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Bioactive Lichen Evaluation: *Parmotrema Perlatum* Targets Pathogenic *Bacillus Species*

A Combined Experimental and Computational Approach to Natural Antibacterial Discovery

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Abstract: *Parmotrema perlatum* (commonly known as black stone flower/kalpasi) is a foliose lichen traditionally used in India and recognized as a source of bioactive secondary metabolites. In this study, GC–MS profiling of *P. perlatum* extracts identified Thujopsene and Resibufogenin as major constituents. Antibacterial screening against *Bacillus species* revealed only weak inhibition, suggesting that active metabolites occur at low concentrations in crude extracts. To complement these results, molecular docking was performed using AutoDock Vina against representative bacterial proteins, including D, D-peptidase Rv3330 (*Mycobacterium tuberculosis*), Cagβ ATPase (*Helicobacter pylori*), dihydrofolate reductase (*Staphylococcus aureus*), and DL-endopeptidase CwlO (*Bacillus subtilis*). Among the tested compounds, Resibufogenin displayed consistently high binding affinities across multiple targets, underscoring its promise as a potential antibacterial lead molecule. Overall, this work demonstrates the value of integrating in vitro assays with computational modeling and highlights *P. perlatum* as an underexplored source of novel antimicrobial agents requiring further isolation, characterization, and preclinical validation.

Keywords: Phytochemical analysis, Molecular docking, *Permotrema parlatum*, Resibufogenin and Thujopsin.

I. Introduction

Bacterial infections caused by pathogens such as *Bacillus* species, *Mycobacterium tuberculosis* (Mtb), *Helicobacter pylori*, and *Staphylococcus aureus* represent a significant global health burden due to their virulence, widespread prevalence, and increasing resistance to conventional antibiotics [3,6,7,9,13,15]. *Bacillus* spp. are rod-shaped, Gram-positive bacteria that form endospores and are commonly found in soil and aquatic environments. While *Bacillus subtilis* is non-pathogenic and widely used as a model organism and probiotic, pathogenic species such as *B. cereus* and *B. anthracis* are linked to foodborne illnesses and anthrax, respectively [7,10]. *M. tuberculosis*, the causative agent of tuberculosis, continues to cause high morbidity and mortality worldwide. The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains has made treatment increasingly difficult, underscoring the urgent need for new therapeutic strategies [3,9,13,15].

H. pylori is a Gram-negative bacterium that plays a key role in chronic gastritis, peptic ulcers, and gastric cancer. Transmission occurs primarily via person-to-person contact, though environmental routes have also been documented. Epidemiological studies report high infection rates, with up to 83% of chronic gastritis cases in some populations testing positive for *H. pylori* [1,16]. *S. aureus*, a clinically significant pathogen, is responsible for a wide spectrum of infections ranging from skin and wound infections to systemic diseases. Its pathogenicity is associated with surface adhesion proteins, extracellular toxins, and enzymes such as coagulase, hyaluronidase, and dihydrofolate reductase (DHFR), which facilitate colonization and immune evasion [6,14].

The global rise of antimicrobial resistance has renewed interest in natural products as alternative therapeutic agents. Lichens, symbiotic organisms composed of fungi and algae or cyanobacteria, produce a diverse range of secondary metabolites including phenolics, depsidones, dibenzofurans, usnic acid, lactones, depsones, quinones, and pulvinic acid derivatives that exhibit antimicrobial activity [12]. *Parmotrema perlatum* (black stone flower, kalpasi), traditionally used in South Asia for its medicinal properties such as diuretic and antimicrobial effects, contains bioactive phytochemicals that represent promising candidates for targeting essential bacterial enzymes and developing natural antibacterial agents [12].

Major bacterial targets investigated include *B. subtilis* DL-endopeptidase CwlO, involved in peptidoglycan remodeling [11]; *M. tuberculosis* D, D-peptidase Rv3330, crucial for cell wall integrity [13]; *H. pylori* Cagβ ATPase, a Type IV secretion system component [19]; and *S. aureus* DHFR, essential for folate metabolism [6]. This study explored the antibacterial activity of *P. perlatum* extracts against *Bacillus* spp. and evaluated their interactions with bacterial targets through molecular docking, aiming to assess their potential as natural antimicrobial agents.

Parmotrema perlatum, commonly known as black stone flower or *kalpasi*, is a foliose lichen classified within the Domain *Eukaryota* and Kingdom *Fungi*. It belongs to the Division *Ascomycota*, which reproduces via ascospores, and is further categorized under the Class *Lecanoromycetes*, Order *Lecanorales*, and Family *Parmeliaceae*. This species is widely distributed across India and has historically been used both as a culinary spice and in traditional medicine, owing to its bioactive compounds and characteristic aroma. Despite its traditional applications, scientific investigations into its chemical composition and therapeutic potential remain limited [12].

Infectious diseases caused by *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Staphylococcus aureus* continue to pose major global health challenges. Tuberculosis alone accounted for approximately 1.25 million deaths in 2023, and the rise of multidrug-resistant strains has complicated treatment, increasing both the cost and difficulty of effective therapy [3,9,15]. Similarly, *H. pylori* is implicated in chronic gastritis, peptic ulcers, and gastric carcinoma [1,4,14], while *S. aureus* exhibits growing resistance to commonly used antibiotics [5,16]. The increasing prevalence of drug-resistant pathogens underscores the necessity of exploring natural sources for novel antimicrobial agents [2].

Lichens, including *P. perlatum*, are known to synthesize a diverse array of secondary metabolites with potential therapeutic applications. These compounds demonstrate antimicrobial, antiviral, enzyme-inhibitory, cytotoxic, antioxidant, wound-healing, and anti-inflammatory properties [12]. *Gas Chromatography–Mass Spectrometry (GC–MS)* is a widely applied analytical technique for profiling such metabolites. In this approach, sample components are separated based on their physicochemical properties using gas chromatography and subsequently ionized and detected in a mass spectrometer to generate characteristic mass spectra. In the current study, *GC–MS* analysis of the ethanolic extract of

P. perlatum provided critical data on its phytochemical profile for downstream molecular docking investigations.

Molecular docking facilitates the prediction of interactions between bioactive compounds and bacterial target proteins. In *M. tuberculosis*, potential targets such as AftB and EmbA have been examined through *in silico* docking to identify compounds capable of inhibiting bacterial growth [4]. In *Bacillus subtilis*, DL-endopeptidase CwlO plays a vital role in cell wall elongation, making it a suitable target for evaluating antibacterial activity [10,11]. For *S. aureus*, dihydrofolate reductase and related enzymes are critical targets for potential inhibitors [6]. In *H. pylori*, the Cagβ ATPase, a key component of the type IV secretion system, has been structurally characterized, allowing assessment of ligand interactions [19]. By combining *GC–MS* phytochemical profiling with molecular docking, this approach enables a rational evaluation of *P. perlatum* as a source of novel antimicrobial agents.

II. MATERIALS AND METHODS:

2.1 Sample Collection:

The Lichen *Parmotrema perlatum* was collected from a local market of Battarahalli, Karnataka, India. A total of 400 grams of fresh lichen material was collected.

2.2 Sample preparation:

Debris such as soil, bark, and other lichen species was removed using forceps. The cleaned lichen was ground into a fine powder using a sterile mortar and pestle and passed through a 60-mesh sieve. The resulting 55.22 g of powder was stored in sterile, airtight containers in a cool, dry place until further use. Gas Chromatography–Mass Spectrometry (GC-MS) was performed on the ethanolic extract to identify bioactive compounds and evaluate its antibacterial potential against *Bacillus* sp., *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Staphylococcus aureus*.

Fig.1: Powdering *Parmotrema perlatum*

Fig.2: Weight of the total sample

2.3 In Vitro Antibacterial Assay:

2.3.1 Materials:

Bacillus subtilis culture, Nutrient Agar (NA), Nutrient broth, *Parmotrema perlatum* powder, Ethanol, Distilled water, Whatman No. 1 filter paper discs (6 mm), Sterile Petri dishes, Sterile cotton swabs, Well borer, Sterile forceps, Shaker incubator, Incubator (37°C), Micropipettes, Sterile tips, Sterile saline or PBS, McFarland Standard (0.5) or spectrophotometer, Sterile distilled water (for control)

2.3.2 Methodology:

An overnight culture of *Bacillus subtilis* was first prepared in nutrient broth to get the bacteria actively growing. To extract the bioactive compounds, ethanolic and aqueous extracts of *Parmotrema perlatum* powder were made using a 10:1 ratio of solvent to plant material and placed on a shaker for 36 hours to ensure thorough extraction. Sterile Whatman No. 1 filter paper discs (6 mm) were then soaked in the ethanolic extract and left in the solvent for the same period.

At the same time, 100 mL of Nutrient Agar was prepared and poured into five sterile Petri dishes. Once the agar had solidified, the surface of each plate was evenly covered with the *Bacillus subtilis* culture using a sterile cotton swab. On two of the plates, the ethanolic extract- soaked discs were gently placed onto the agar using sterile forceps. For another two plates, small wells were made in the agar, into which the aqueous extract was added. One plate was kept as a negative control, with sterile water added instead of the extract. All plates were incubated at 37°C for 48 hours, and the bacterial growth was observed for clear zones around the discs or wells, indicating antibacterial activity.

2.4 Databases:

2.4.1 Protein Data Bank

The Protein Data Bank (PDB) serves as a comprehensive repository of three-dimensional structures of biological macromolecules [2]. In this study, four bacterial protein structures were retrieved from the RCSB PDB in .pdb format for molecular docking analyses: DL-endopeptidase CwlO from Bacillus subtilis (PDB ID: 8WT4) [2,10,11], D,D-peptidase Rv3330 from Mycobacterium tuberculosis (PDB ID: 4PPR) [2,13], Cagβ ATPase from Helicobacter pylori (PDB ID: 6JHO) [2,19], and dihydrofolate reductase (DHFR) from Staphylococcus aureus (PDB ID: 2W9S) [2,6]. These proteins were selected as targets to assess the binding interactions and potential inhibitory effects of phytochemicals derived from Parmotrema perlatum.

2.4.2 PubChem Database

PubChem is an open-access database providing comprehensive information on the chemical properties and biological activities of small molecules. As part of the NCBI Entrez system, it is organized into three interconnected databases. In this study, the chemical structures of Thujopsene and Resibufogenin were retrieved from PubChem [8]in SMILES format and subsequently converted into PDB format for use in molecular docking experiments[8].

2.5 Software

2.5.1 AutoDock Vina

AutoDock Vina is an open-source software tool used for molecular docking, helping predict how small molecules bind to a receptor protein. It uses the PDBQT file format for both input and output, similar to AutoDock. In this study, Thujopsene and Resibufogenin retrieved from PubChem [8] were docked with selected receptor proteins using AutoDock Vina [17] to assess their binding interactions and potential antibacterial activity.

III. RESULT

The GC-MS analysis of the ethanolic extract of Parmotrema perlatum revealed 38 bioactive compounds, including fatty acid derivatives, esters, sugars, phenols, terpenes, and steroid-like molecules. Among these, Thujopsene [17] and Resibufogenin [17] were selected for molecular docking studies. Using AutoDock Vina [17], these compounds were virtually screened against four bacterial target proteins: DL- endopeptidase CwlO from Bacillus subtilis (PDB ID: 8WT4) [2,7], D, D-peptidase Rv3330 from Mycobacterium tuberculosis (PDB ID: 4PPR) [13], Cagβ ATPase from Helicobacter pylori (PDB ID: 6JHO) [19], and dihydrofolate reductase (DHFR) from Staphylococcus aureus (PDB ID: 2W9S) [6].

3.1 Thujopsene [8]

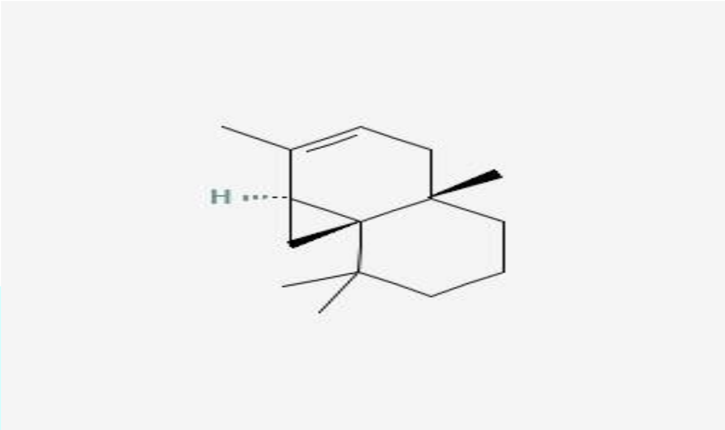


Fig.3: Thujopsene

3.2 Resibufogenin [8]

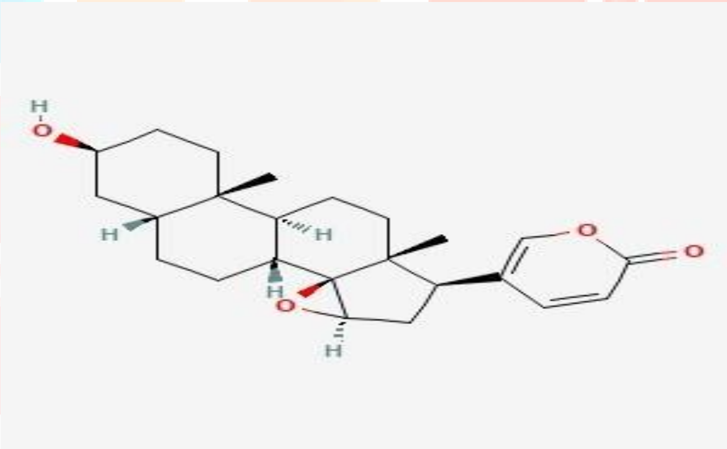


Fig.4: Resibufogenin

3.3 Protein and Compound Retrieval:

The three-dimensional crystal structures of selected bacterial target proteins were obtained from the Protein Data Bank (PDB) for molecular docking studies. These proteins are functionally important and expressed throughout the bacterial life cycle, making them suitable targets for antimicrobial evaluation.

For *Mycobacterium tuberculosis*, D, D-peptidase Rv3330 (PDB ID: 4PPR) was used, solved at 2.00 Å resolution [13]. This enzyme is essential for cell wall synthesis, and its complex with the β-lactam antibiotic meropenem provides insight into active site interactions. The Cagβ ATPase from *Helicobacter pylori* (PDB ID: 6JHO), resolved at 2.60 Å [19], functions as the ATPase motor of the type IV secretion system, important for virulence factor transport. For *Staphylococcus aureus*, dihydrofolate reductase (DHFR) (PDB ID: 2W9S), determined at

1.80 Å resolution [6], is critical for folate metabolism supporting DNA and protein synthesis. From *Bacillus subtilis*, DL-endopeptidase CwlO (PDB ID: 8WT4), solved at 2.06 Å [2,7], plays a key role in peptidoglycan remodeling during cell wall elongation and maintenance.

A total of 38 phytochemicals identified in the ethanolic extract of *Parmotrema perlatum* through GC-MS analysis were selected for in silico evaluation. Their chemical structures were retrieved in SMILES format from

PubChem [8] and converted into PDBQT format. Among these, Thujopsene [8] and Resibufogenin [8] were prioritized based on their docking scores and pharmacological relevance. These compounds were docked with the selected bacterial proteins using AutoDock Vina [17], and their interactions were analyzed to predict potential antibacterial activity.

3.4 Binding Site Identification

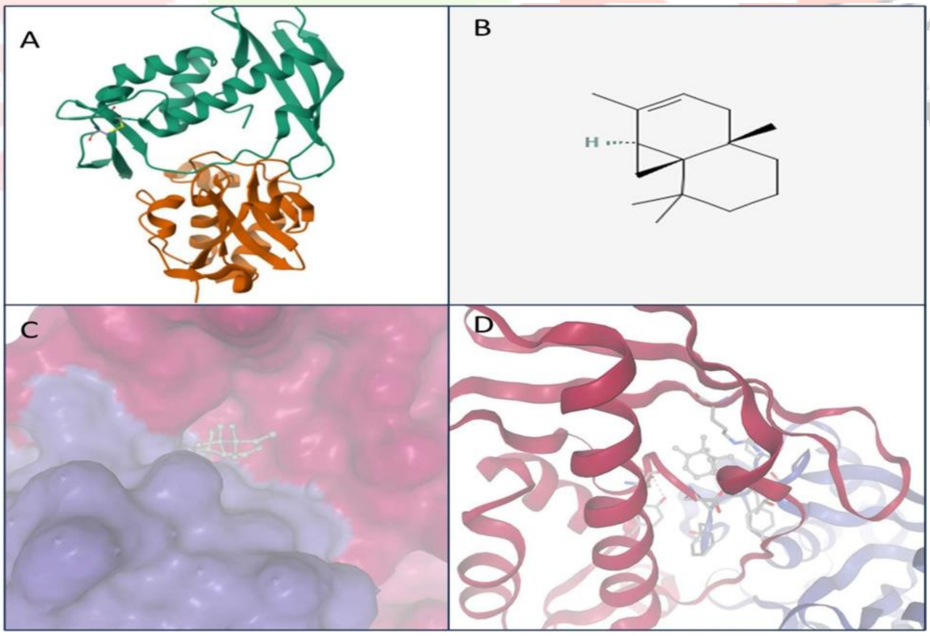
Potential active sites on the target proteins were predicted using DoGSiteScorer [18] via the Proteins Plus web server, helping to identify likely binding pockets for docking studies.

3.5 Molecular Docking Analysis

Molecular docking was carried out using AutoDock Vina [17] to evaluate the binding interactions of Thujopsene [8] and Resibufogenin [8] with selected bacterial target proteins. The proteins included D, D-peptidase Rv3330 from *Mycobacterium tuberculosis* (PDB ID: 4PPR) [13], Cagβ ATPase from *Helicobacter pylori* (PDB ID: 6JHO) [19], and dihydrofolate reductase (DHFR) from *Staphylococcus aureus* (PDB ID: 2W9S) [6]. To incorporate a non-pathogenic model organism, DL-endopeptidase CwlO from *Bacillus subtilis* (PDB ID: 8WT4) [2,7] was also included. The predicted binding affinities, measured in kcal/mol, are summarized in Tables 1 and 2.

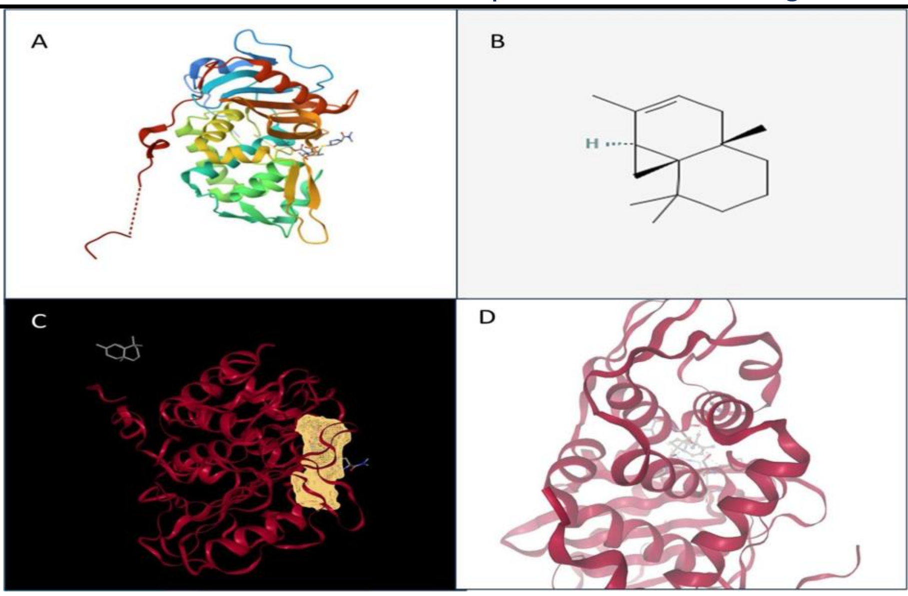
Table 1: Binding Affinity (ΔG) of Thujopsene with Target Proteins from Pathogenic Bacteria Identified via AutoDock Vina

Compound	Target Protein	Organism	PDB ID	Binding Affinity (kcal/mol)
Thujopsene	Dihydrofolate Reductase (DHFR)	*Staphylococcus aureus*	2W9S	–6.009
Thujopsene	DL-endopeptidase CwlO	*Bacillus subtilis*	8WT4	–5.817
Thujopsene	Cagβ ATPase	*Helicobacter pylori*	6JHO	–5.413
Thujopsene	D, D-peptidase Rv3330	*Mycobacterium tuberculosis*	4PPR	–4.762



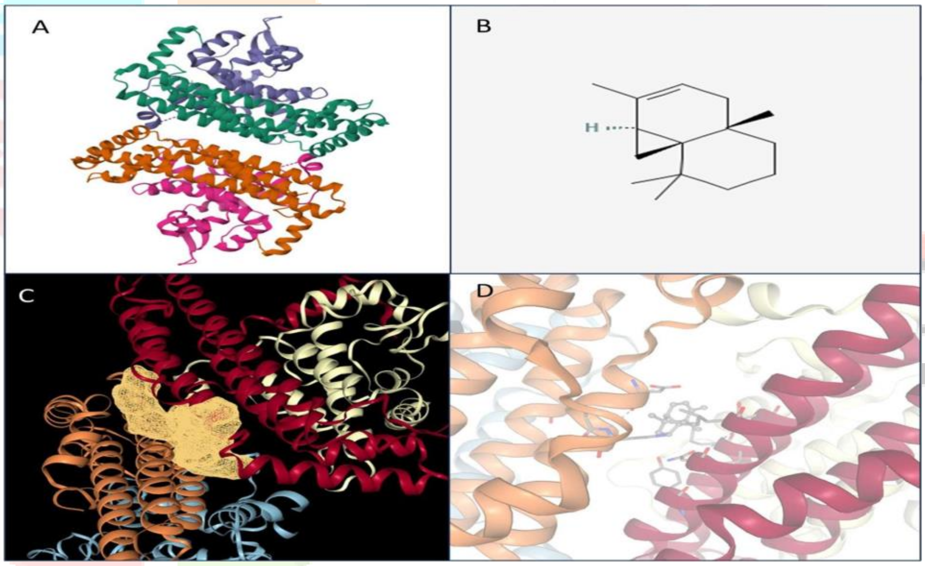
A. Protein - DL-endopeptidase CwlO **B.** Ligand- Thujopsene, **C.** Ligand binding pocket, **D.** Protein-ligand interaction

Fig.5: Docking interaction of Thujopsene with DL-endopeptidase CwlO (PDB ID: 8WT4) from Bacillus subtilis.



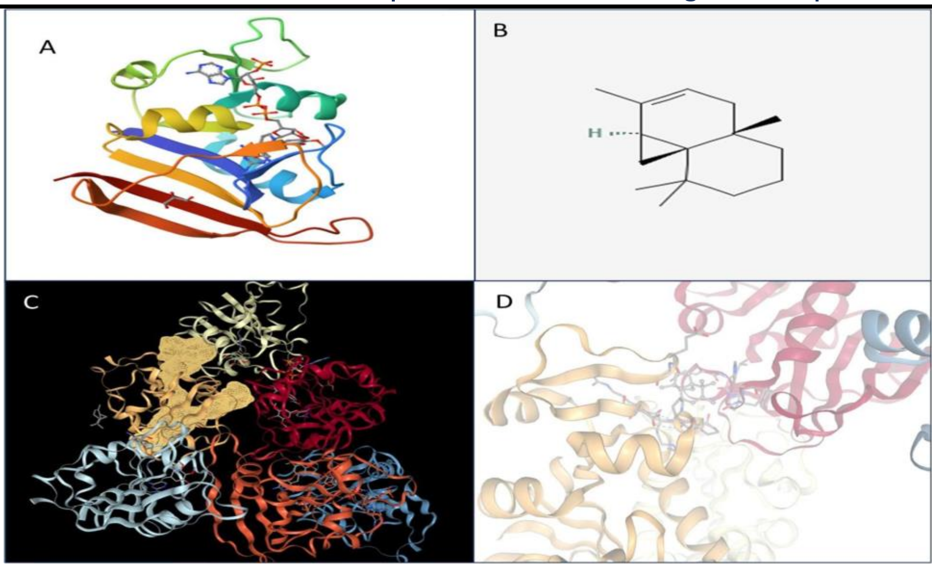
A. Protein - D, D-peptidase Rv3330 **B.** Ligand- Thujopsene, **C.** Ligand binding pocket, **D.** Protein-ligand interactions

Fig.6: Molecular Docking Visualization of Thujopsene with D, D-peptidase Rv3330 (PDB ID: 4PPR) from *Mycobacterium tuberculosis*



A. Protein- Cagβ ATPase **B.** Ligand- Thujopsene, **C.** Ligand binding pocket, **D.** Protein-ligand interactions

Fig.7: Molecular Docking Visualization of Thujopsene with Cagβ ATPase (PDB ID: 6JHO) from *Helicobacter pylori*

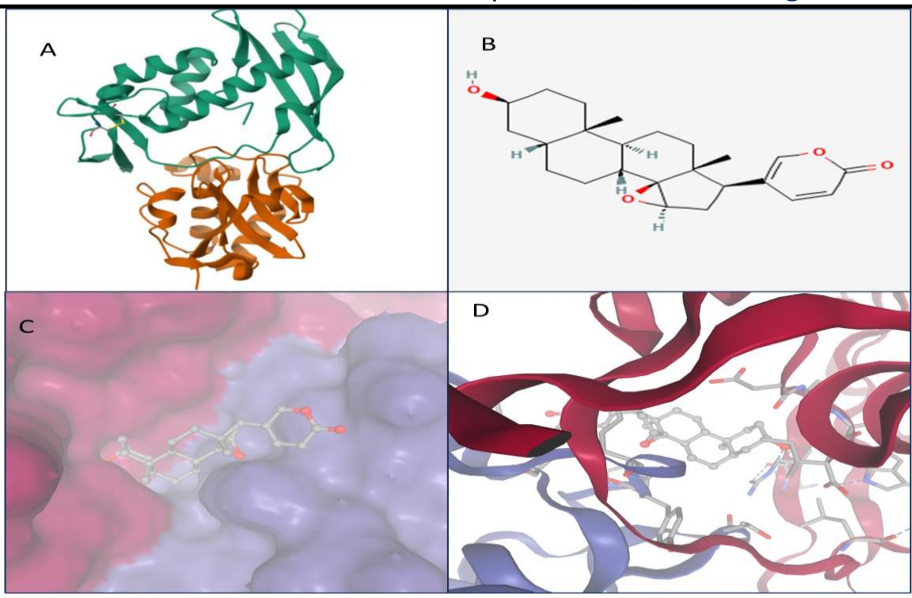


A. Protein- Dihydrofolate Reductase (DHFR) **B.** Ligand- Thujopsene, **C.** Ligand binding pocket, **D.** Protein-ligand interactions

Fig.8: Molecular Docking Visualization of Thujopsene with Dihydrofolate Reductase (PDB ID: 2W9S) from *Staphylococcus aureus*

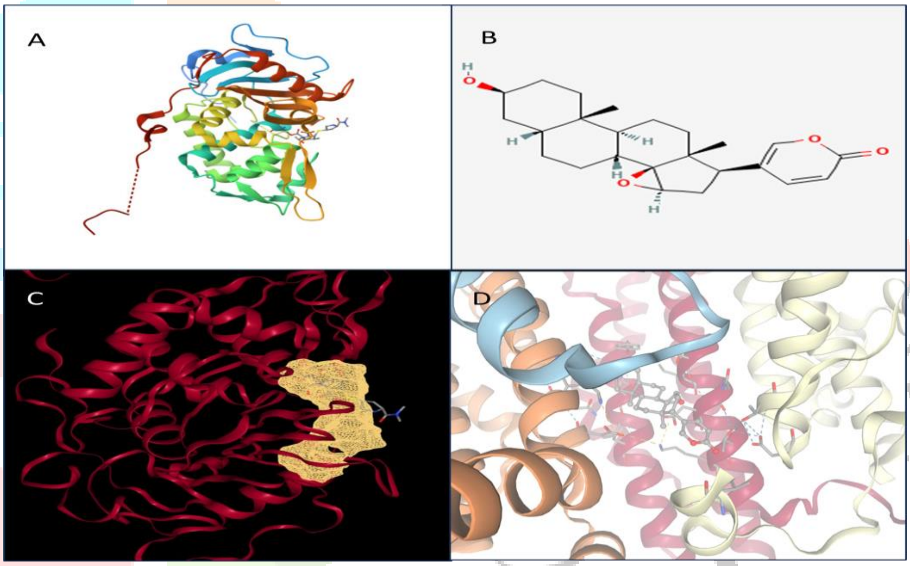
Table 2: Binding Affinity (ΔG) of Resibufogenin with Target Proteins from Pathogenic Bacteria Identified via AutoDock Vina

Compound	Target Protein	Organism	PDB ID	Binding Affinity(kcal/mol)
Resibufogenin	Dihydrofolate Reductase (DHFR)	*Staphylococcus aureus*	2W9S	–8.098
Resibufogenin	Cagβ ATPase	*Helicobacter pylori*	6JHO	–7.708
Resibufogenin	DL-endopeptidase CwlO	*Bacillus subtilis*	8WT4	–6.308
Resibufogenin	D, D-peptidase Rv3330	*Mycobacterium tuberculosis*	4PPR	–5.062



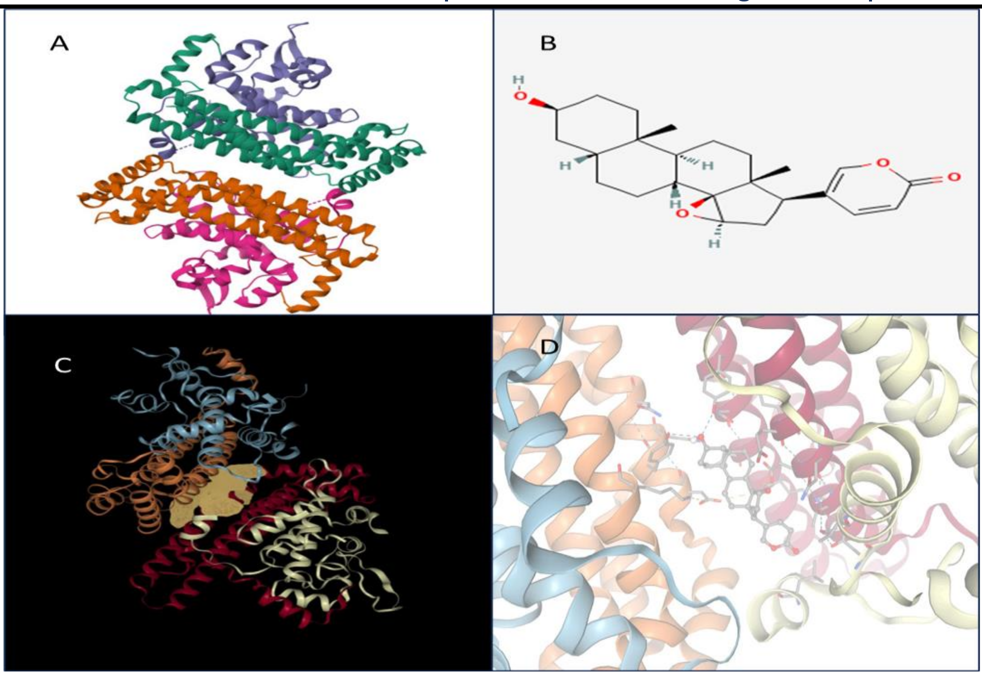
A. Protein - DL-endopeptidase CwlO **B.** Ligand - Resibufogenin, **C.** Ligand binding pocket, **D.** Protein-ligand interactions

Fig.9: Docking interaction of Resibufogenin with DL-endopeptidase CwlO (PDB ID: 8WT4) from *Bacillus subtilis.*



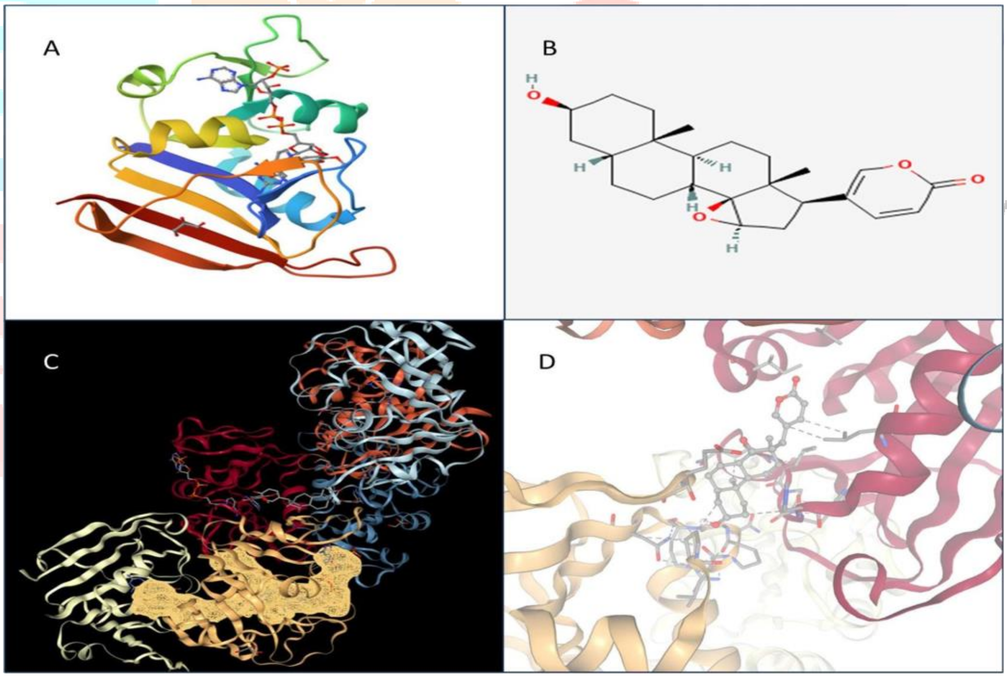
A. Protein - D, D-peptidase Rv3330 **B.** Ligand - Resibufogenin, **C.** Ligand binding pocket, **D.** Protein-ligand interactions

Fig.10: Molecular Docking Visualization of Resibufogenin with D, D-peptidase Rv3330 (PDB ID: 4PPR) from *Mycobacterium tuberculosis*



A. Protein - Cagβ ATPase **B.** Ligand - Resibufogenin, **C.** Ligand binding pocket, **D.** Protein-ligand interactions

Fig.11: Molecular Docking Visualization of Resibufogenin with Cagβ ATPase (PDB ID: 6JHO) from *Helicobacter pylori*



A. Protein - Dihydrofolate Reductase (DHFR) **B.** Ligand - Resibufogenin, **C.** Ligand binding pocket, **D.** Protein-ligand interactions

Fig.12: Molecular Docking Visualization of Resibufogenin with Dihydrofolate Reductase (PDB ID: 2W9S) from *Staphylococcus aureus.*

Table 3: Antibacterial Activity of *P. perlatum* Extracts Against *Bacillus subtilis*

Sample	Bacterial Strain	Zone of Inhibition (mm)	Mean ± SD
Aqueous extract	*Bacillus subtilis*	13, 12	12.5 ± 0.71
Ethanolic extract	*Bacillus subtilis*	10	10.0 ± 0.00

3.6 Results of Antibacterial Assay

The aqueous and ethanolic extracts of *Parmotrema perlatum* were tested for antibacterial activity against *Bacillus subtilis*. Both extracts showed mild inhibition, with the aqueous extract producing a zone of inhibition of about 12.5 mm and the ethanolic extract a smaller zone of around 10 mm. These results suggest only limited antibacterial potential under the tested conditions. The lower activity observed may be linked to differences in extraction methods, the levels of bioactive compounds, or natural variation in lichen material. While some inhibitory effect was evident, the findings indicate that crude extracts on their own may not be sufficiently effective, highlighting the need for further studies using purified fractions, higher concentrations, or possible synergistic approaches.

IV. DISCUSSION AND CONCLUSION

This study explored the antimicrobial and drug-like potential of *Parmotrema perlatum* extracts through both in vitro and in silico approaches. The in vitro assays demonstrated only modest zones of inhibition against selected bacterial strains, indicating limited direct antibacterial action under the tested conditions. However, these findings are not unexpected, as crude extracts may contain a complex mixture of active and inactive compounds, where synergistic or concentration-dependent effects are not fully expressed.

By contrast, in silico molecular docking revealed notable interactions between bioactive compounds of *P. perlatum* and bacterial targets relevant to pathogenicity and resistance, including proteins from *Helicobacter pylori*, *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Bacillus subtilis* [1,3,4,11,12,14]. These results align with previous reports that lichen-derived secondary metabolites often act on specific molecular pathways rather than producing strong broad-spectrum effects in crude form.

Interestingly, docking studies highlighted a potential for certain metabolites to disrupt bacterial enzymes such as dihydrofolate reductase and penicillin-binding proteins, both of which are established antibiotic targets. This observation suggests that *P. perlatum* could serve as a source of lead compounds for the development of adjunct or novel therapeutics. Moreover, the limited in vitro inhibition recorded against *Bacillus* sp. [7,10,11] provides preliminary evidence that certain bacterial groups may be more susceptible, though further testing is required to validate this finding.

Overall, this work highlights the importance of integrating in vitro and in silico methods in natural product research. While direct antibacterial effects of crude extracts may appear modest, computational screening provides valuable insight into potential molecular mechanisms and supports the case for compound isolation and optimization.

In conclusion, the findings suggest that *Parmotrema perlatum* harbours secondary metabolites with promising antimicrobial potential at the molecular level. Although crude extracts displayed limited in vitro activity, docking results underscore the possibility that purified compounds may demonstrate stronger effects. Future work should focus on fractionation of the extract, evaluation of synergistic combinations with existing antibiotics, and mechanistic studies to establish precise modes of action. Such efforts will be essential to fully unlock the therapeutic potential of *Parmotrema perlatum* in addressing the global challenge of antimicrobial resistance [3].

V. FUTURE ASPECT

This study gives a good starting point for exploring the antibacterial properties of *Parmotrema perlatum*, especially its active compounds like Thujopsene and Resibufogenin. To understand their true potential, future research could focus on isolating these compounds in pure form and testing them at different concentrations. It would also be useful to try different extraction methods and test the extracts against more types of bacteria, including those that are resistant to antibiotics.

More advanced studies like checking if these compounds work better when combined with existing antibiotics, or testing their safety in living systems can provide deeper insights. Exploring how these compounds behave in real biological environments and improving their delivery through techniques like nano-formulations may also make them more effective. Overall, combining both lab experiments and computer-based studies could bring us closer to developing new treatments from natural sources like lichens.

VI. ACKNOWLEDGEMENT

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