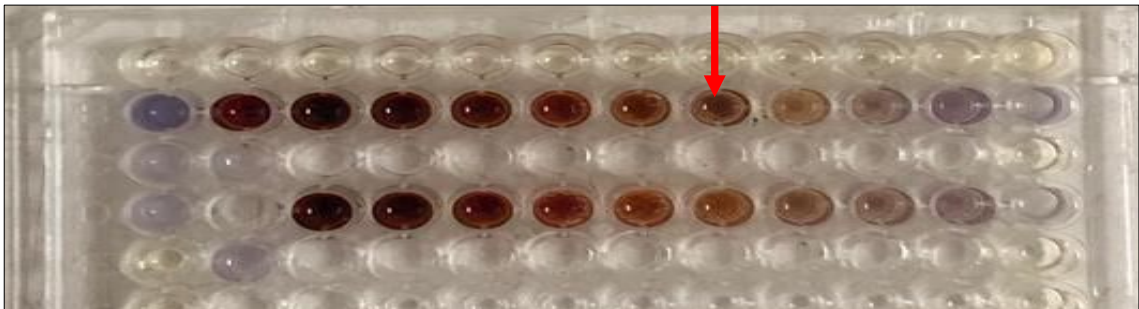


Fig 2: MIC of 1 mg/ml against *S. aureus*

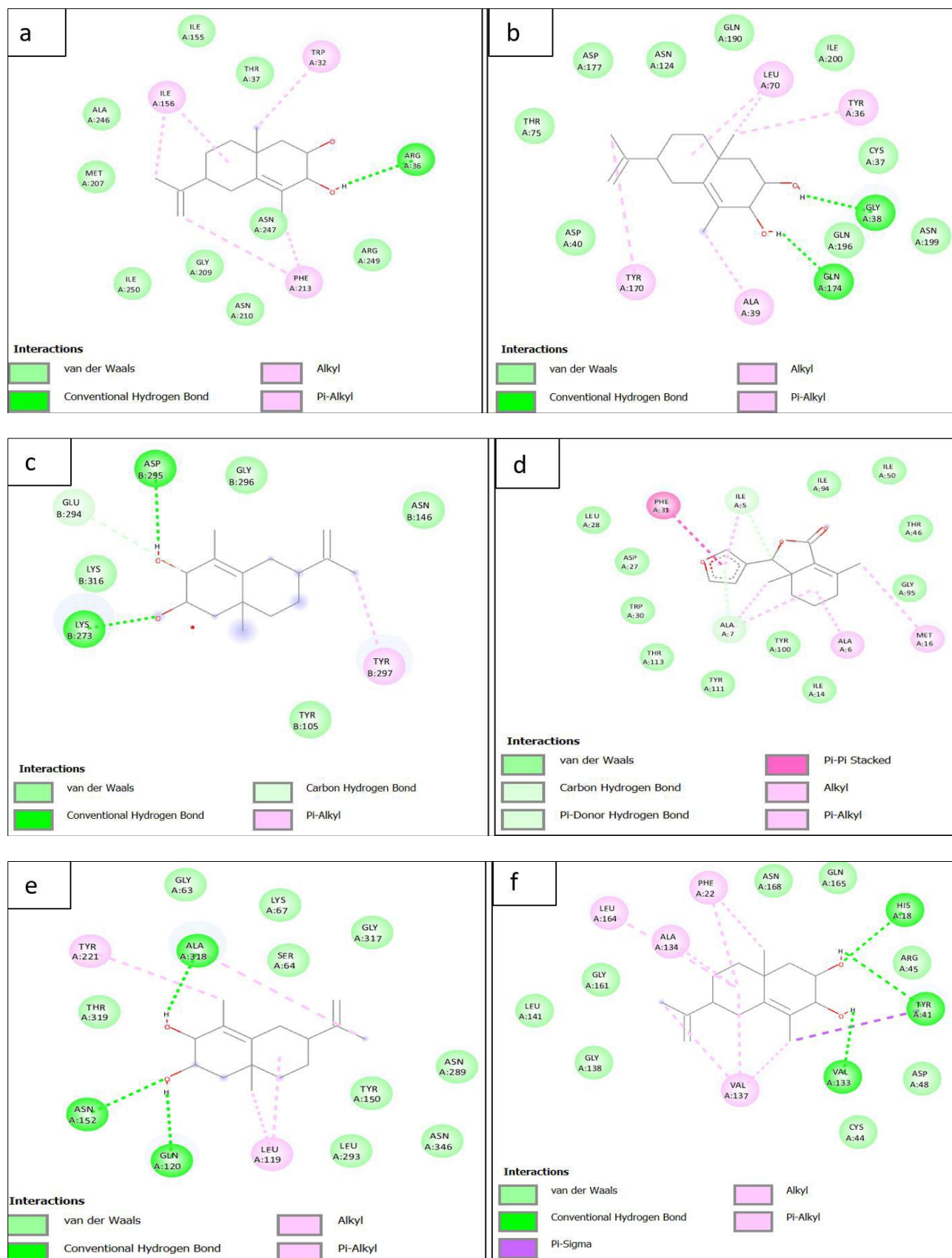


Fig 3: a, b, c, d, e and represents interaction of ligand CID 535221 with 1HNJ, 1JIJ, 4CJN, 7NAE, 1IEM and 2ZCO

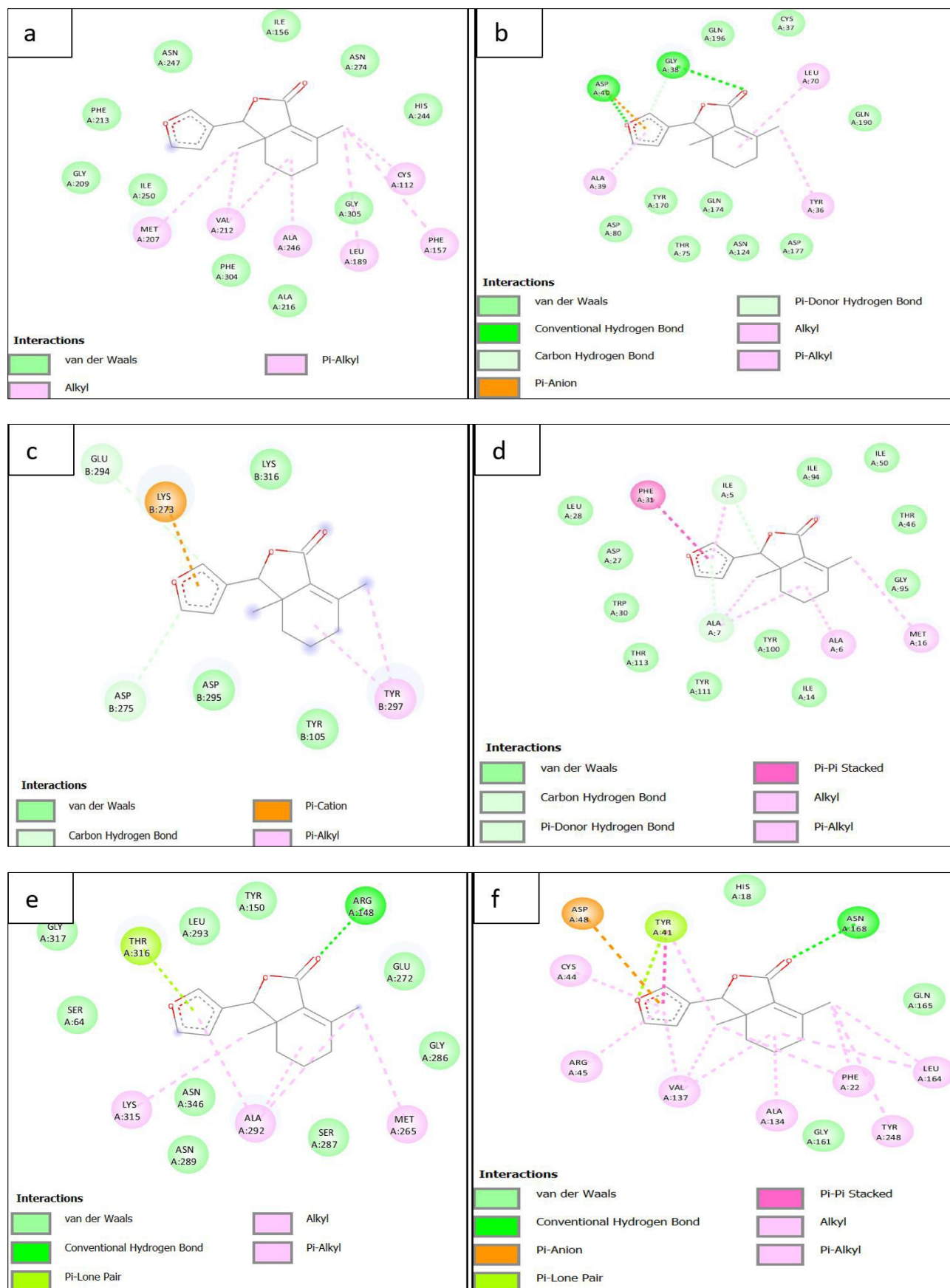


Fig 4: a, b, c, d, e and f represent interaction of ligand CID 500095 with 1HNJ, 1JIJ, 4CJN, 7NAE, 1IEM and 2ZCO

Bioactive constituents of bark extract

GC-MS analysis of the extract showed presence of wide range of bioactive molecules (Table 4a&b) such as glycerin, 6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalene-2,3-diol; lidocaine; Isobenzofuran-1(3H)-one,

3-(3-furyl)-3a,4,5,6-tetrahydro-3a,7-dimethyl; Hexadecanoic acid, methyl ester; Dibutyl phthalate; Z-2-Octadecen-1-ol acetate; 9,12-Octadecadienoic acid, methyl ester; 9-Octadecenoic acid (Z)-, methyl ester; 11-Octadecenoic acid, methyl ester; Methyl stearate; (E)-9-

Octadecenoic acid ethyl ester; trans-Z-. alpha. -Bisabolene epoxide; Glycidyl palmitate; Eicosanoic acid, methyl ester; Z-17-Nonadecen-1-ol acetate; Glycidyl palmitoleate;

Glycerol 1-palmitate; 9-Octadecenoic acid (Z) and 2-hydroxy-1-(hydroxymethyl) ethyl ester.

Table 4a: Constituents identified by GC-MS analysis.

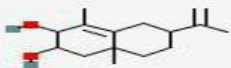
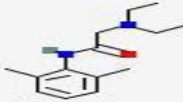
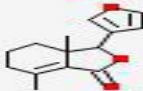
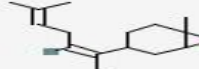
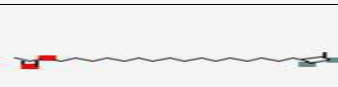
Compound Name	PubChem ID	% Area	
Glycerin	753	4.31	
6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalene-2,3-diol	535221	1.18	
Lidocaine	3676	1.73	
Isobenzofuran-1(3H)-one, 3-(3-furyl)-3a,4,5,6-tetrahydro-3a,7-dimethyl	500095	0.96	
Hexadecanoic acid, methyl ester	8181	8.75	
Dibutyl phthalate	3026	2.01	
Z-2-Octadecen-1-ol acetate	5363098	1.56	
9,12-Octadecadienoic acid, methyl ester	5284421	6.25	
9-Octadecenoic acid (Z)-, methyl ester	5364509	34.08	
11-Octadecenoic acid, methyl ester	5364432	2.09	

Table 4b: Constituents identified by GC-MS analysis.

Compound Name	PubChem ID	% Area	
Methyl stearate	8201	14.81	
(E)-9-Octadecenoic acid ethyl ester	5364430	0.93	
trans-Z-. alpha. -Bisabolene epoxide	5363099	2.23	

Glycidyl palmitate	347736	2.21	
Eicosanoic acid, methyl ester	14259	1.80	
Z-17-Nonadecen-1-ol acetate	5363225	0.91	
Glycidyl palmitoleate	23624909	7.31	
Glycerol 1-palmitate	14900	2.14	
9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester	5319879	1.77	

Predicted bacterial targeted-protein & drug interaction

The results of *in silico* analysis after the screening of compounds through PyRx (drug computational software), a compound viz. CID 535221 and CID 500095 showed very good activity against targeted proteins of *E. coli* (PDB ID 1IEM, 7NAE, 1HNJ) and *S. aureus* (PDB ID 2ZCO, 4CJN, 1JII). These compounds were individually docked against respective proteins for further validation by AutoDock 4.2 software. They showed specificity and binding affinity with that of the receptors under 3D modeling system (Fig. 3 & 4). The binding energy and inhibition constant of both compounds against targeted proteins are represented in tabulated form for comparison (Table 5). Drug-likeness (Table 6) was performed through PreADMET software.

In general, the cross-species transfer of bacteria has been made easier by the historical growth of agriculture as well as by the current industrialization and globalization of the livestock industry. This dissemination has most likely had a significant impact on the creation of diseases. *S. aureus* and *E. coli* both are opportunistic pathogens. These organisms are responsible for causing many diseases in animals. It is already known that *S. aureus* and *E. coli* are one of the predominant causes of mastitis which led to tremendous economic loss to the farmers. Along with this, *S. aureus* is responsible for causing different infections encompassing superficial to deep skin infections as well as septicemia (Mala *et al.*, 2021) [15]. Also, *E. coli* infection is a substantial cause of neonatal mortality in calves due to septicemia or enteric infection (Peek *et al.*, 2018) [21]. Overuse use of antibiotics against these pathogens leads to the development of antibiotic-resistant strains. So, there is an urgent need to develop alternative compounds with higher potency, lower side effects, and sustainability.

Melia azedarach plant comes under the family Meliaceae. It is commonly grown in areas of sub- Himalayan region 2000 m above sea level. This plant possesses a remarkable history of being used in Ayurveda. Bark of *Melia azedarach* is known to be used in diarrhoea, piles, fever, loss of appetite, rheumatic pain and ulcers (Sharma and Singla, 2013). According to the present study, the minimum inhibitory concentration of *Melia azedarach* hydro-ethanolic bark extract was 1 mg/ml against *S. aureus* and 8 mg/ml against *E. coli* which depicts that hydro-ethanolic bark extract

possesses significant antibacterial activity against both organisms. However, according to Hemdan *et al.* (2023) [8] MIC value of the methanolic leaf extract of *Melia azedarach* plant against *S. aureus* and *E. coli* was 75 mg/ml and 50 mg/ml corresponding to each organism. Which was much higher than the present reported value of MIC. This finding revealed that hydro-ethanolic bark extract possesses much higher antibacterial activity than methanolic leaf extract. Khatoon *et al.* (2014) [11] reported antibacterial potential of *M. azedarach* L. bark against human pathogens at fixed concentration 2mg/ml using n-hexan and methanolic extracts. They reported n-hexane extract exhibited highest zone of inhibition against *S. flexneri*, least against *K. pneumoniae* and resistant to *S. mitis*, *S. aureus*, *B. subtilis*. While methanolic extract showed highest zone of inhibition on *S. mitis*, least against *K. pneumoniae* and moderate against *S. typhi*, *S. aureus* and *B. subtilis*. It has been cleared that the solvent system influences the release of bioactive constituents that contributing to pharmacological activity against the bacterial strains. Another study, methanol extract of *Melia azedarach* leaves showed least MIC value (22.4 µg/ml) against *B. cereus*, while ethanol extract showed 37.6 µg/ml against *P. aeruginosa*. Against *S. aureus* and *E. coli* showed comparatively efficient MIC value 38.7 µg/ml and 39.6 µg/ml in methanol and ethanol extract, respectively (Sen and Batra, 2012) [32]. When compared these finding with present study suggested that the plant parts and type of solvent or fraction has influence over biochemical constituents. Their activity varies with the type of bacterial strains. When compared with report of Khatoon *et al.* (2014) [11], present study hydro-ethanolic extracts showed comparatively better activity than the n-hexan and methanolic fractions.

A previous study on GC-MS analysis of the flower of *Melia azedarach* showed the presence of fatty acids like hexadecenoic acid methyl ester and 9-octadecenoic acid (Z) which possess antibacterial activity against various bacteria (Muhammad *et al.*, 2015) [18]. Another study showed that the presence of 6-Isopropen yl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol in essential oil from *Ocimum gratissimum* was one of the responsible constituents for antimicrobial activity (Salvi *et al.*, 2022) [30]. The GC MS analysis of hydroalcoholic extract of bark from *M.*

azedarach in this study also showed presence of above-mentioned constituents. So, the antibacterial activity reported in this study may be due to presence of these bioactive compounds in hydroalcoholic bark extract from *M. azedarach*. *In vitro* assay along with *in silico* prediction showed that extract from *Melia azedarach*'s bark possess a significant antibacterial activity against *S. aureus* and *E. coli*. The presence of bioactive compounds like organic acids, fatty acids and terpenoid identified by GC-MS may be given credit for exhibition of antimicrobial activity in the extract.

In-silico study was carried out to screen the phytochemical constituents distinguished by the GC-MS analysis against various proteins of *S. aureus* and *E. coli* such as 2ZCO, 4CJN, 2FNP and 1IEM, 1HNJ, 7NAE, respectively. 2ZCO (Carotenoid dehydrogenase synthase) is responsible for survival of *S. aureus* inside the host cell through the antioxidant activity of staphyloxanthin. Penicillin binding protein 2a (4CJN) is commonly involved in the synthesis of the bacterial cell wall by performing the role of transpeptidase activity (Shalaby *et al.*, 2020) [33]. *S. aureus* tyrosyl-tRNA synthetase (1JIJ) possesses a central role in protein synthesis (Qiu *et al.*, 2001) [24-25]. AmpC beta-lactamase of *E. coli* (1IEM) is responsible for causing the inactivation of beta-lactam antibiotic (Power *et al.*, 2011) [22]. beta-Ketoacyl-acyl carrier protein synthase- III (1HNJ) possesses key role in fatty acid biosynthesis which is essential part of bacterial membrane lipid and therefore important in viability of bacteria (Qiu *et al.*, 2001) [24-25]. dihydrofolate reductase (7NAE) is responsible for trimethoprim resistance in gram-negative bacteria (Krucinska *et al.*, 2022) [12]. So, it would be phenomenal to target these proteins to develop a new compound that possesses good efficacy and lower side effects. According to the *in-silico* results, it was found that phytochemical

constituents identified by GC-MS had great activity against selected proteins of *S. aureus* (PDB ID 2ZCO, 4CJN, 1JIJ) and *E. coli* (1IEM, 7NAE, 1HNJ). Here, 6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6, 7,8,8a-octahydronaphthalene-2,3-diol and Isobenzofuran-1(3H)-one, 3-(3-furyl)-3a,4,5,6-tetrahydr o-3a, 7-dimethyl showed best result against 2ZCO, 4CJN, 1JIJ, 7NAE, 1HNJ and 1IEM protein. Besides the good binding energies, these two best constituents also formed the hydrogen bond and alkyl bond with the active site amino acid residue which was presented in the binding pocket of the targeted protein. The interacting amino acids in a hydrogen bond, alkyl bond, and van der Waals interaction formed by ligand with the active site of targeted proteins was noticed (Table 7 and 8). This finding supports that both constituents possess good interacting capacity against selected target proteins. So, in support of the above discussion, we can say that these compounds can be further studied and may be used for the development of an alternative to the currently used antibiotics.

The present study based on the findings of the GC-MS analysis, *in vitro* antibacterial study, and *in silico* studies, suggests that AEBE from *M. azedarach* has potential antibacterial activity against both the organisms *S. aureus* and *E. coli*. The *in-silico* study showed that protein and ligand possess good binding activity. There is no effective cure without side effects predicted through ADME studies. So, these phytochemical constituents can be used as an initial compound for formulation development and/or a series of derivatizations may be done for improvement of antibacterial efficacy. Further, efficacy studies should be done based on individual compounds against different microbes to establish the mechanism of action and efficacy. Furthermore, *in vivo* antibacterial efficacy study may be carried out for proper dose determination against *S. aureus* and *E. coli* using a suitable animal model system.

Table 5: Results of AutoDock 4.2

Ligand	Free binding energy (kcal/mol)					
	1IEM	7NAE	1HNJ	2ZCO	4CJN	1JIJ
535221	-5.58	-7.76	-6.52	-7.07	-4.78	-7.99
500095	-7.25	-7.94	-7.10	-7.17	-5.38	-7.43
	Inhibition Constant, Ki (uM)					
	1IEM	7NAE	1HNJ	2ZCO	4CJN	1JIJ
535221	81.35	2.04	16.71	6.52	312.61	1.39
500095	4.88	1.51	6.29	5.55	114.45	3.55

Table 6: Drug likeness prediction of two best ligands

Drug likeness prediction	CID 535221	CID 500095
Rule of five	Suitable	Suitable
Lead like rule	Suitable if Binding affinity (>0.1 microM)	Suitable if Binding affinity (> 0.1 microM)
Lead like rule violation	0	0
Lead like rule violation field	-	-
Rule of five violation	0	0
CMC like rule	qualified	qualified
CMC like rule violation	0	0
MDDR like rule	Mid structure	Mid structure
MDDR like rule violation field	No ring, No rotatable bond	No rotatable bond
MDDR like rule violation	2	
WDI like rule	In 90% cutoff	In 90% cutoff
WDI like rule violation	0	0

Table 7: Interaction of ligand CID 500095 with all target proteins.

Target protein	Interactions		
	Hydrogen bond	Alkyl	Van Der Waals
2ZCO	ASN A:168	CYS A:44, ARG A:45, VAL A:137, ALA A:134, PHE A:22, TYR A:248, LEU A:164.	HIS A:18, GLN A:165, GLY A:161.
4CJN	GLU B:294, ASP B:275	TYR B:297	ASP B:295, TYR B:105, LYS B:316
1JIJ	GLY A:38, ASP A:40	LEU A:70, ALA A:39, TYR A:36,	GLN A:196, CYS A:37, GLN A:190, ASP A:80, TYR A:170, THR A:75, ASN A:124, ASP A:177
1IEM	ARG A:148	LYS A:315, ALA A:292, MET A:256	GLY A:317, LEU A:293, TYR A:150, SER A:64, ASN A:346, ASN A:289, SER A:287, GLY A:286, GLU A:272
1HNJ	-	MET A:207, VAL A:212, ALA A:246, LEU A:189, PHE A:157, CYS A:112	ALA A:216, PHE A:304, ILE A:250, GLY A:209, PHE A:213, ASN A:247, ILE A:156, ASN A:274, HIS A:244
7NAE	ILE A:5, ALA A:7	ALA A:6, MET A:16	LEU A:28, ASP A:27, TRP A:30, THR A:113, TYR A:111, ILE A:14, TYR A:100, ILE A:94, ILE A:50, THR A 46, GLY A:95

Table 8: Interactions of ligand CID 535221 with all target proteins.

Target protein	Interactions		
	Hydrogen bond	Alkyl	Van Der Waals
2ZCO	ASN A:168	CYS A:44, ARG A:45, VAL A:137, ALA A:134, PHE A:22, TYR A:248, LEU A:164	HIS A:18, GLY A:16, GLN A:165, GLY A:161
4CJN	ASP B:295, LYS B:273	-	GLU B:294, GLY B:296, LYS B:316, ASN B:146, TYR B:105
1JIJ	GLY A:38, GLN A:174	LEU A:70, TYR A:36, ALA A:39, TYR A:170	ASP A:40, THR A:75, ASP A:177, ASN A:124, GLN A:190, ILE A:200, CYS A:37, GLN A:196, ASN A:199
1IEM	ALA A:318, ASN A:152, GLN A: 120	TYR A:221, LEU A:119	GLY A:63, LYS A:67, SER A:64, GLA A 317, TYR A:150, ASN A:289, ASN A:346, LEU A:293, THR A:319.
1HNJ	ARG A:36	ILE A:156, TRP A:32PHE A:213	ILE A:155, THR A:37, ASN A:247, ALA A:246, MET A:207, ILE A:250, GLY A:209, ASN A:210, ARG A:249
7NAE	ILE A:5, ILE A:94, TYR A:100, ASP A:27, ALA A:7	ALA A:6, MET A:16	ILE A:14, GLY A:15, ILE A:50, THR A:46, GLY A:95, GLY A:96, TRP A:22, LEU A:28, THR A:113, PHE A:31

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Declaration of Interest Statement:

I declare that this research work has no financial interests or relationships that could be construed as a potential conflict of interest regarding the research.

Conflict of Interest Statement

Title of Manuscript: Unravelling the Antibacterial Potential of Hydroethanolic Bark Extract from *Melia azedarach*: *In vitro* and *In Silico* Investigation

Authors: Dr Marmik Dhebar, Dr. Natasha Yadav, Dr. Sushree Sangita, Dr Sivaraman, Dr. M.K Lonare, Dr. S.P.S Saini

Conflict of Interest Disclosure

The authors declare that there are no conflicts of interest associated with this manuscript. Specifically:

1. Financial Disclosure

- The research was conducted without any financial support or sponsorship from commercial entities or

organizations that could influence the outcomes or interpretation of the study.

2. Personal Relationships

- The authors have no personal relationships with individuals, organizations, or institutions that could be perceived as affecting the objectivity of the research.

3. Professional Affiliations

- The authors are not affiliated with any organizations or institutions that might be perceived to have a vested interest in the results of this study.

4. Consulting and Advisory Roles

- The authors have not served as consultants or advisors for any companies or organizations related to the subject matter of this research.

5. Patent and Financial Interests

- The authors do not hold any patents or financial interests related to the hydroethanolic bark extract from *Melia azedarach* or its components.

Novelty statement

This study presents a novel investigation into the antibacterial potential of *Melia azedarach* bark extract by combining traditional *in vitro* assays with advanced *in silico* techniques:

- Combined Approach:** The novelty lies in employing a comprehensive approach that bridges the gap between *in vitro* observations and potential molecular targets. This combined strategy offers a more refined understanding of the extract's antibacterial properties.
- In Silico Exploration:** Incorporating *in silico* analysis into the research design is a relatively new approach for exploring the antibacterial potential of *Melia azedarach* extracts. This computational aspect allows for targeted

exploration of potential bioactive compounds within the extract. By combining these elements, our research offers a more mechanistic understanding of the antibacterial properties of *Melia azedarach* bark extract, paving the way for future investigations into its potential as a natural antibacterial source.

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