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Antibacterial Activity of Compounds Identified from the Active Fractions of Secang Wood (*Caesalpinia sappan*)

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ABSTRACT

Sappan wood, also known as 'Secang' wood (*Caesalpinia sappan*), has traditionally been used as an antimicrobial agent. This study aimed to identify the chemical composition of sappan wood extract and evaluate its antibacterial activity. Sappan wood was extracted by maceration in methanol, followed by Vacuum Liquid Chromatography (VLC), resulting in five primary fractions (A – E) which were further analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS/MS). The antibacterial activity of the fractions was evaluated against *Escherichia coli* and *Staphylococcus aureus* using the microdilution method. Molecular docking was performed by docking ligands identified in Fraction E against target proteins; *β*-ketoacyl-acyl carrier protein synthase (*β*-ketoacyl-ACP synthase) from *E. coli* (PDB ID: 1FJ4) and tyrosyl-tRNA synthetase (TyrRS) from *S. aureus* (PDB ID: 1JIJ) using AutoDock Tools v1.5.6. LC-MS/MS analysis of the fractions identified various compounds belonging to the terpenoid, steroid, phenolic, and alkaloid groups of phytochemicals. All the fractions exhibited strong antibacterial activity against the test pathogenic bacterial (*E. coli* and *S. aureus*) with minimum inhibitory concentration (MIC) ranging from 2 – 8 µg/mL. Of all the fractions, Fraction E showed the highest antibacterial activity against the two organisms tested, with MIC values of 2 µg/mL against both organisms. Molecular docking study showed that the compound isosalsoline from Fraction E of sappan wood extract has the most promising antibacterial activity against *S. aureus* and *E. coli*. These findings indicate that sappan wood has the potential as alternative source of natural antibacterial compounds that can be used for the treatment of bacterial infections, especially those caused by antibiotics-resistant bacteria.

Keywords: *Caesalpinia sappan*, Antibacterial, Fraction, Secondary Metabolite

Introduction

Microbial infections still remain a global health issue, including those caused by bacteria, viruses, and fungi.[1] Bacteria such as *Escherichia coli* and *Staphylococcus aureus* may induce skin infections that can lead to serious systemic infections, as well as fungal and viral infections, which also result in significant health issues.[2,3] The increasing global mobility also supports the more widespread and rapid transmission of pathogens, thus, an effective antimicrobial agent is increasingly necessary.[4]

Antimicrobial agents, such as antibiotics and antifungals, are commonly used to treat microbial infection. The mechanism of action of antibiotics include the inhibition of bacterial cell wall synthesis, inhibition of protein synthesis, and disruption of DNA metabolism.[5] However, excessive use of antibiotics has led to antibiotic resistance, where bacteria become immune to the drugs.[6] Antibiotic resistance is a serious threat to global health, making the treatment of infections difficult and increasing the risk of complications.[7] Medicinal plants offer an alternative potential as a natural source for current antimicrobial drugs.[8] Many plant-based bioactive substances exhibit antimicrobial activity through mechanisms different from those of conventional antibiotics,[9] which is significant for mitigating antibiotics resistance. Plant-based compounds have various mechanisms of action, including the damaging of microbial cell membranes, inhibition of key enzymes, and modulation of the body's immune response.[10]

One of the plants offering potential as source of antimicrobial agent is sappan wood (*Caesalpinia sappan*).[11] Sappan wood has been traditionally used for the treatment of many diseases, which has encouraged recent studies to explore its antimicrobial potential.[12] Sappan wood extract have proven its effectiveness against pathogens, including both gram-positive and gram-negative bacteria,[13] which calls for more research into its effectiveness as antimicrobial agent. Its active compounds include xantone, coumarin, chalcones, flavones, homoisoflavonoids and brazilin,[14-18] especially in its wood where brazilin, sappanone B, protosappanin A, alpinetine, and 3-deoxysappanone B have been identified.[19,20] Sappan wood's antibacterial activity is driven by its chemical constituents. Brazilin primarily shows potential as an antibacterial agent, which is prominent for the treatment of infections,[21] and its presence in sappan wood can also inhibit bacterial and fungal growth.[22,23] Methanol and ethyl acetate fractions of sappan wood have shown activity against *Escherichia coli* and *Staphylococcus aureus*.[24] Research in this area is expanding, particularly regarding its active compounds against antibiotic-resistant pathogens. Therefore, this study aimed to assess the chemical

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composition of sappan wood through extraction, chromatographic and spectroscopic techniques and evaluate their antibacterial activity using in vitro and in silico methods. Through this study, additional information on chemical composition of sappan wood, and its molecular mechanism as an antibacterial agent is a novelty that complements previous studies, thereby offering support for its use as an herbal product in the future.

Materials and Methods

Plant collection, identification, and authentication

Fresh sappan wood was collected from Mekarsari Village Plantation, South Konawe Regency, Southeast Sulawesi Province, Indonesia (4°19'41.8"S 122°25'28.9"E) in March 2024. The plant material was identified and authenticated as *Caesalpinia sappan* at the Phytochemistry and Pharmacognosy Laboratory, Politeknik Bina Husada Kendari, Indonesia. Herbarium specimen with voucher number HERB/2024-017 was deposited.

Preparation and extraction of plant material

A 1 kg sample of sappan wood was chopped and oven-dried at 40°C for 48 h, then pulverized into fine powder. The powdered sample (500 g) was extracted by maceration in methanol (2.5 L) at room temperature for 72 hours with constant stirring. The extract was filtered using Whatman No. 1 filter paper, and the filtrate was concentrated in a rotary evaporator (Stuart RE300, USA) at 50°C to obtain a thick extract (25 g). The concentrated extract was stored at 4°C until further analysis.

Fractionation

The extract (20 g) was fractionated by Vacuum Liquid Chromatography (VLC), using a stationary phase of silica gel 60GF254 (Merck, 250 g), and a mobile phase of gradient solvent system of n-hexane:ethyl acetate (9:1, 8:2, 5:5, 2:8), followed by 100% ethyl acetate. Eluates (150 mL each) were collected to produce five fractions (A – E) which were further analyzed using Thin Layer Chromatography (TLC) with a solvent system of n-hexane:ethyl acetate (4:6). The TLC chromatogram was visualized under UV radiation at 254 nm and 366 nm. Fractions A-E were concentrated using a vacuum rotary evaporator (Stuart RE300, USA) for further analysis.

Identification of chemical constituents

Chemical constituents of the fractions were analyzed using Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) (Waters, USA). Samples of the fractionated extract were dissolved in methanol and passed through a 0.22 µm membrane filter before analysis. The LC-MS/MS system was equipped with a C18 column (2.1 × 50 mm, 1.7 µm) and operated with a mobile phase of aqueous acetonitrile (with 0.1% formic acid) in a gradient manner, with a flow rate set at 0.3 mL/min and a column temperature of 40°C. The MS was operated in electrospray ionization (ESI) positive and negative modes, with Multiple Reaction Monitoring (MRM) detection to identify and quantify target compounds. Data analysis was performed using mass compound identification software based on comparisons to a database or reference mass spectrum.[25]

Evaluation of antibacterial activity

Antibacterial activity test was done using microdilution method, according to previous literature.[7,26] *E. coli* and *S. aureus* cultures were prepared in Mueller Hinton Broth (MHB) and adjusted to 0.5 McFarland standard, about 1.5 x 10^6 CFU/mL. Each well of a 96-well plate was filled with 100 µL MHB. The fractions to be tested, including standard antibiotic (positive control) were serially diluted in 96-well plate to produce gradient concentrations (1 - 512 µg/mL), broth without bacteria, and bacteria without the test fraction were used as the negative control, growth control, respectively. A 10 µL suspension of bacteria was added to each well except for negative control to obtain a final concentration of 10^6 CFU/mL. Plates were incubated in 37°C for 18-24 h. After incubation, the absorbance of each sample was measured at 625 nm using a UV-Vis Spectrophotometer (Thermo Scientific™ Genesys™ 50, USA). Lower or equal absorbance to the negative control indicates no bacteria growth and this was regarded as the Minimum Inhibition Concentration (MIC).

Molecular docking study

The 3D structures of the target enzymes; β-ketoacyl-acyl carrier protein synthase (β-ketoacyl-ACP synthase) from *E. coli* (PDB ID: 1FJ4) and tyrosyl-tRNA synthetase (TyrRS) from *S. aureus* (PDB ID: 1JIJ) were obtained from the Protein Data Bank.[27,28] The 3D structure of the compounds; valine, isosalsoline, and 3-Tert-butyl-4-methoxyphenol from Fraction E of sappan wood extract were downloaded from the PubChem database. The protein targets and compounds structures were prepared using AutoDock Tools v1.5.6 (The Scripps Research Institute, USA, released in 2011) according to standard protocols.[29,30] Protein targets structure preparation include deletion of bound water and residual molecules, addition of hydrogen atoms (protonation), and addition of Kollman charges.[30] The docking process was performed using AutoDock Vina v1.1.2 software (The Scripps Research Institute, USA, released in 2011).[31] The docking was simulated at the binding sites of β-ketoacyl-ACP synthase (site coordinates: x = 4.302; y = -19.867; z = 0.617) and TyrRS (site coordinates: x = -11.273; y = 13.817; z = 86.08). To validate the docking process, a redocking experiment was conducted by re-docking the native ligands (with ID TLM and 629) into β-ketoacyl-ACP synthase and TyrRS, respectively. The validity of the protocol was confirmed as the resulting root mean square deviation (RMSD) value was below 2 Å.[32] Final analysis involved visualizing the interaction between the target enzyme and the compound from Fraction E of sappan wood using Discovery Studio Visualizer v17.2.0.16349 (Dassault Systèmes, France, release in 2017).

Results and Discussion

Extraction and fractionation

Drying sappan wood at low temperature (about 40°C) aims to reduce moisture content and maintain the stability of active compound(s). Sample pulverization aims to enlarge the contact surface during extraction, which is essential for facilitating the extraction of active compounds from plant tissue. Maceration with stirring in methanol is preferred in this study due to its simplicity for room temperature setups, which still allows for an accelerated diffusion process with easy maintenance. Additionally, methanol is flexible as a solvent for extracting various compounds, including polyphenols, alkaloids, terpenoids, steroids, and flavonoids. The fractionation of 25 g of the methanol extract yielded five fractions: 1.7 g (A), 2.2 g (B), 4.8 g (C), 10.5 g (D), and 5.3 g (E). The TLC analysis showed differences in the compounds composition of the various fractions (Figure 1). The TLC profile were influenced by the polarity of the compounds, where compounds with lower polarity were identified in the earlier fractions, and those with higher polarity were found in the later fractions.

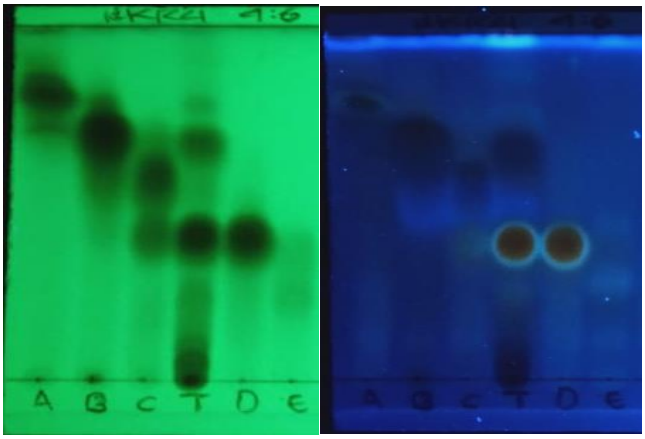

Figure 1: TLC chromatogram of Fractions A – E of sappan wood methanol extract. Mobile Phase: n-Hexane:Ethyl acetate (4:6)

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Chemical constituents

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is a highly sensitive and selective analytical method used to identify and quantify chemical compounds in complex substances. In this analysis, compounds in the sample are separated by their polarity using liquid chromatography (LC) resulting in compound chromatograms (Figure 2), which is further analyzed using a mass spectrometer that identifies compounds based on their mass-to-charge ratio (m/z). The tandem mass spectrometry (MS/MS) analysis allows deep detection by further fragmenting the ions to give more detailed information regarding the molecule's structure, thus identify chemical compounds more accurately (Figure 3). The LC-MS/MS identification (Table 1) showed that the fractions contained chemicals from the terpenoid, steroid, phenolic, and alkaloid groups.

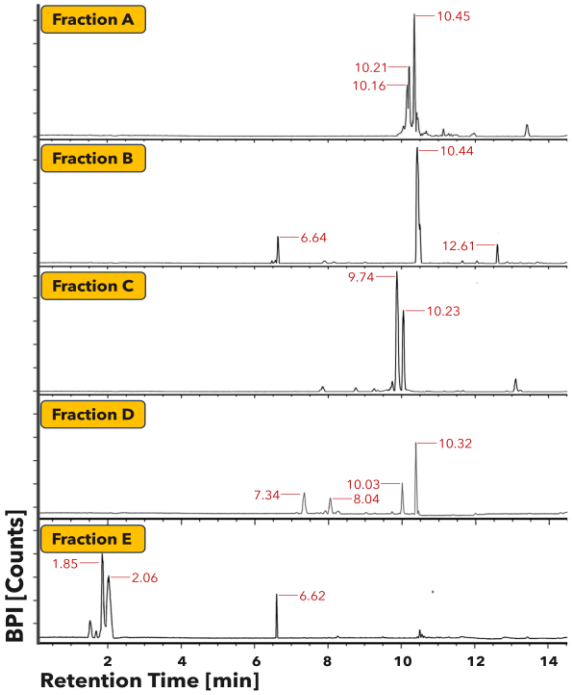

Figure 2: LC-MS/MS chromatograms of Fractions A – E of sappan wood methanol extract

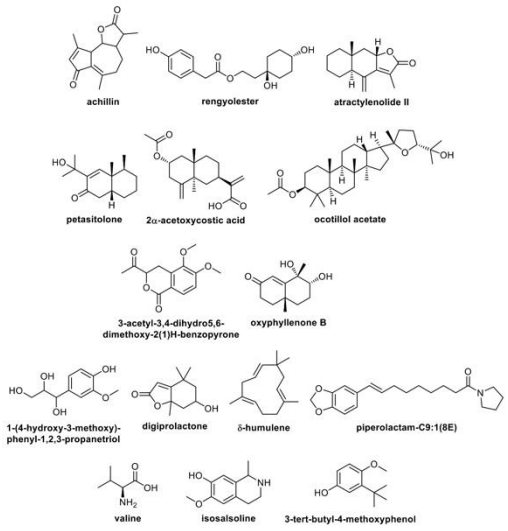

Figure 3: Structure of compounds identified from the LC-MS/MS analysis of Fractions A – E of sappan wood methanol extract

Table 1: Chemical composition of fractions of sappan wood methanol extract

Component Name	Observed RT (min)	Observed $[M+H]^+$ (m/z)	Neutral mass (Da)	Formula	Detector Counts	Group
Fraction A						
Achillin	10.16	247.1328	246.12559	$C_{15}H_{18}O_3$	441917	Terpenoid
Rengyolester	10.21	295.1543	294.14672	$C_{16}H_{22}O_5$	181750	Phenolic
Atractylenolide II	10.45	233.1536	232.14633	$C_{15}H_{20}O_2$	207242	Terpenoid
Fraction B						
Petasitolone	6.64	237.1845	236.17763	$C_{15}H_{24}O_2$	29755	Terpenoid
2α-Acetoxycostic acid	10.44	293.1749	292.16746	$C_{17}H_{24}O_4$	691580	Terpenoid
Ocotillol acetate	12.61	503.4092	502.40221	$C_{32}H_{54}O_4$	79369	Steroid
Fraction C						
3-Acetyl-3,4-dihydro 5,6-dimethoxy-2(1)H-benzopyrone	9.74	251.0893	250.08412	$C_{13}H_{14}O_5$	33716	Terpenoid
Oxyphyllenone B	10.23	211.1342	210.12559	$C_{12}H_{18}O_3$	150091	Terpenoid
Fraction D						
1-(4-Hydroxy-3-methoxy)-phenyl-1,2,3-propanetriol	7.34	215.0932	214.08412	$C_{10}H_{14}O_5$	36561	Phenolic

Digiprolactone	8.04	197.1168	196.10994	$C_{11}H_{16}O_3$	181524	Terpenoid
δ-Humulene	10.03	203.1790	202.17215	$C_{15}H_{22}$	103941	Terpenoid
Piperolactam-C9:1(8E)	10.32	330.1906	329.19909	$C_{20}H_{27}NO_3$	565074	Alkaloid
Fraction E						
Valine	1.85	118.0858	117.07898	$C_5H_{11}NO_2$	23351	Alkaloid
Isosalsoline	2.06	194.1172	193.11028	$C_{11}H_{15}NO_2$	221388	Alkaloid
3-Tert-butyl-4-methoxyphenol	6.62	181.1218	180.11503	$C_{11}H_{16}O_2$	235056	Phenolic

Antibacterial activity

The five fractions of sappan wood methanol extract showed varying activity against the test bacterial strains. Their minimum inhibitory concentrations (MICs) are shown in Figure 4. MIC describes the lowest concentration of an antimicrobial substance that can inhibit the growth of microorganisms. The MIC values of the various fractions varied from 2 to 8 µg/mL. Fractions B, D and E showed the highest activity against *S. aureus* with MIC of 2 µg/mL for each fraction, while fraction E exhibited the highest activity against *E. coli* with MIC of 2 µg/mL. The inhibitory activity of these fractions may be attributed to their chemical components, which have been shown to exert antibacterial activity through various mechanisms.

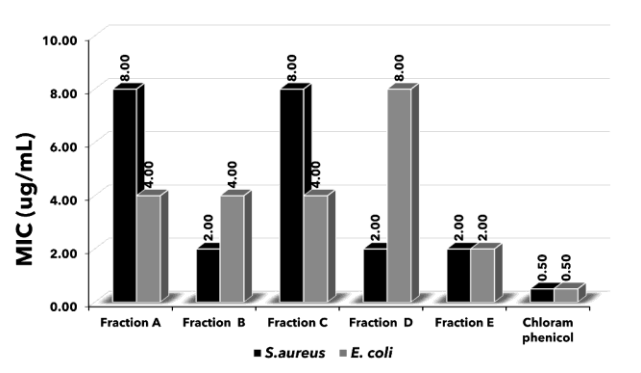

Figure 4: Antibacterial activity of fractions of Sappan wood methanol extract

Terpenoids are known to damage the integrity of the bacterial cell wall by altering its membrane permeability, which leads to lysis, leakage of vital cellular components, and ultimately induces cell death.[33] Terpenoids also inhibit important biosynthetic pathways of bacterial growth, including enzymes related to protein synthesis and DNA replication, such as the inhibition of NADH dehydrogenase, an enzyme important in the bacterial respiratory system.[34] Furthermore, terpenoids are capable of disrupting carbohydrate and fatty acid metabolic pathway in bacteria, resulting in limited energy production needed for bacteria growth and function.[35] Terpenoid compounds such as 3-acetyl-3,4-dihydro5,6-dimethoxy-2(1)H-benzopyrone, digiprolactone and δ-humulene are able to inhibit various pathogenic bacteria both gram-positive and gram-negative bacteria growth through similar mechanism. In particular, digiprolactone has been shown to exert strong effect against several strains that are commonly known to be resistant to conventional antibiotics.[36,37] Other terpenoid compounds such as achillin, atractylenolide II, petasitolone, and oxyphyllenone B have also shown potential biological activity, considering their resemblance to other sesquiterpenoid that are widely known to possess various pharmacological properties, including antimicrobial activity.[38]

Steroids show antibacterial activity through several mechanisms, primarily by disrupting the bacterial cell membrane. Steroids integrate into the lipid bilayer of the bacterial membrane, thereby increasing its permeability and decreases its integrity, which leads to lysis and cell death in both gram-positive and gram-negative strains. Steroids also exhibit another important characteristic: the inhibition of efflux pumps, which are mechanisms that allow bacteria to excrete toxic substances through dedicated proteins, including antibiotics. Steroids are able to block this protein pump, and increase bacterial susceptibility to antibacterial agents.[39,40]

Ocotillol acetate has shown promising antibacterial activity, especially against gram-positive bacteria such as methicillin- resistant *S. aureus* (MRSA). Several ocotillol-derivatives exert antibacterial activity against MRSA with MICs as low as 2 µg/mL, confirming their potential as candidates for further development as antimicrobial agents. The potent antibacterial activity of the ocotillol-derivatives against resistant strains also emphasizes their benefit in addressing antibiotic resistance issues.[41]

Phenolic compounds show more targeted antibacterial activity through several mechanisms, primarily by damaging the membrane of the bacterial cell wall, inhibiting important enzymes involved in virulence, and disrupting biofilm production. Furthermore, phenolic compounds may interfere with intracellular molecule transport, inducing the leakage of important ions, such as potassium, which leads to membrane instability.[42] Phenolic compounds are also known to act synergistically with antibiotics, enhancing their effectiveness against resistant bacteria.[43] The 3-tert-butyl-4-methoxyphenol compound, also known as BHA (Butylated Hydroxyanisole), has been studied for its antimicrobial activity, particularly in inhibiting the growth of pathogenic microorganism. The compound has demonstrated effective antimicrobial activity against various strains, including *E. coli* and *S. aureus* through cell membrane disruption, leading to cell leakage and death.[44]

Alkaloids show significant antibacterial activity through several mechanisms, primarily by disrupting bacterial cell membrane, leading to the leakage of important ions and metabolites, damaging the structural integrity of the cell, and eventual cell death. In addition, alkaloids inhibit DNA and RNA synthesis in bacteria cell, disrupting the replication and transcription processes.[37] The findings from the present study indicate that all five fractions of sappan wood methanol extract showed good antibacterial activity against the two pathogenic bacteria tested, with fraction E showing the highest activity.

Molecular docking results

The antibacterial potential of Fraction E from sappan wood methanol extract was evaluated through docking simulations of the identified compounds; valine, isosalsoline, and 3-Tert-butyl-4-methoxyphenol against two target enzymes; tyrosyl-tRNA synthetase (TyrRS) from *S. aureus* and *β*-ketoacyl-acyl carrier protein synthase (β-ketoacyl-ACP synthase) from *E. coli*. The docking process was meticulously performed, achieving an RMSD value of 0.68 Å for the redocking of TLM on β-ketoacyl-ACP synthase from *E. coli* (Figure 5A) and 1.43 Å for the redocking of 629 on TyrRS from *S. aureus* (Figure 5B). This high precision in docking validates the reliability of the interaction between compounds from fraction E and protein targets of *E. coli* and *S. aureus*. The simulation showed that isosalsoline had the highest antibacterial potential with binding energies of -6.9 kcal/mol for TyrRS (*S. aureus*) and -7.3 kcal/mol for β-ketoacyl-ACP synthase (*E. coli*) which indicated a stable interaction between isosalsoline with both enzymes. On the contrary, 3-Tert-butyl-4-methoxyphenol and valine exhibited lower affinity against both target enzymes, with binding energies of -6.3 kcal/mol and -4.4 kcal/mol, respectively against *E. coli*

and -6.2 kcal/mol and -5.2 kcal/mol, respectively against *S. aureus*. This finding indicates a weaker interaction compared to isosalsoline, although they were still able to interact with the enzyme's active site. As a comparison, Chloramphenicol, known for its strong in vitro antibacterial activity, presents docking results consistent with its biological activity. Chloramphenicol showed high affinity, with binding energies of -8.3 kcal/mol against β-ketoacyl-ACP synthase (*E. coli*) and -7.2 kcal/mol against TyrRS (*S. aureus*). This higher affinity reflects an optimum interaction within the enzyme's active pocket.

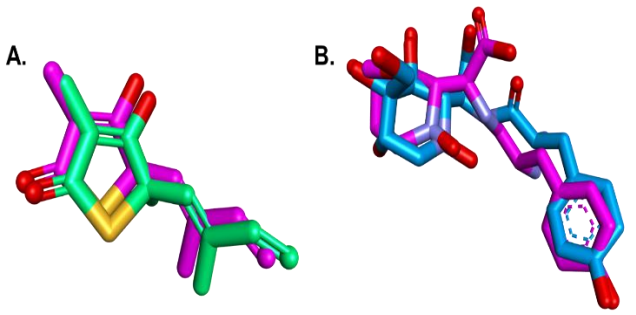

Figure 5: Overlay of the 3D conformation of the co-crystallized native ligand (pink) and the docked TLM (green) on β-ketoacyl-ACP synthase from *E. coli*, and the docked 629 (blue) on TyrRS from *S. aureus*

In *E. coli*, the compounds of Fraction E generally interacted with β-ketoacyl-ACP synthase through hydrogen bonds, particularly with the residue Thr300 (Figure 6A-D). Interestingly, isosalsoline showed additional interaction through hydrogen bond formation with the residues Asp265 and Asn396, which significantly contributed to the stability of the compound-enzyme complex (Figure 6C). In comparison, chloramphenicol formed hydrogen bonds with four active residues: Ala271, Thr300, Thr302, and Gly393 (Figure 6A), which reflected more diverse interaction patterns within the active pocket of the enzyme. Additionally, hydrophobic interaction also plays an important role in compound binding to β-ketoacyl-ACP synthase. Isosalsoline and chloramphenicol both showed hydrophobic interaction with residues Pro272 and Ala271, which contributed to the stability and binding affinity of the compounds to the enzyme. Moreover, 3-Tert-butyl-4-methoxyphenol formed three hydrophobic interactions with residues Pro272, His298, and Phe392, showing their potential to optimize binding through nonpolar interaction with the hydrophilic pocket of the enzyme (Figure 6B).

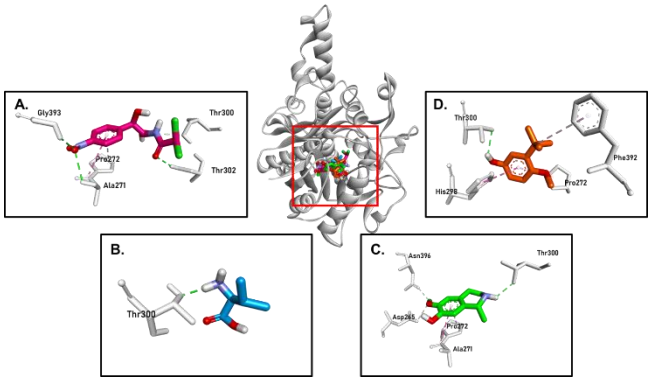

Figure 6: Molecular interaction of (A) Chloramphenicol, (B) Valine, (C) Isosalsoline, and (D) 3-Tert-butyl-4-methoxyphenol against β-ketoacyl-ACP synthase from *E. coli*

In *S. aureus*, chloramphenicol demonstrated complex molecular interactions with the TyrRS (tyrosyl-tRNA synthetase) enzyme, forming six hydrogen bonds with the amino acid residues Tyr36, Gly38, Asp40, His50, Asp80, and Gln196 (Figure 7A). A hydrogen bond with the residue Asp40 was also observed in the 3-Tert-butyl-4-methoxyphenol compound (Figure 7D), indicating a similar interaction mechanism in the active site of the enzyme.

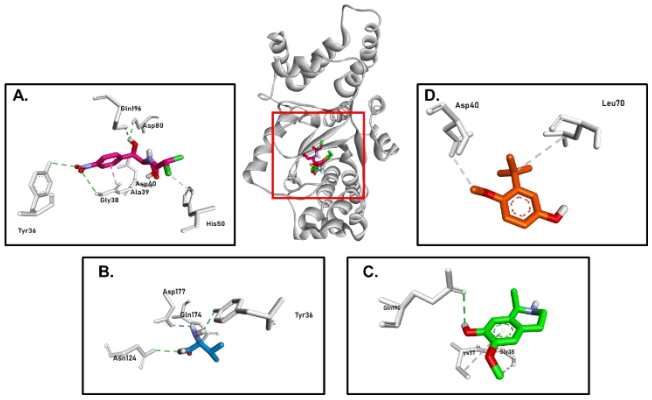

Figure 7: Molecular Interaction of (A) Chloramphenicol, (B) Valine, (C) Isosalsoline, and (D) 3-Tert-butyl-4-methoxyphenol against TyrRS from *S. aureus*

Isosalsoline interacted with TyrRS through hydrogen bond formation with the residues Gly38 and Gln190 (Figure 7C). This interaction pattern was distinct from that of chloramphenicol, showing a specific preference for certain residues, which affected the stability and ligand orientation within the active pocket. On the other hand, valine formed three unique hydrogen bonds with the residues Asn124, Gln174, and Asp177 (Figure 7B), indicating a different binding pattern compared to the other compounds. Aside from hydrogen bond interaction, hydrophobic interaction was also observed in the target *S. aureus*. Chloramphenicol, isosalsoline, and 3-Tert-butyl-4-methoxyphenol each formed only one hydrophobic interaction with different residues; Gly38, Cys37, and Leu70. This difference indicates that nonpolar interactions play a secondary role in compound binding with TyrRS.

Conclusion

Sappan wood or 'secang' wood (*Caesalpinia sappan*) contained various active compounds that have strong antibacterial potential, especially against *E. coli* and *S. aureus*. Identified compounds, such as terpenoids, steroids, phenolics and alkaloids have demonstrated various mechanisms in inhibiting bacterial growth. Based on the docking results, Isosalsoline from Fraction E of the sappan wood extract was the main contributor to the antibacterial activity of sappan wood against *E. coli* and *S. aureus*. Therefore, sappan wood has the potential as an alternative source of natural bioactive compounds that could be developed as antimicrobial agents for combatting the challenges of antibiotic resistance.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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