

In vitro antibiofilm activity of *Aquilaria malaccensis* leaf extracts and literature-derived docking studies on LasR and RhIR of *Pseudomonas aeruginosa*

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Research Article

Keywords: *Aquilaria malaccensis*, Antibiofilm, Quorum sensing, *Pseudomonas aeruginosa*, Molecular docking

Posted Date: January 7th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-7082118/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background: *Pseudomonas aeruginosa* biofilm-related infections are a major problem owing to their resistance to common antibiotics. *Aquilaria malaccensis*, with its high phytochemical content, has exhibited some antibacterial activity; nevertheless, its antibiofilm activity has not yet been explored.

Methods: The leaves of *A. malaccensis* were sequentially extracted with hexane, ethyl acetate, and 80% ethanol. The antibiofilm activity of the extracts was assessed using a crystal violet-based microtiter plate assay against clinical isolates of biofilm-producing *Pseudomonas aeruginosa*. Due to resource limitations, GC-MS analysis was not performed; however, literature-reported sesquiterpenes were chosen for molecular docking experiments based on AutoDock Vina. The target proteins for quorum-sensing inhibition were LasR (PDB: 2UV0) and RhIR (PDB: 8DQ0).

Results: The hexane extract possessed the greatest antibiofilm activity (98.32% inhibition at 150 mg/mL), followed by ethyl acetate (75.10%), whereas the ethanol extract did not. Docking analysis indicated that α -bisabolol and jinkoh-eremol had strong affinities for LasR (−8.9 and −8.7 kcal/mol) and RhIR (−9.1 and −8.7 kcal/mol), overcoming the native ligands. These compounds also established multiple hydrogen bonds and hydrophobic interactions, suggesting potential disruption of bacterial quorum sensing.

Conclusion: The antibiofilm activity of *A. malaccensis* leaf extracts, and notably the hexane fraction, can be attributed to sesquiterpenes, such as α -bisabolol and jinkoh-eremol. This study provides the first evidence of the plant's potential antibiofilm activity and indicates its potential as a source of natural quorum-sensing inhibitors.

INTRODUCTION

The leaves of *Aquilaria malaccensis* are a rich source of secondary metabolites which has diverse pharmacological value. The methanol extract of the leaves contains flavonoids, triterpenoids, steroids and phenolics, while in its n-hexane fraction consists of triterpenoids and steroids. On the contrary, the methanol-water extract characteristically features compounds, for example, flavonoids, triterpenoids and phenolic compounds (Hidayah et al., 2023). These bioactive constituents containing phenols, terpenoids, flavonoids, glycosides, tannins, and steroids/triterpenoids demonstrate high antioxidant activity, validating their traditional medicinal uses (Ghiffari & Fevria, 2024). Based on its diverse secondary metabolite profile, the leaves of *A. malaccensis* also exhibit remarkable antimicrobial potential. The ethyl acetate fraction has shown effective antibacterial activity compared to the n-hexane fraction with MIC values as low as 0.2% targeting bacteria associated with skin infections (Sompa et al., 2024). While in another study, ethanol extracts have a notable inhibition against *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Escherichia coli*, where bioautographic analysis has identified active compounds responsible for these effects (Marsaoly, 2024). In addition, the ethanol extract shows significant activity against *Escherichia coli* and *Staphylococcus aureus*, showing zones of inhibition up to 13.34 mm, which further supports the leaves' therapeutic potential (Misrahanum et al., 2022). More experiments reveal that extraction methods and solvents have effects on the yield and antibacterial activity of the leaf extracts. The soxhlet method with ethanol solvent extracted the highest yield (18.4%) and exhibited better phenolic and tannin content, which translates to better antibacterial properties, especially against Gram-positive *S. aureus* (Santoso et al., 2022). Crude extracts showed a large amount of inhibition zones, namely 14 mm against *Acinetobacter baumannii* and 9.7 mm against *Klebsiella pneumoniae*, proving that it could be potential in the fight against drug-resistant pathogen (Jihadi et al., 2020). Flavonoids such as quercetin, kaempferol, and myricetin are specifically known for their anti-inflammatory, antioxidant, and antimicrobial activities. GC-MS analysis has also revealed unique compounds like hesperetin and lupeol, which contribute to anti-inflammatory activities. Moreover, extract of the leaves has shown a bacteriostatic effect in addition to some antimicrobial activities against *Candida albicans*, and *Propionibacterium acnes* by possible synergic action between tannin and flavonoids, respectively (Batubara et al., 2021). Among phenolic acids, sissotrin and lupeol have been identified in *Aquilaria* for the first time, while glucopyranosyl sinapate and coumaric acid illustrate the anti-inflammatory and oxidative stress benefits attributed to this plant. Similarly, the sterols such as β -sitosterol and stigmasterol, the terpenoids like nerolidol and α -amyrin, and fatty acids such as linoleic acid, among others, contribute to its medicinal values. The presence of unique compounds like mangiferin and genkwanin-5-O-beta-primeveroside may also indicate potential applications in the management of diabetes, oxidative stress, and neuroprotection (Kakino & Hara, 2016). *A. malaccensis* leaves show outstanding chemical diversity and therapeutic potential of the identified metabolites. Amongst these findings were bioactive compounds n-hexadecanoic acid, eudesmol, and oxo-agarospirol, elucidated through advanced spectroscopic techniques, including NMR, MS, UV, and FTIR (Asma & Wil, 2019; Eissa et al., 2020). These compounds, along with friedelanol and

friedelin, which were extracted with hexane and dichloromethane, respectively, indicate great pharmacological potential in the leaves of this species. Advanced methodologies used, starting from solvent extractions (ethanol, methanol, and hexane) down to the detection tools LC-QTOF-MS and GC-MS, point toward the strict approach in profiling these metabolites. Such a comprehensive chemical inventory not only enhances our pharmacological understanding of the promises of *A. malaccensis* leaves but places them at the doorstep of therapeutic incorporation. These results also show that *A. malaccensis* leaves are a robust source for antibacterial products, thus emphasizing their worth in combating infectious diseases. At the time of writing this paper, there are no study reported on the antibiofilm activity of *A. malaccensis* leaf extracts against common bacterial pathogens, much less the identification of compounds with potential antibiofilm activity. This study evaluates the potential of the leaf extract to inhibit the initial adhesion of biofilm-forming bacteria and compares the chemical compounds from the literature that are docked into known biofilm formation proteins.

METHODOLOGY

In vitro studies

Solvent extraction of leaves and preliminary phytochemicals tests

Mature *Aquilaria malaccensis* leaves were collected from the plantation area of the university campus (**Figure S1**), washed, dried and powdered. Soxhlet extraction was carried out sequentially and exhaustively with hexane, ethyl acetate and 80% (v/v) ethanol. The resulting extracts were dried by evaporation and the extract yields were calculated. The extracts were further assessed qualitatively for the phytochemicals present. These included flavonoids, tannins, saponins, alkaloids, steroids, and glycosides using standard methodology. Each extract was also scanned within the spectrum of 200-800nm in a UV-Visible Spectroscopy (Systronics Double Beam Spectrophotometer, 2202) using the respective pure solvents as the blank.

Determination of antibacterial activity

Antibacterial activity, by agar-well diffusion assay, was tested against Gram-positive bacteria - *Staphylococcus epidermidis* (ATCC-35984), *Enterococcus* species (8061), and Gram-negative bacteria – *Escherichia coli* (106 & 99), *Klebsiella pneumoniae* (ATCC-BAA-1705), *K. pneumoniae* (357), *Pseudomonas aeruginosa* (479 & 2728), *Acinetobacter baumannii*, and *Proteus mirabilis*. Non-ATCC strains were clinical isolates collected from a local hospital. The focus of this study lies in testing the antibiofilm activity of the leaf extracts, only *Pseudomonas aeruginosa* was selected for further evaluation. The negative control used was 40% DMSO which was also used to dissolve the leaf extracts.

Biofilm formation assay

Both biofilm formation assay and antibiofilm activity of the leaf extracts were tested by the indirect, semi-quantitative crystal violet inference assay (Das et al., 2017). The biofilm formation assays were conducted in triplicate using borosilicate test tubes. Briefly, 3 mL of autoclaved tryptic soy broth (TSB) supplemented with 2% glucose was inoculated with bacterial isolates at 1×10^6 CFU/mL. The broth was then incubated for 24 hours at 37°C and then discarded; the tubes were then washed with autoclaved distilled water. Biofilms were stained using 5 mL of 1% crystal violet for 15 minutes before being washed with distilled water three times to remove excess stain. The stain in biofilms were solubilized with 5 mL of 33% glacial acetic acid, and the optical density was measured at 570 nm. A blank control was included in all assays. Tube method was carried out to assess the ability of *Pseudomonas aeruginosa* isolates to form biofilms. From the observations, isolates with best biofilm forming ability were selected for the antibiofilm studies.

Anti-adherence antibiofilm activity

To evaluate the antibiofilm activity of the leaf extracts, a microdilution assay was carried out by following the same protocol as the biofilm formation assay (Das et al., 2017). In brief, 100 µL of autoclaved media was added to each well, making the final working volume 200 µL. The wells were inoculated with *Pseudomonas aeruginosa* isolates at 1×10^6 CFU/mL, and the plant extracts were

added at concentrations of 10, 50, 100, and 150 mg/mL for hexane and ethyl acetate extracts, and 0.1, 0.5, 1, and 1.5 g/mL for ethanolic extracts. The endpoint evaluation was performed as mentioned earlier.

Phytocompounds and Ligand preparation

Identification of the phytocompounds of the extracts showing antibiofilm activity was beyond the resources. Therefore, compounds that were previously reported from published literature of extracts prepared from *A. malaccensis* leaves were listed out. The compounds were selected from the following phytochemical classes - terpenoids (11), monoterpenes (4), sesquiterpenes (19), and oxygenated sesquiterpenes (15); each of them reported to be present in the leaves of this species (Asma & Wil, 2019). These ligands were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) (Wang et al., 2009) in Structure Data File (SDF) format. These ligands were converted to Protein Data Bank (PDB) format using Open Babel. This conversion ensured compatibility with downstream computational tools.

Prediction of ADME Properties Using SwissADME

The pharmacokinetic and drug-likeness properties of the selected compounds were evaluated using the SwissADME web tool (<http://www.swissadme.ch/>), developed by the Swiss Institute of Bioinformatics. The SMILES representations of the compounds were inputted into the platform to predict key physicochemical properties (e.g., molecular weight, LogP, LogS, hydrogen bond donors/acceptors, and TPSA) and drug-likeness based on Lipinski's Rule of Five, Veber's Rule, and other filters (Ghose, Egan, Muegge). Pharmacokinetic predictions included gastrointestinal absorption, blood-brain barrier permeability, and cytochrome P450 enzyme interactions (CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4). The BOILED-Egg model and bioavailability radar were used for visual analysis of absorption, distribution, and drug-likeness (Daina et al., 2017). Results were systematically analyzed to identify compounds with favorable profiles, with data presented in tabular and graphical formats. This streamlined, *in silico* approach provided insights into the suitability of compounds as potential drug candidates.

Protein Preparation

Structures of target proteins were extracted from Protein Data Bank (PDB) (<https://www.rcsb.org/>) (Bernstein et al., 1977). These are *Pseudomonas aeruginosa* LasR quorum-sensing receptor (PDB: 2UV0) and quorum-sensing regulator, RhlR (PDB: 2DQ0). Protein preparation for molecular docking was carried out using AutoDockTools (ADT) (<https://autodock.scripps.edu/>) (Morris et al., 2009). The PDB files were loaded into ADT, and water molecules, heteroatoms, and bound ligands were removed. The protein is protonated to ensure proper charge states at physiological pH. Polar hydrogens are added, and appropriate partial charges are assigned using the Kollman method to optimize the 3D structure of the protein. The structure is saved in PDBQT format, including atomic charges and rotatable bonds, ready for docking simulations.

Molecular Docking Studies

Molecular docking was conducted to determine the ligand-target protein binding interactions. The target proteins' binding sites were determined through PyMOL (Delano, 2002) by analyzing the interactions between the proteins and their native ligands available in the PDB. Rigid docking simulations were carried out using AutoDock Vina (<https://autodock.scripps.edu/>) (Trott & Olson, 2010), allowing the prepared ligands to dock into the predefined active sites of the proteins. This docking procedure shed light on the binding mechanisms of the test ligands by comparing the interactions with the protein with the native ligands. The results provided insights into the affinity of ligand-protein binding and interaction patterns, crucial in the evaluation of the efficacy of the test compounds. After performing the docking simulations, AutoDock Vina provides a score representing the binding affinity, usually expressed in kcal/mol. A more negative binding energy would indicate a stronger binding affinity between the ligand and the protein. The lowest binding energy conformation was considered to be the most favorable, which means the most stable interaction between the ligand and the protein. The used values of binding energy evaluated and compared the relative strength of interaction of the test ligands with the target protein and thus, provide a potential insight into them as effective binders.

RESULTS

In vitro studies

Solvent extraction of leaves and preliminary phytochemicals tests

The extraction of *A. malaccensis* leaves using different solvents included hexane, ethyl acetate, and 80% (v/v) ethanol, with differing efficiencies; highest yield was shown by ethanol 40%, followed by hexane (12.3%) and then ethyl acetate (10%) (**Table 1**). The differences in yield percentages indicate variations in solvent polarity, influencing the extraction efficiency of bioactive compounds from agarwood leaves. Phytochemical screening shows that flavonoids and tannins are present in ethanolic and ethyl acetate extracts and absent in the hexane extract with ethanol being one of the most effective solvents for extracting phenolic compounds (**Table 1**). Alkaloids are found only in the hexane extract by Wagner's test, and steroids were present in both ethanol and hexane extracts, implying a higher affinity of non-polar solvents with these secondary metabolites. Steroids were present in both ethanol and hexane extracts but absent in the ethyl acetate extract. Glycosides were not detected in any of the solvent extracts. These findings suggest that ethanol is the most effective solvent for extracting phenolic compounds such as flavonoids and tannins, while hexane extracts predominantly contain alkaloids and steroids. UV-Vis spectrophotometric analysis further confirmed the presence of diverse phytoconstituents, with a consistent absorption peak at 468.8 nm across all extracts (**Figure S2**) indicating shared chromophores. Additional peaks for the hexane extract appeared at 363.2, 533.6, 608.0, and 668.0 nm; ethyl acetate extract showed peaks at 363.2, 497.6, 608.0, and 648.8 nm; while the ethanolic extract exhibited peaks at 363.2, 468.8, 497.6, and 660.8 nm likely corresponding to flavonoids and other phenolic compounds.

Antibacterial Activity of *A. malaccensis* Extracts

The antibacterial potential of hexane, ethyl acetate, and ethanolic extracts of *A. malaccensis* leaves, and their minimum inhibitory concentration was evaluated using agar well diffusion methods, and microtiter plate assay respectively. The extracts demonstrated antimicrobial activity against both Gram-positive (*S. epidermidis* ATCC-35984, *Enterococcus* spp.) and Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *P. mirabilis*). Among the tested extracts, the ethyl acetate extract exhibited the highest antibacterial activity, with an inhibitory zone of 20 mm against *P. aeruginosa* 479, followed by 15 mm against *K. pneumoniae* ATCC-BAA-1705. The hexane extract was also effective, particularly against *Enterococcus* spp. and *P. mirabilis* (9 mm inhibition zones). In contrast, ethanolic extract displayed minimal antibacterial activity, with inhibition observed only against *A. baumannii* (9 mm) and *P. mirabilis* (8 mm) (**Table S1**).

Antibiofilm Activity of *A. malaccensis* Extracts

The antibiofilm activity of *A. malaccensis* leaf extracts was assessed against clinical isolates of *P. aeruginosa* using microtiter plate assay. The biofilm formation assay confirmed strong biofilm development by *P. aeruginosa*. Hexane and ethyl acetate extracts demonstrated dose-dependent biofilm inhibition (**Figure S3**). Hexane extract at 150 mg/mL exhibited the highest inhibition of 98.73%, followed by ethyl acetate extract at 150 mg/mL with 53.64% inhibition (**Table 2**). Lower concentrations of 50 mg/mL and 10 mg/mL showed significantly reduced antibiofilm efficacy, with only 24.52% and 13.13% inhibition, respectively, for hexane extract (**Figure 1**). Notably, the ethanolic extract failed to inhibit biofilm formation of *P. aeruginosa*, suggesting that non-polar solvents (hexane, ethyl acetate) are more effective in biofilm inhibition than polar solvents.

In silico Analysis

SwissADME Screening and Drug-Likeness Assessment

The selected terpenoids from *Aquilaria malaccensis* were first subjected to ADME profiling using SwissADME to evaluate their pharmacokinetic properties and compliance with Lipinski's Rule of Five. Among the screened compounds, only those without violations were considered for molecular docking studies. These compliant compounds included α -Bisabolol, Jinkoheremol, β -Agarofuran, Agarospirol, and other oxygenated sesquiterpenes and terpenoids (**Table 3**). These compounds exhibited high gastrointestinal (GI) absorption, favorable blood-brain barrier (BBB) permeability, and acceptable lipophilicity ($\text{LogP} < 5$), indicating their potential as bioavailable quorum-sensing inhibitors. Conversely, several sesquiterpenes and triterpenoids, such as α -Selinene, β -Selinene, α -Guaiene, and Betulinic Acid, violated Lipinski's rule due to excessive lipophilicity ($\text{LogP} > 5$) or poor GI absorption, thereby disqualifying them from further docking analysis.

Molecular Docking of Compliant Terpenoids

The AutoDock Tools and AutoDock Vina were used to predict the binding affinities and molecular interactions of compliant terpenoids with LasR (PDB: **2UV0**) and RhIR (PDB: **8DQ0**) through a rigid docking approach where the grid box was placed exactly at the native ligand-binding site for precise comparisons. The native ligands (3-oxo-C12-acylhomoserine lactone for LasR and N-Butanoyl-L-homoserine lactone for RhIR) were docked with the purpose of establishing a baseline binding energy cut-off. Among the tested terpenoids (**Table 3**), α -Bisabolol (-8.9 kcal/mol), and Jinkoheremol (-8.7 kcal/mol) exhibited the highest binding affinities to LasR. Similarly, α -Bisabolol (-9.1 kcal/mol), and Jinkoheremol (-8.7 kcal/mol) had significant binding to RhIR, indicating their potential as quorum-sensing inhibitors (**Table 4** and **Figure 2**).

Interaction Analysis Using LigPlot+

Analysis of the docked complexes' hydrogen bonding and hydrophobic interactions was performed with LigPlot+, highlighting key molecular interactions in **Table 4** and **Figure 2**. For LasR, Jinkoheremol established hydrogen bonding with Tyr64 and Asp73, stabilizing it within the binding pocket. α -Bisabolol was hydrogen bonded to Tyr72 and Asp81, and the strong binding of the molecule is supplemented by hydrophobic contacts with Trp96, Phe101, and Leu116 in RhIR. Docking and interaction analysis indicate that α -Bisabolol, and Jinkoheremol are potential quorum-sensing inhibitors and further studies for their validation in *in vitro* and *in vivo* settings.

DISCUSSION

The antibacterial and antibiofilm activities of *A. malaccensis* leaf extracts reveal a strong link between solvent polarity, bioactivity. Differences in the effectiveness of the extracts for antimicrobial action point toward the selective extraction of bioactive compounds, as reflected by hexane and ethyl acetate being the most active extracts. The ethyl acetate extract showed the strongest antibacterial activity, especially against *P. aeruginosa*, which indicates that medium-polarity antimicrobial compounds such as flavonoids and tannins may be present. The ethanolic extract, though having the highest yield, had minimal antibacterial activity, suggesting that though ethanol extracts a wide range of compounds, it may not selectively isolate compounds with strong antimicrobial activity. The hexane leaf extract of *A. malaccensis* was highly active against *P. aeruginosa* (~98% inhibition at 150 mg/mL), while the ethyl acetate extract was moderate and the ethanolic extract was inactive. This indicates that non-polar bioactive molecules, probably alkaloids and steroids, interfere with the formation of the biofilm matrix or interfere with quorum sensing mechanisms. Ethyl acetate extract showed significant biofilm inhibition, which reinforced the role of flavonoids and tannins in disrupting biofilm integrity. However, the ethanolic extract failed to inhibit biofilm formation, further supporting the hypothesis that effective antibiofilm compounds are better extracted using non-polar or medium-polarity solvents. The differential activity of these extracts underscores the importance of solvent selection in optimizing the isolation of functionally relevant phytochemicals.

Literature based GC-MS studies of *A. malaccensis* (and allied agarwood) attest that its leaves and oils are rich in sesquiterpenes. For instance, the volatile oil of *A. malaccensis* contains α -selinene (~10.99%) and β -selinene (~8.01%) (Tajuddin & Yusoff, 2010). These compounds, however, were not docked with the target proteins as they screened out in the drug-likeness assessment. Both β -selinene (~0.1%) and γ -muurolene (trace) were found in the hexane leaf extracts, with traces of jinkoh-eremol (~0.1%) (Adam et al., 2018). α -Bisabolol (a sesquiterpene alcohol with antimicrobial activity) has also been reported in agarwood oils and *Aquilaria* leaves. For example, α -bisabolol-abundant essential oils from *Nectandra* spp. (closely related sesquiterpenes) prevent *P. aeruginosa* biofilm formation (Farias et al., 2019). Therefore, these sesquiterpenes (α - and β -selinene, γ -muurolene, α -bisabolol, and jinkoh-eremol) are established constituents of the *A. malaccensis* leaf/wood (Adam et al., 2018; Haslina et al., 2018) and might be responsible for the observed activity. To investigate this mechanism, we performed molecular docking of these terpenoids and sesquiterpenes against the *P. aeruginosa* quorum-sensing receptors LasR and RhIR. The findings (**Table 4**) revealed that α -bisabolol and jinkoh-eremol strongly interacted with both proteins. For instance, α -bisabolol interacted with LasR with -8.9 kcal/mol and RhIR with -9.1 kcal/mol, while the native homoserine lactone signals (3-oxo-C12-HSL for LasR, N-butanoyl-HSL for RhIR) interacted with -8.6 kcal/mol and -7.0 kcal/mol respectively. Jinkoh-eremol interacted similarly well with -8.7 kcal/mol to both the receptors. These figures are on par with or better than those of the native ligands, suggesting possible competitive inhibition of QS binding. By inhibiting LasR/RhIR signaling, which has been reported to control biofilm formation in *P. aeruginosa*, these sesquiterpenes could inhibit bacteria from developing mature biofilms. High affinities indicated interference with the adhesion pathways of bacteria as well as with intracellular signaling pathways, and may weaken structural integrity of the biofilm.

α -Bisabolol-enriched oils have been reported to significantly inhibit *P. aeruginosa* biofilms *in vitro* (Farias et al., 2019). The strong antibiofilm activity of the hexane extract was attributed to its sesquiterpene composition. Standard leaf analyses indicate that *A. malaccensis* yields α -selinene, β -selinene, γ -muurolene, α -bisabolol, and jinkoh-eremol (Tajuddin & Yusoff, 2010). Our docking experiments indicated that these compounds should be able to bind and inhibit the *P. aeruginosa* QS regulators LasR and RhIR, thus inhibiting biofilm formation. Combining the *in vitro* biofilm inhibition assays with literature-documented compound profiles through *in silico* findings confirms that these leaf sesquiterpenes are responsible for the reported antibiofilm activity.

Thus, these findings indicate a strong potential therapeutic application of *A. malaccensis* extracts to combat biofilm-associated infections, which are notoriously resistant to conventional antibiotics. The future would lead to the isolation of the most active components, exploration of the molecular mechanisms, and studies on synergistic effects with antibiotics. Further *in vivo* studies on pharmacokinetics and toxicity will lie ahead in advancing such extracts as a natural antibiofilm agent.

CONCLUSION

This study provides evidence of strong antibiofilm activity of *Aquilaria malaccensis* leaf extracts against *Pseudomonas aeruginosa*, with the hexane extract exerting the greatest inhibition ($98.32 \pm 0.58\%$ at 150 mg/mL). The ethyl acetate extract exhibited moderate inhibition, whereas the ethanolic extract was inactive. These results imply that non-polar to moderately polar phytochemicals, particularly sesquiterpenes, are responsible for this activity. Literature-based GC-MS analyses led to the selection of possible terpenoids and sesquiterpenes found in the leaves of *A. malaccensis*. After screening the selected compounds for drug-like properties, 19 compounds showed promise. Molecular docking of these compounds with quorum-sensing proteins LasR and RhIR showed high binding affinities (up to -9.1 kcal/mol) comparable to or superior to those of native ligands especially of the two compounds - α -bisabolol and jinkoh-eremol, validating their potential as quorum sensing inhibitors. The synergistic *in vitro* and *in silico* evidence validates that *A. malaccensis* leaf compounds, especially sesquiterpenes, can be formulated as novel natural antibiofilm compounds.

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Tables

Table 1: Yield and Phytochemical Composition of Agarwood Leaf Extracts

Leaf Extract (Solvent)	Yield Percentage (%)	Flavonoids	Tannins	Saponins	Alkaloids		Steroids	Glycosides
					Mayer's Test	Wagner's Test		
80% Ethanolic	40.0	Present (Red color formed)	Present (Dark green color formed)	Absent (Foam formation was unstable)	Absent (No cream precipitate)	Absent (No reddish-brown precipitate)	Present (Yellow with green fluorescence)	Absent (No rose pink/red color)
Hexane	12.3	Absent (No red/orange color)	Absent (No dark green/blue color)	Absent (Foam formation was unstable)	Absent (No cream precipitate)	Present (Reddish brown precipitate)	Present (Yellow with green fluorescence)	Absent (No rose pink/red color)
Ethyl Acetate	10.0	Present (Red color formed)	Absent (No dark green/blue color)	Absent (Foam formation was unstable)	Absent (No cream precipitate)	Absent (No reddish-brown precipitate)	Absent (No color change observed)	Absent (No color change observed)

Footnotes:

1. Yield Percentage (%) = (Extracted material weight / Initial plant material weight) × 100

Table 2. Antibiofilm Activity of *Aquilaria malaccensis* Extracts Against *Pseudomonas aeruginosa*

Extract Type	Concentration (mg/mL)	% inhibition	IC50 value	<i>p</i> -value
Hexane	150	98.32 ± 0.58	71.2 mg/mL	9.15 × 10 ⁻¹²
	100	96.19 ± 1.07		
	50	16.02 ± 1.42		
	10	3.78 ± 2.68		
Ethyl Acetate	150	75.10 ± 1.49	8.64 mg/mL	0.0035
	100	59.49 ± 2.26		
	50	86.50 ± 2.93		
	10	45.30 ± 18.61		
Ethanolic	1500	0 ± 2.57	Not determined	0.103
	1000	6.27 ± 7.86		
	500	0 ± 2.23		
	100	0 ± 3.23		

Footnote:

p-values indicate statistical significance, with values less than 0.05 considered significant. For highly significant results (*p* < 0.001), exact *p*-values are provided in scientific notation where applicable. Non-significant values (*p* > 0.1) are reported explicitly.

Table 3. SwissADME Screening of Terpenoid Compounds from *Aquilaria malaccensis*

Compound	Lipinski compliance	No. of violations	GI Absorption	BBB permeability	LogP (XLogP3)	TPSA
Monoterpenes						
Limonene	Yes	0	Low	Yes	4.57	0
Linalool	Yes	0	High	Yes	2.97	20.23
Sesquiterpenes						
Rotundone	Yes	0	High	Yes	3.66	17.07
β -Agarofuran	Yes	0	High	Yes	3.13	9.23
Oxygenated Sesquiterpenes						
10-epi- γ -Eudesmol	Yes	0	High	Yes	3.39	20.23
Bulnesol	Yes	0	High	Yes	2.91	20.23
Epoxybulnesene	Yes	0	High	Yes	3.89	12.53
Isoaromadendrene Epoxide	Yes	0	High	Yes	3.76	12.53
Selina-3,11-Dien-14-ol	Yes	0	High	Yes	3.95	20.23
Selina-3,11-Dien-9-One	Yes	0	High	Yes	3.36	17.07
Spathulenol	Yes	0	High	Yes	3.11	20.23
α -Bisabolol	Yes	0	High	Yes	3.79	20.23
α -Cadinol	Yes	0	High	Yes	3.34	20.23
α -Eudesmol	Yes	0	High	Yes	3.5	20.23
β -Elemol	Yes	0	High	Yes	4.41	20.23
Terpenoids						
Jinkoh-eremol	Yes	0	High	Yes	3.54	20.23
Karanone	Yes	0	High	Yes	3.87	17.07
Nerolidol	Yes	0	High	Yes	4.83	20.23
Oxo-agarospirol	Yes	0	High	Yes	2.92	37.3

Table 4. Molecular Docking Analysis of Selected Compounds with LasR (PDB: 2UV0) and RhIR (PDB: 8DQ0) in *Pseudomonas aeruginosa* Quorum Sensing Pathways

Compound	LasR (PDB: 2UV0)			RhIR (PDB: 8DQ0)		
	Binding Energy	Hydrogen bonds	Hydrophobic interactions	Binding Energy	Hydrogen bonds	Hydrophobic interactions
	(Kcal/mol)			(Kcal/mol)		
3-oxo-C-(12)-acylhomoserine lactone	-8.6	Trp56, Trp60, Asp73, Ser129	Leu36, Tyr47, Arg61, Tyr64, Ala70, Thr75, Val76, Cys79, Trp88, Trp93, Phe101, Leu110, Leu125, Gly126, Ala127	na	na	na
N-Butanoyl-L-homoserine lactone	na	na	na	-7.0	Tyr72, Asp81	Tyr64, Ala83, Ile84, Trp96, Leu116, Thr121, Val133, Ser135
α -Bisabolol	-8.9	-	Leu36, Gly38, Leu40, Tyr47, Ile52, Tyr56, Arg61, Tyr64, Asp73, Thr75, Val76, Cys79, Leu125, Ala127, Ser129	-9.1	Tyr72, Asp81	Ala44, Val60, Tyr64, Leu69, Trp96, Phe101, Trp108, Leu116, Ser135
Jinkoh-eremol	-8.7	Tyr64, Asp73	Leu36, Gly38, Leu39, Leu40, Tyr47, Ala50, Val76, Leu125, Gly126, Ala127, Ser129	-8.7	-	Ala44, Val60, Tyr64, Trp68, Tyr72, Asp81, Ala83, Ile84, Trp96, Phe101, Leu107, Ala111, Thr121, Val133, Ser135

Footnote:

- Binding energy values are given in kcal/mol, where more negative values indicate stronger binding affinity.
- Hydrogen bond interactions include residues involved in direct polar interactions with the ligand.
- Hydrophobic interactions represent nonpolar contacts contributing to ligand stability within the binding site.
- "na" indicates that the ligand was not docked or binding data was unavailable for the given receptor.

Figures

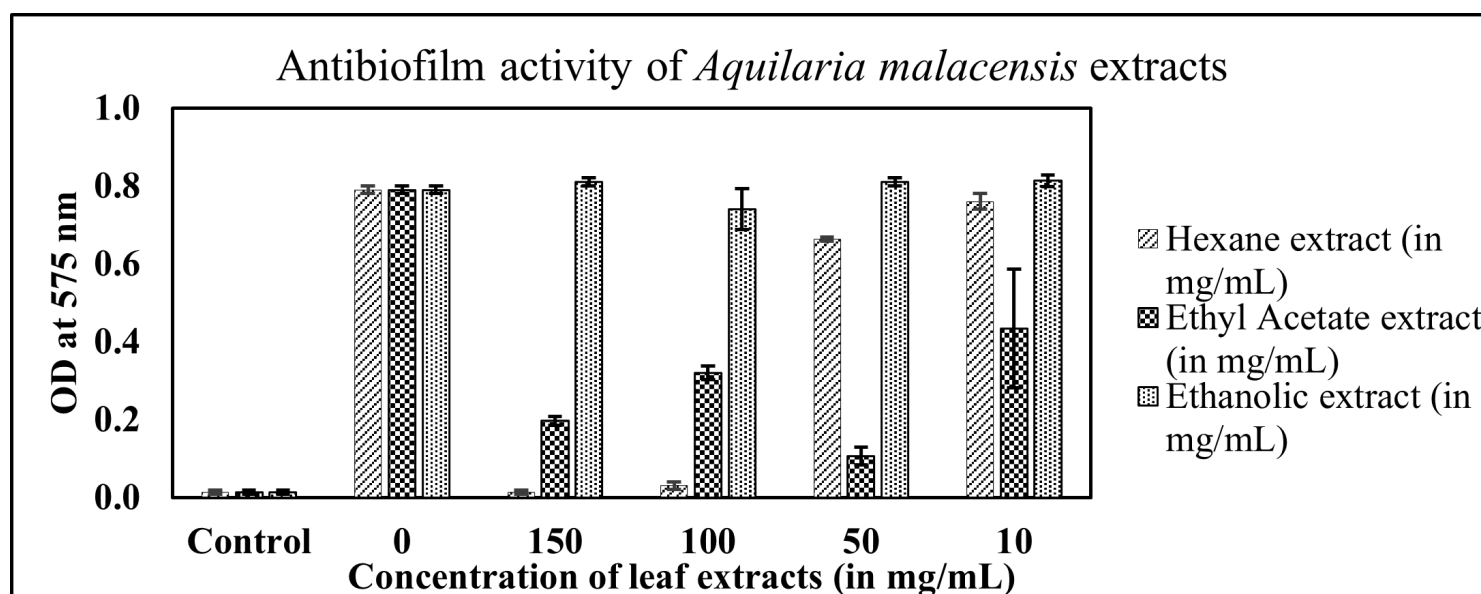


Figure 1

Antibiofilm Activity of *Aquilaria malaccensis* Leaf Extracts. The figure illustrates the antibiofilm activity of hexane, ethyl acetate, and ethanolic extracts of *Aquilaria malaccensis* at varying concentrations (10, 50, 100, and 150 mg/mL). The biofilm formation was measured using optical density (OD) at 575 nm.

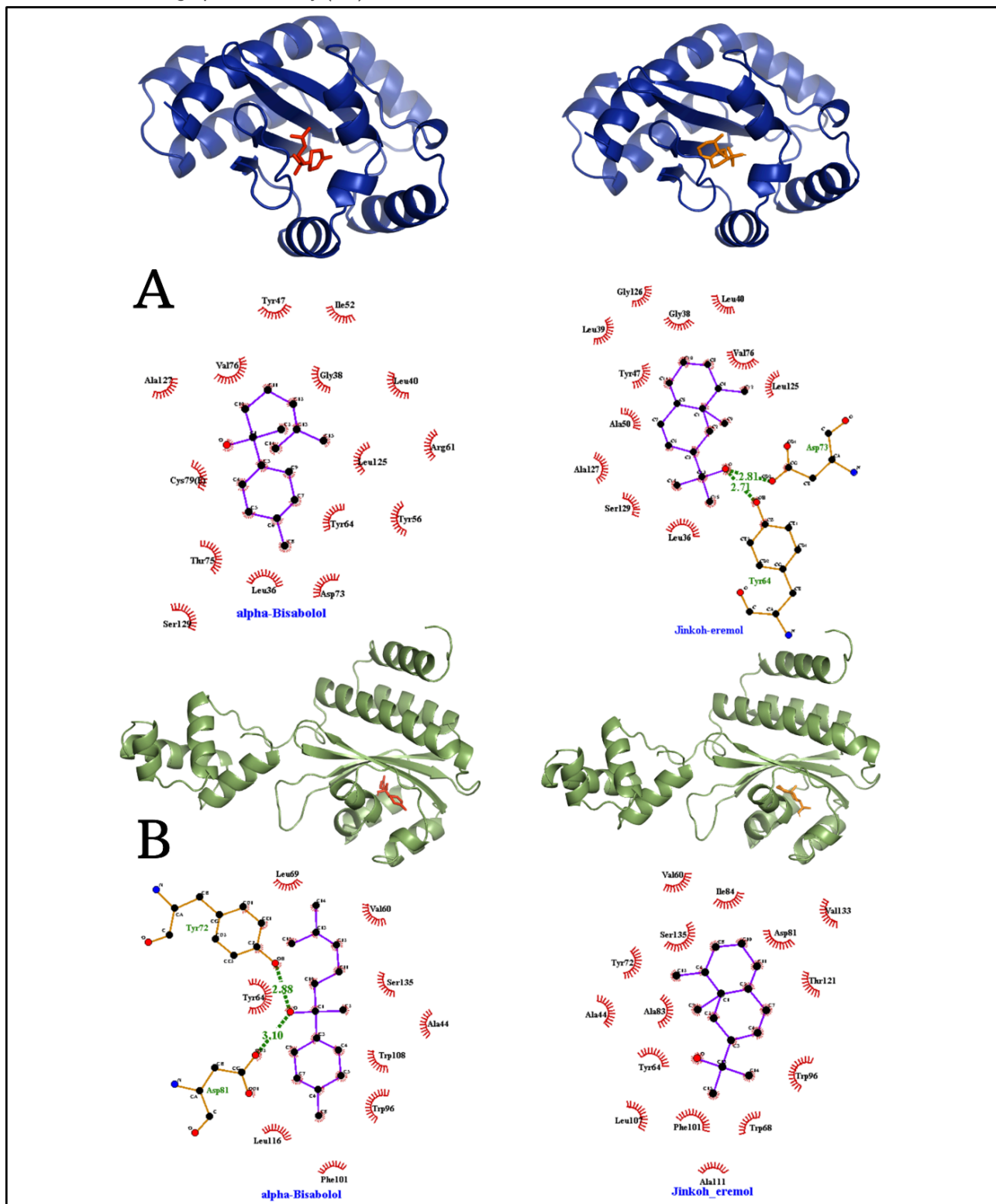


Figure 2

Docking poses of α -Bisabolol (left) and Jinkoh-eremol (right) to the target proteins – (A) LasR (PDB: 2UV0) and (B) RhlR (PDB: 8DQ0) and the interacting amino acid residues with the ligands.

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