Extraction and Identification of Phytoconstituent obtained from *Streblus as per* Lour Root Bark as an Antibacterial Agent

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Abstract

Streblus asper Lour has been used in Ayurveda to treat microbial infections. The current research looks at the quantity of Lupeol in a Streblus asper root bark (SARB) extract, as well as its antibacterial activity and in silico studies. The SARB contain lupeol, which is having antibacterial activity. Time course assay, disc diffusion method, minimum inhibitory concentration (MICs) using broth dilution method, and pH

sensitivity assay were used to assess the antibacterial activity of SARB extract against gram-positive and as well as gram-negative bacteria.In silico molecular docking studies, Lupeol has good binding activity against MurE E. coli than MurE S. aureus. E. coli was inhibited by the plant extract SARB at MIC ≥625 µg ml-1, whereas S. aureus was inhibited at MIC ≥1250 µg ml-1. The SARB demonstrated a remarkable MBC against the tested organism. In case of time response assay, suggested that plant extract completely inactivated E. coli and S. aureus within 6 hrs and 8 hrs respectively. The amount of lupeol present in SARB was found to be 4.63% (w/w) through RP-HPLC, which indicated significant amount of lupeol present in the extract. Our study suggests that *Streblus asper* can be useful as a potential natural antimicrobial agent.

Key words: *Streblus asper,* Ayurveda, RP-HPLC, *In silico*, Antibacterial
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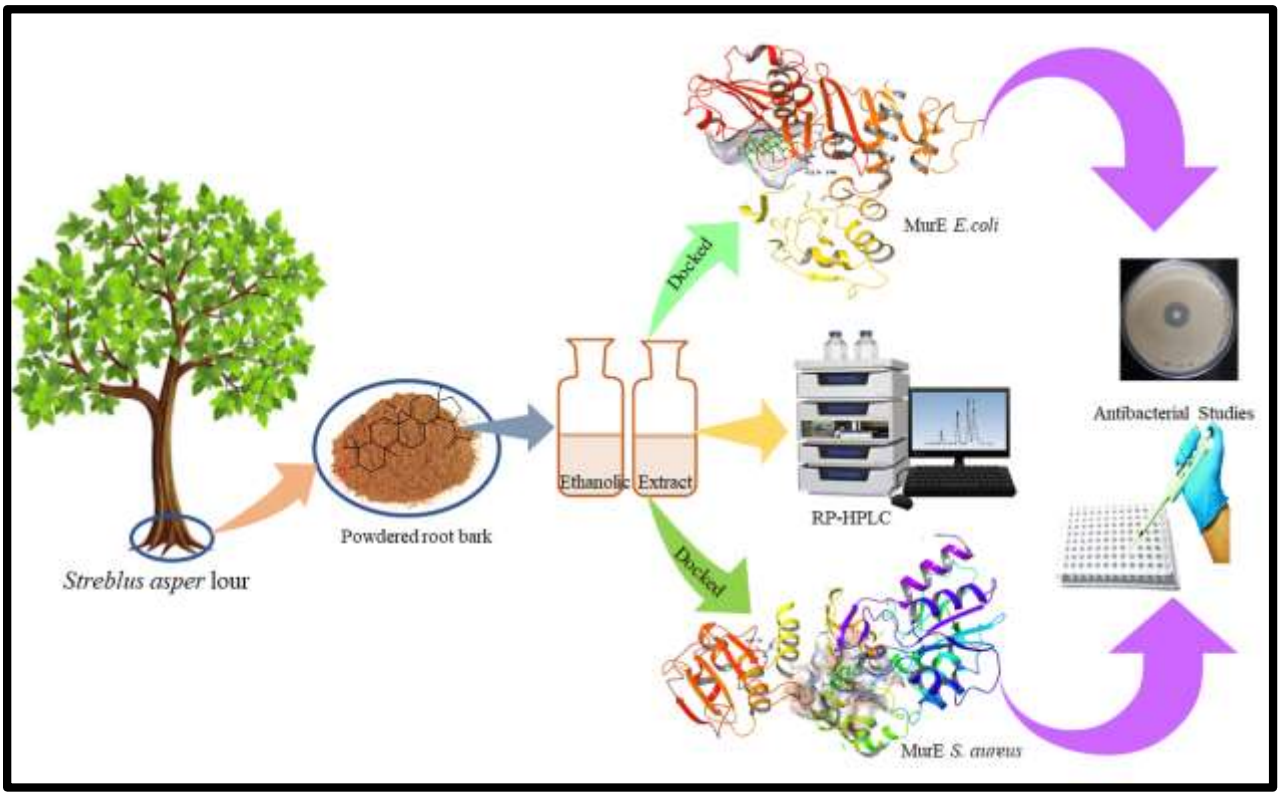


Introduction

The development of antibiotic resistance is increasing turn into a global public risk [1, 2]. This crisis is specifically arising from the excessive use of antibiotics [3, 4]. Therefore to reduce the use of antibiotics and infection can be treated with suitable drugs [5]. Antibiotic resistance bacteria and genes are now common in our environment due to the widespread usage of antibiotics. As a result, the likelihood of transmission from the environment to humans is skyrocketing [6]. Thequest for a new antimicrobial lead molecule is a global challenge, as microbes are becoming resistant to the conventional antimicrobials as drug resistance and microbes may develop resistance even without any exposure to a drug [7]. Therefore, to overcome these problems, the quest for new leads from natural resources is required. So, alternative antimicrobials from botanicals serve to lessen side effects and their combination helps to develop synergistic

actions that avoid resistance. [8,9]. Many synthetic antibacterial medications are already available, but they are expensive, toxic, and contribute to drug resistance mutations. As a result, there is a need for low-cost, readily available natural antimicrobials with few side effects.A polysaccharide chain, Peptidoglycan, consists of irregular N-acetyl muramic acid (MurNAc) and N-acetyl glucosamine sugar residues (GlcNAc) which are cross-linked with pentapeptide side chains committed to MurNAc residues. The order of pentapeptide in Gram-positive is UDP-MurNAc-L-Ala-ᵞ-D-Glu-L-Lys-D-Ala-D-Ala and in Gram-negative is UDP-MurNAc-L-Ala-ᵞ-D-Glu-m-DAP-D-Ala-D-Ala the only difference is in MurE ligase which aids in the addition of L-Lys and m-DAP [10]. The MurE protein of *Staphylococcus aureus is* composed of a three-domain in which Domain 1 covers 1 to 98 residues, 99 to 332 residues follow domain 2, and domain 3 extends from residues 333 to 493. The UMT lysine-binding residues were Asp-406 and Glu-460 [11]. Similarly, MurE of *Escherichia coli* consists of three domains. Domain 1 comprises 1-88 amino acids, 90–338 residues were observed in Domain 2, and Domain 3 consists of 340 amino acids. The UMT binding site was present in domain 1 [12].

Streblus asper Lour is one of the most extensively used ethnomedicinal plants in Indian Ayurveda for treating piles, leprosy, diarrhoea, elephantiasis, cancer, and dysentery. Streblus asper Lour (Moraceae) is primarily found in Southeast Asia, including Sri Lanka, India, Philippines, Malaysia, and Thailand. The root of the plant is used to treat syphilis in Indian traditional medicine. Cardenolide, α-amyrin, lupeol, and β-sitosterol are present in the plant's roots.[13,14]. Among these, lupeol exhibit several pharmacological activities, includingantiprotozoal, anti-inflammatory, antimicrobial, and antitumor activity [15]. Ample evidence suggested that *Streblus asper* Lour is widely used in microbial infection. The purpose of this study is to evaluate the antibacterial activity and to detect the chemical constituent, Lupeol, using the standardization method with the help of RP-HPLC.

Materials and methods

Chemicals and reagents

The chemicals and reagents which are used to perform the work are listed below:

Table 1. The list of chemicals and reagents

Chemicals and reagents	Company
Lupeol (standard)	Sigma Aldrich (St. Louis, MO, USA)
DMSO (Cell culture grade) Glacial acetic acid Ethanol (HPLC grade) Acetonitrile	Merck Ltd. (Mumbai, India)
Nutrient Agar (NA) Muller Hinton Agar (MHA)	Himedia, India
Streptomycin	Sisco Research Laboratory, India

Plant material collection and extraction

The S. asper rootbark (SARB) was obtained from the hilly region of West Bengal in the winter of 2020. Dr. S. Rajan, a famous field botanist in the Indian state of Tamilnadu, identified and authenticated the obtained plant sample. Following authentication, the plant specimen sample was stored in the Bengal College of Pharmaceutical Sciences and Research (BCPSR) Herbarium in Durgapur, India, for future reference. SASB has the vide voucher specimen number BCPSR/201/1405. The plant components were collected and dried in the shade before being crushed into powder using a mechanical grinder. The powder was then extracted by soaking it in ethanol (95% v/v) at 25°C. To get the crude extract, the extract was collected, filtered, and the solvent was

evaporated at 45°C using an Eyela Rotary Evaporator (Japan). The percentage yield was estimated and kept for future uses at 4°C.

RP-HPLC analysis

Instrumentation

A 600-controller pump and a multiple-wavelength ultraviolet-visible detector were included in the RP-HPLC system (Waters, Milford, MA, USA). The HPLC system included an AF2489 in-line degasser and a Rheodyne 7725i injector with a 20 µl loop. Empower 2 software programs with an external calibration method were used to do the quantitative analysis. Furthermore, the mobile phase was filtered using a Millipore membrane with a pore size of 0.45 µm. The extract and standard samples were filtered with 0.45 µm syringe filters (NYL).

Chromatographic conditions

RP-HPLC analysis was performed for the estimation of lupeol in SARB. At ambient (25ºC) conditions, a reversed-phase C18 column (Waters Spherisorb 5 mm ODS2, 250 mm × 4.6 mm, 5 µm particle size) was utilized to generate chromatographic separation. The mobile phase was made using acetonitrile and water containing 1% glacial acetic acid (95:5 v/v). For each sample, the flow rate was set at 1 ml/min and the run period was fixed for 30 minutes, with the λ maxset at 215 nm.

In silico docking studies

After the literature survey, we targeted the lupeol molecule to know whether it has antibacterial activity or not, so we perform *In silico* docking studies in Mur*E. coli* and *S. aureus*.

Ligand preparation

Lupeol molecule was outlined using ChemSketch, and optimization was done using LigPrep module in Schrödinger 2017-2. The development of various ionization states of each ligand was carried out at pH 7.0, and then energy minimization was carried out using the OPLS 3 force field [16] until the root mean square deviation (RMSD) was reached upto 0.01 Å.The 3D structures thus obtained were then used for docking studies.

Protein preparation and binding energy calculation

The protein data bank was used to get the crystal structures of MurE S. aureus (PDB ID: 4C13, resolution 1.9 Å) and E. coli (PDB ID: 1E8C, resolution 2 Å). The resulting protein was prepared using the Protein Preparation Wizard (Schrödinger 2017-2) [17].At pH 7.0, the protein structures were protonated, and water molecules were removed. Prime (v4.3, Schrödinger 2017-2) was used to build the misplaced side-chain atoms as well as the structural breaks [18]. The protein structure was minimized and validated using the Ramachandran plot (**Figure 1**). Further OPLS 3 [16]force field was generated, and the UDP-MurNAc-L-Ala-γ-D-Glu-meso-A2pm (UMT) pocket has been chosen for *E. coli* to create a grid by removing the UMT substrate and for Mur*E. aureus* L-lysine binding pocket was selected. The Lupeol molecule was docked in the catalytic pocket (**Figure 3**) by extra precision (XP) mode using Glide (version 8.0). All docking scores were presented in **Table2.** The binding free energy was calculated using the molecular mechanics–generalized born surface area (MM–GBSA) approach using Prime in Schrödinger 2017–2.

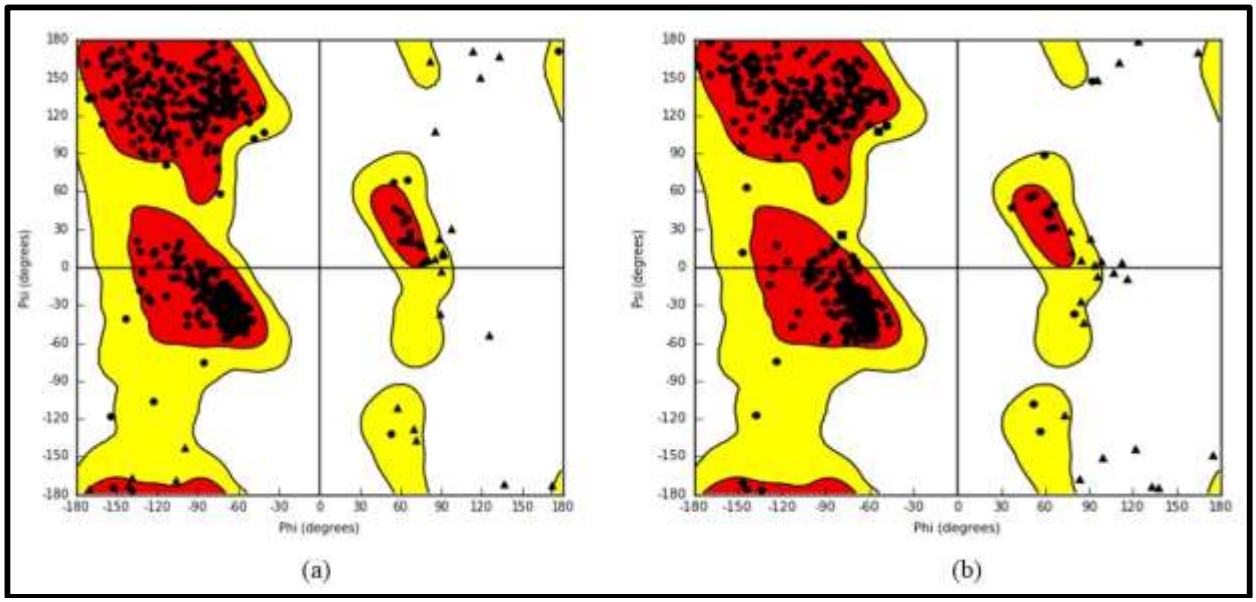


Figure 1. Ramachandran plot of (a) MurE*E. coli* (b) MurE*S. aureus*

ADME studies

The pharmacokinetic studies of a drug play a crucial role in absorption, distribution, metabolism, and excretion(ADME). Schrodinger software's QikProp module was used to conduct an ADME study to determine features such as human oral absorption percentage, logS, logP, blood-brain barrier, MDCK studies, Rule of five, and so on [19].

Antibacterial assay

Preparation of stock solution

The stock solution of Streptomycin (Sisco Research Laboratory, India) was 10 µg/ml (w/v). The antibacterial assay was evaluated using DMSO % (v/v) as a solubilizing solvent for test samples and as a control. SARB extract stock solution was produced at a final concentration of 5000 µgml-1. The required varied concentrations of plant extract against various bacterial tests were prepared from this stock solution.

Bacterial strains and culture condition

The Gram-positive (Staphylococcus aureus ATCC 29213) and gram-negative (Escherichia coli ATCC 25922) bacteria were chosen as standard strains according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards [20]. At 4°C, the bacterial cultures were grown using Nutrient Agar (NA) or Nutrient Broth (NB) (Himedia, Mumbai, India), with the subculture obtained every 4 weeks.

Disc diffusion method

The antibacterial assay of the crude extract and the biomarker both were conducted using the disc diffusion method [20]. The base plate was made by distributing 10 ml of sterilized Muller Hinton Agar (MHA) (pH 7.2 ± 0.2 at 25°C) into a sterile petri dish (9 cm in diameter, Borosil) and allowing it to settle for some time. The bacterial suspension (5×105 CFU/ml) in a volume of 100 µl was placed on each base plate.To soak the 20 µl of each test sample (50-2000 g/disc), sterile paper discs (6 mm) were utilized. After air-drying the discs, they were placed on each base plate and incubated for 24 hours at 37 ± 2°C. Streptomycin was employed as a positive control for gram-negative and gram-positive bacteria, with a disc concentration of 10 µg/ml. The diameter of the zone of bacterial growth inhibition was measured (mm). For the zone of inhibition, an average of three measurements were made in various orientations [21]. The performed experiment was carried out in triplicate.

Determination of minimum inhibitory concentration (MICs) and minimum bactericidal concentration (MBCs)

According to CLSI guidelines [20], the broth micro-dilution method was applied to determine MIC values. Suspending one isolated colony from each base plate containing 5ml of MHB was used to prepare bacterial cultures. After 24 h of the proper incubation period, each suspension was diluted with 0.5 Mac Farmland standard to prepare the final inoculum population (5×105 CFU/ml). Colony morphology and the gram stain technique were used to determine the accuracy of the mother culture throughout the test. Using a 96-well microtiter plate, test samples were serially diluted two times by using the known stock solution with MHB. Each bacterial inoculum in equal volume was placed in each well of a microtiter plate containing 0.05 ml of serial dilutions of test samples incubated at 37 ± 2°C for 24 hours. The lowest concentration of a substance that has a significant impact on the inhibition of the observable growth of bacteria inmedia is termed MIC. The growth of the bacteria was shown through turbidity and a pellet formation on the bottom of the well. MICs were determined probability as the first well, where no pellet become visible [22]. The calculation was done by comparing the absorbance of sample wells and control wells by using Spectra-max M5 (USA) at 405 nm wavelength. The MBCs were performed by adding 50 µl of the suspensions from the wells in 25 ml fresh MHB, and suspensions were incubated at 37°C for 48 h. The MBC was determined as the lowest concentration of the test sample that inhibited the complete growth (100%) of microorganisms.

pH sensitivity assay

The effect of pH on the antibacterial activity of SARB extract was evaluated by pH sensitivity assay [23]. Overnight the broth cultures of *S. aureus* and *E. coli* at different pH levels (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) were prepared by using 0.1N HCl and 5M NaOH and swabbed on MHA plates with the related pH level. The antibacterial activity was analyzed by the disc diffusion method. Streptomycin was used as a positive control for gram-negative and gram-positive bacteria.

Time course assay

The rapidity and duration of antibacterial activity of the test samples were performed by time-kill analysis [24]. Overnight broth cultures of bacterial strains were adjusted to 5×10^5 CFU/ml concentration and treated with SARB extract at a concentration of 0×MIC, 0.5×MIC, 1×MIC, 1.5×MIC, and 2×MIC. Control tubes were also prepared without SARB extract. Then 100 µl of the sample was withdrawn and poured on MHA plates at regular intervals (0, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h). Each plate was incubated at 37°C for 24 h, and CFU was calculated. All the experiment was done in triplicates.

Statistical analysis

Data expressed as mean Inhibition zone diameter ± SEM. The results recorded were statistically analyzed by one-way ANOVA using Graph-Pad InStat Version 5.0 (GraphPad Prism Software, Inc., USA).

Results and discussion:

Determination of lupeol in plant extract through RP-HPLC

The mean retention time ($R_{t)}$ was observed at 15.4 ± 0.06 min for lupeol by comparing standard (**Figure 2a**) and extract chromatograms (**Figure 2b**). The calibration range of lupeol was found to be 10-1000 µg/ml, with the linear equation Y= 26513X + 62826, with the coefficient of determinants (r^2) of 0. 996. The amount of lupeol found in SARB was 4.63% (w/w).

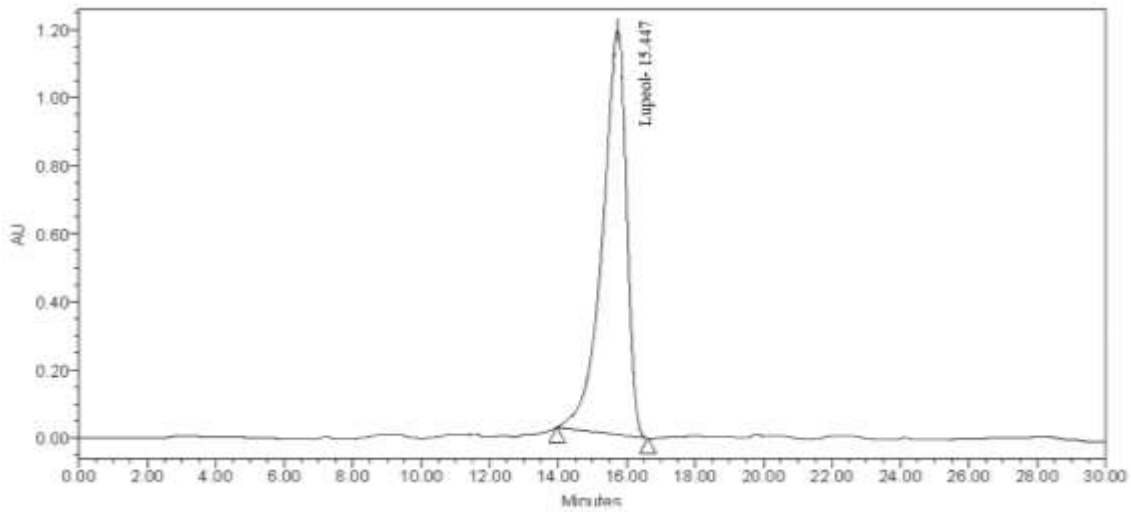


Figure 2a. RP-HPLC Chromatogram of Lupeol Standard

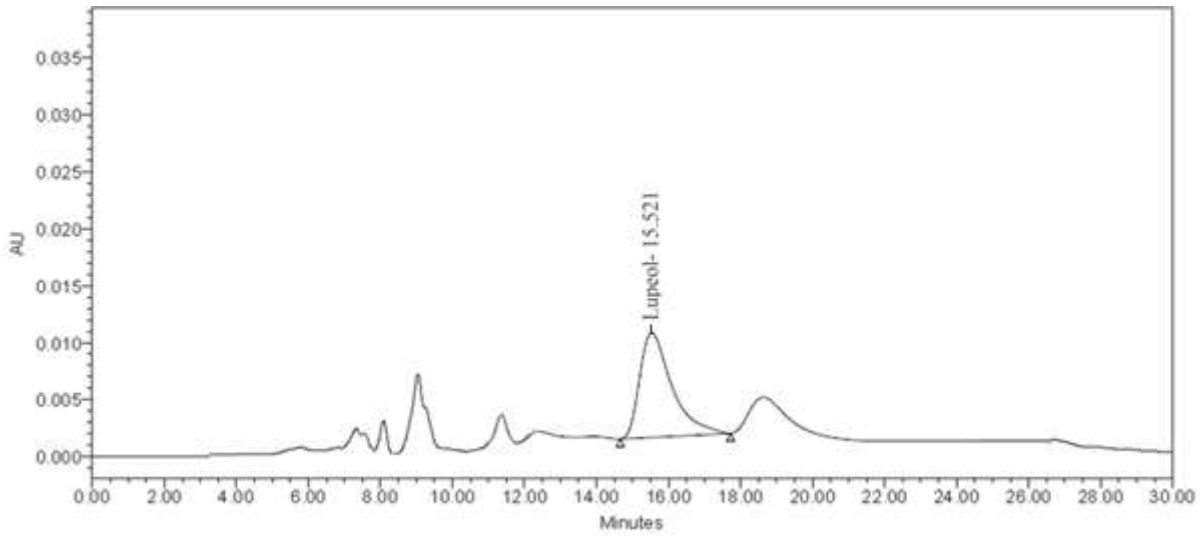


Figure 2b. RP-HPLC Chromatogram of Lupeol in *Streblus asper* Ethanol Extract

Molecular docking studies

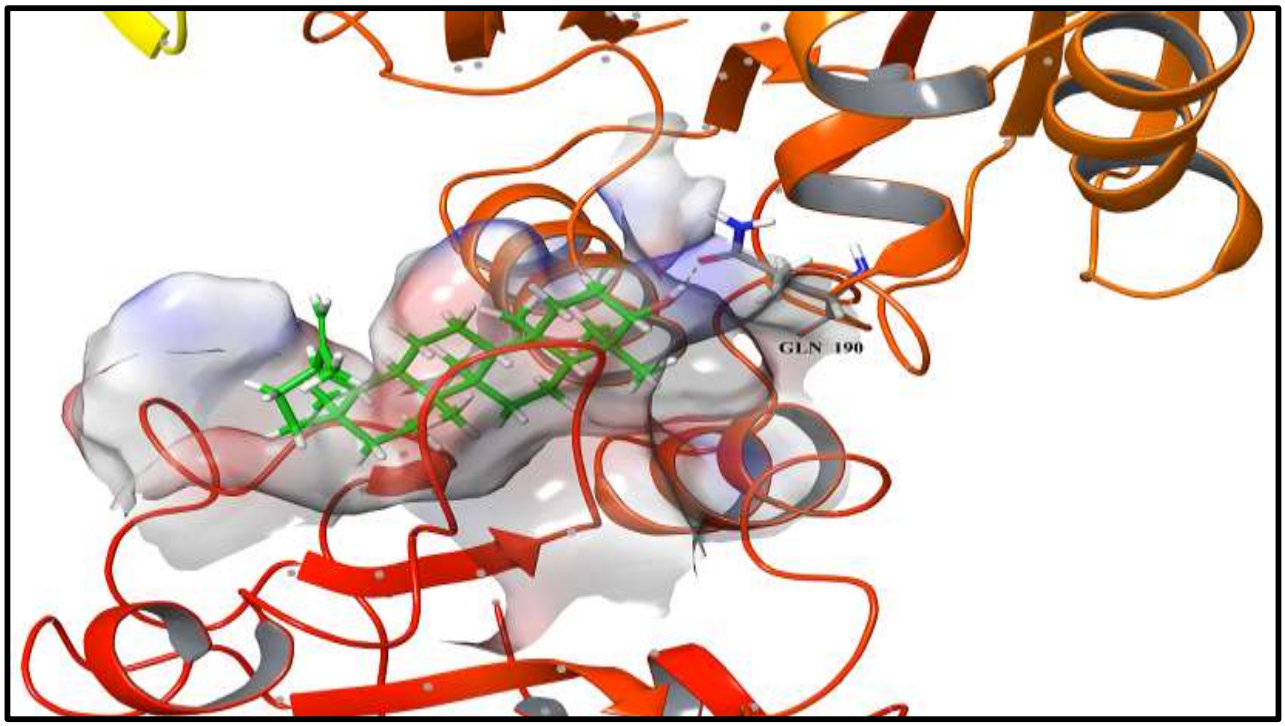
It is evident from **Figure 3a and Figure 4a**that Lupeol has good binding activity with an MM/GBSA [25] score of -81.02kcal/mol against Mur*E. coli* (shown in **Table 2**), where GLN190 is interacting with the OH group and forming a hydrogen bonding. In contrast, for Mur*ES. aureus* **(Figure 3b and Figure 4b),** the OH group of Lupeol is interacting with ASP29, and the binding score was found to be -53.97 kcal/mol showing the binding at Domain 1. In general, most of the ligands van der Waals and Coulomb energy terms favour ligand binding. Further, the high negative value in Mur*E. coli* compared to MurE S. aureus indicates that van der Waals term is the driving force for binding inhibitors.

Table 2. Molecular docking and binding free energy (MM/GB-SA) calculation (kcal/mol) of Lupeol molecule in the catalytic pocket of Mur*E. coli* and Mur*ES. aureus*.

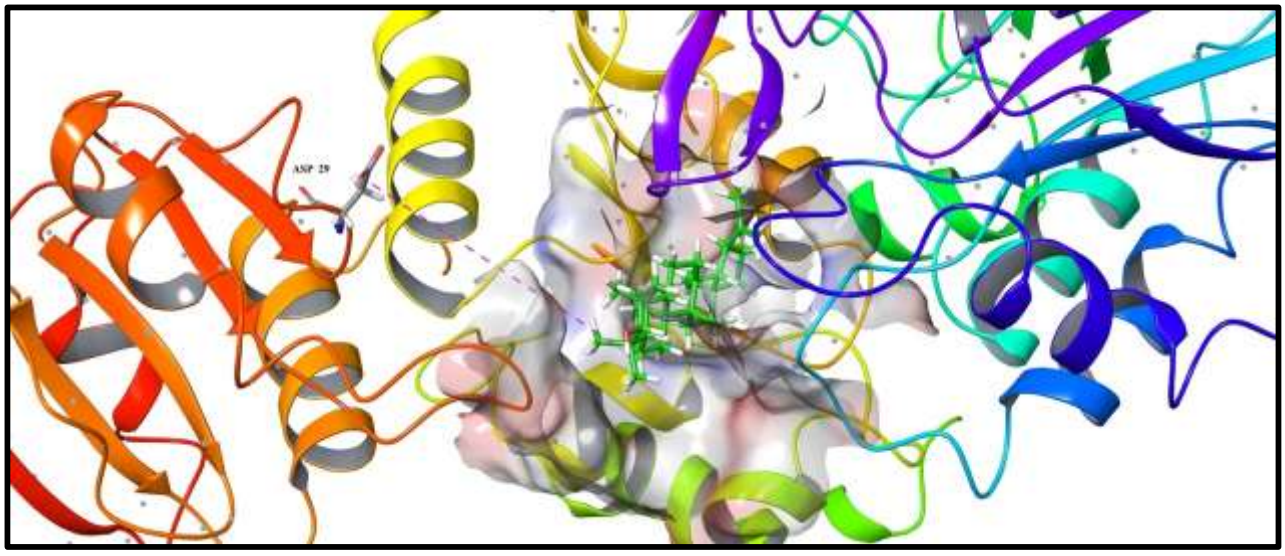
	Compound (Lupeol)	
	Mur*E. coli* (PDB: 1E8C)	**Mur*ES. aureus* (PDB: 4C13)**
$^{a}G_{score}$	-3.91	-2.599

$^{b}G_{energy}$	-27.704	-22.945
$^{c}G_{emode}$	-31.445	-22.999
$^{d}XP_{HBond}$	-0.7	-0.698
$^{e}\Delta G_{Bind}$	-81.02	-53.97
$^{f}\Delta G_{Cov}$	-21.68	-4.07
$^{g}\Delta G_{vdW}$	-58.57	-40.39
$^{h}\Delta_{Coul}$	26.79	0.3
$^{i}\Delta G_{H\text{-}bond}$	3.66	-0.71

$^{a}G_{score}$: glide score; $^{b}G_{energy}$: glide energy; $^{c}G_{emodel}$: glide model energy; $^{d}XP_{HBond}$: extra-precision hydrogen bond reward; $^{e}\Delta G_{bind}$: binding free energy; $^{f}\Delta_{cov}$: covalent energy; $^{g}\Delta_{vdW}$: van der Waals energy; $^{h}\Delta_{Coul}$: Coulomb energy; $^{i}\Delta$H-bond: hydrogen bond energy contribution.



(a)



(b)

Figure 3. 3D ligand interaction diagram of Lupeol in the catalytic pocket of *E. coli* (a) and *S. aureus* (b)

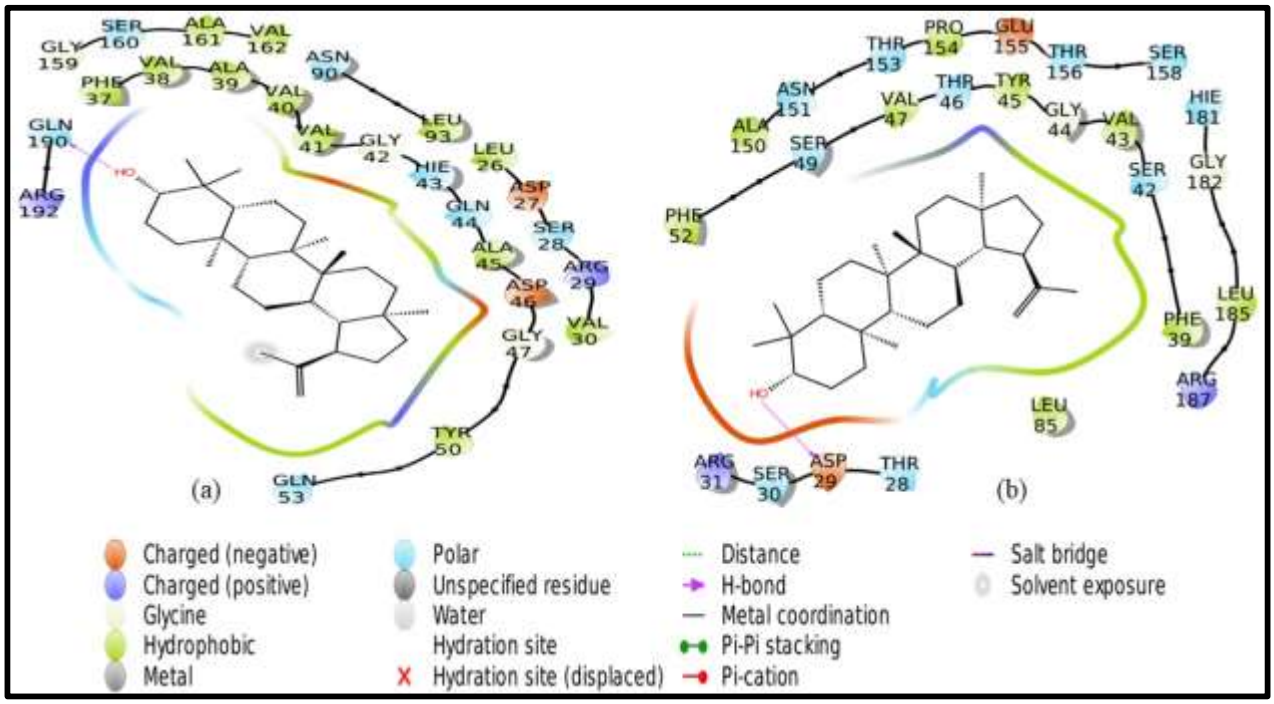


Figure 4. 2D Ligand interaction diagram of lupeol in the catalytic pocket of (a) MurE*E. coli* (PDB: 1E8C) (b) MurE*S. aureus* (PDB: 4C13)

ADME studies

The ADME studies of Lupeol were performed by using QikProp (**Table 3**) to identify the absorption, distribution, metabolism, excretion, and toxicity studies. Physiochemical properties such as logPo/w and logS play a crucial role in a drug acting on the target. Another physicochemical property of BBB makes the drug efficient for permeability. The result of Lupeol doesn't lie in the range for logPo/w, logS. The human oral absorption percentage was found to be 100 percent which reveals good absorption.

Table 3. ADME/Toxicity studies of best binding molecules with receptor(s)

Compounds	logPo/w (–2.0 – 6.5)	logS (–6.5 – 0.5)	logHERG (below –5)	QPPCaco (<25poor, >500great)	logBB (–3.0–1.2)	MDCK (<25 poor, >500 great)	Human Oral Absorption (%) (>80% is high, <25% is poor)	Rule of Five (maximum is 4)	CNS (–2 inactive, +2 active)
Lupeol	7.043	-7.907	-3.768	4441.181	0.119	2478.673	100	1	1

Antibacterial activity

MIC, MBC and ZOI of *S. asper* extract

The SARB extract showed notable inhibitory activity against two bacterial (*E. coli* and *S. aureus*) strains with MIC below 2000 µg ml^{-1} (**Table 4**). The SARB extract inhibited *S. aureus* and *E. coli* with MIC >1250 µgml^{-1} and >625 µgml^{-1} (**Table 4**), respectively. Hence, the results indicated that SARB showed potent (15.4 mm at ≥625 µg/ml concentration) antibacterial activity against *E. coli*. In contrast, the plant extract possesses a moderate to a poor degree (12.2 mm at ≥1250 µg/ml concentration) of antibacterial activity against *S. aureus.* The MBC assay, using 2 to 3-fold MIC, presented that lower concentrations of the SARB extract had bacteriostatic activity, but at higher concentrations had bactericidal activity (**Table 5**) due to the active principle present in the extract.**Figures 5a and 5b** depicted the ZOI of *E. coli* and *S. aureus* respectively.

Table 4. Zone of inhibition and MIC value of *Streblus asper* extract

Name of Bacteria	Ethanolic extract of *Streblus asper*		Streptomycin (Standard)	
	MIC (µg/ml)	ZOI (mm)	MIC (µg/ml)	ZOI (mm)
S. aureus	≥1250	12.2 ± 0.05	≥50	16 ± 0.05
E. coli	≥625	15.4 ± 0.05	≥12.5	19 ± 0.05

Table 5. MBC value of *Streblus asper* extract

Plant extract	Bacteria MBC (µg/ml)	
	S. aureus	***E. coli***
S. asper	2500	1250
Standard	100	25

Figure 5a. ZOI against*E. coli*of SARB extract

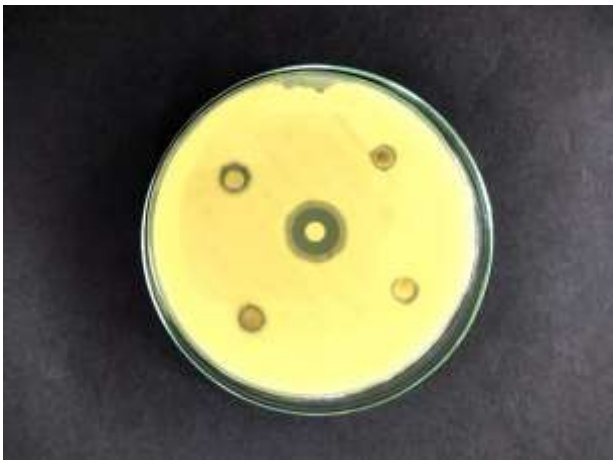
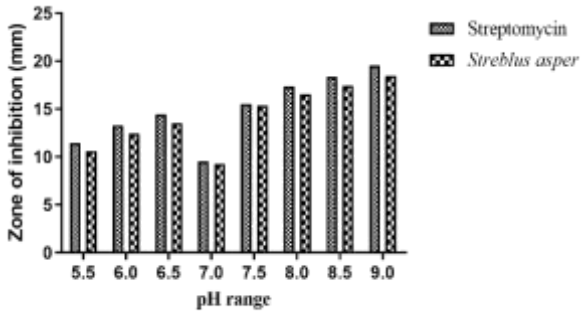


Figure 5b. ZOI against *S. aureus*of SARB extract

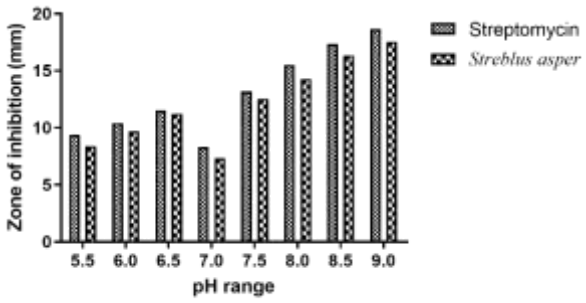
Figures 5a and 5b. Both figures are showing the ZOI against *E.coli* and *S. aureus* where Streptomycin was used as standard

Effect of pH on the antibacterial activity

The antibacterial activity of SARB extracts increased gradually with increasing the pH of the medium. The highest zone of inhibition (18.7 mm) against *E. coli* was observed at pH 9 (**Figure 6a**). The effects of pH on the zone of inhibition by the SARB extracts against *E. Coli* and *S. aureus* are shown in **Figure 6(a-b)**. The bacterial growth in the control discs loaded with DMSO was unaffected by the changes in pH.Streptomycin has shown the highest zone of inhibition 19.5 mm, at pH 9 compared with other pH ranges.



Effect of pH against *Escherichia coli*



Effect of pH against *Staphylococcus aureus*

Figure 6. Effect of pH (a) against *E. coli* and (b) against *S. aureus*

Time course assay

Treatment with SARB extract (MBC) exhibited a bactericidal effect on the test organisms. The SARB extract completely inactivated the *E. coli S. aureus*and population within 6 hrs and 8 hrs respectively. These results suggested that the SARB extracts ultimately reduce *E. coli* strain reproducing capability within 2-6 hrs of exposure (**Figure7**) and reduce *S. aureus*strain reproducing capability within 2-8 hrs of exposure (**Figure 8**).

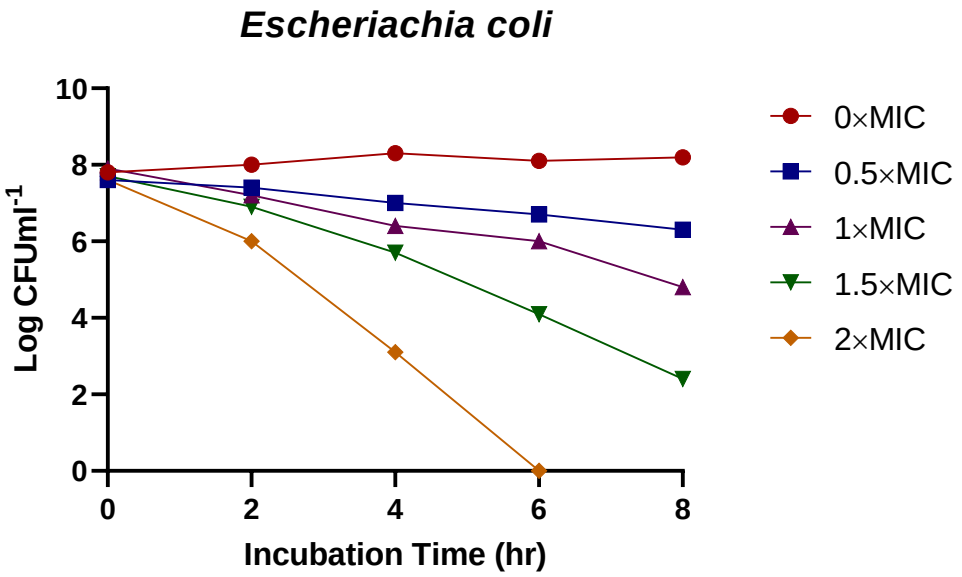


Figure 7. Graph showing time course assay for *E. coli* between incubation time (hrs) vs Log CFU ml^{-1}

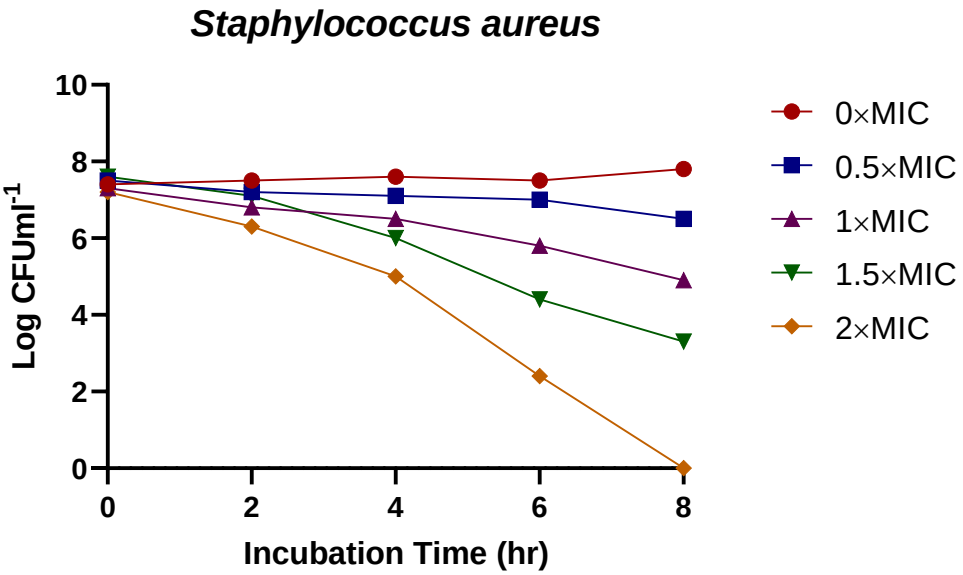


Figure 8. Graph showing time course assay for *S. aureus* between incubation time (hrs) vs Log CFU ml^{-1}

Conclusion

The study suggested that SARB extract has potential antibacterial agents against *S. aureus* and *E. coli*. The study also exposed that *Streblus asper* containsa significant amount of lupeol. This scientific exploration will help to identify effective antimicrobial agents from *Streblus asper* root, which may be clinically investigated to treat infectious diseases and may be useful in pharmaceutical preparations.

Conflict of interest

The authors declare that they have no conflict of interest.

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