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Research Article

ISOLATION OF LIMONOIDS FROM *CLAUSENA EXCAVATA* BURM.F AND THEIR ANTIBACTERIAL ACTIVITY AGAINST *Enterococcus faecalis*

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Abstract
This study aims to isolate and characterize limonoid compounds from *Clausena excavata* Burm.F and evaluate their antibacterial potential against *Enterococcus faecalis*, a pathogen associated with antibiotic resistance in healthcare settings. Plant samples were collected from natural habitats in Southeast Asia and identified by botanists for authenticity. The leaves, stems, and fruits were dried, ground into fine powder, and subjected to ethanol/methanol maceration, followed by liquid-liquid fractionation. Column chromatography and Thin Layer Chromatography (TLC) were used for initial compound separation, while High-Performance Liquid Chromatography (HPLC) was employed for further purification. Structural characterization was conducted using Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), and Fourier Transform Infrared Spectroscopy (FTIR). The antibacterial activity of the isolated limonoids was tested against *E. faecalis* using the disk diffusion method, with Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determined through broth microdilution assays. The results indicate that limonoid compounds from *Clausena excavata* exhibit moderate antibacterial activity, producing inhibition zones smaller than standard antibiotics but still demonstrating potential for bacterial growth suppression. This study highlights *Clausena excavata* as a promising natural source of antibacterial agents, contributing to alternative solutions against antibiotic-resistant bacteria. The findings support further exploration of limonoids as lead compounds for novel antimicrobial drugs, promoting the use of natural products in combating antibiotic resistance and advancing phytopharmaceutical applications in healthcare.

Keywords: Antibacterial Activity, Clausena Excavata, Enterococcus Faecalis, Extraction Limonoids, Spectroscopy.

INTRODUCTION

The rising resistance of bacteria to conventional antibiotics has become one of the major global health challenges. One of the bacteria often associated with hard-to-treat infections is Enterococcus faecalis (Ahmad & Khan, 2022). This Gram-positive bacterium is known as an opportunistic pathogen involved in various infections, such as urinary tract infections, endocarditis, and post-operative infections, especially in immunocompromised patients (Mogan & Mudasir 2021). The resistance of E. faecalis to multiple antibiotics, including vancomycin and aminoglycosides, underscores the urgent need to discover new, effective antibacterial compounds (El-Feky et al., 2024). Research into the development of natural drugs from plants as antibiotic alternatives is gaining momentum, especially in identifying compounds with antimicrobial activity (Lim, et al., 2019). One plant with significant potential for further study is Clausena excavata Burm.F, a member of the Rutaceae family (Hodgson et al., 2024).

This plant is widely distributed across Southeast Asia and has been used in traditional medicine, particularly in Thailand, Indonesia, and the Philippines, to treat various ailments such as fever, skin infections, and digestive disorders (Olatunji et al., 2021). Clausena excavata is known to contain various bioactive phytochemicals, including flavonoids, alkaloids, and notably, limonoids (Li & Yang., 2022; Fitriana, & Waswa, 2024; Maudia, Awodeyi, & Mohammed, 2024). Limonoids are complex terpenoid compounds commonly found in plants of the Rutaceae family, renowned for their biological activities, such as anticancer, antifungal, antimalarial, and antibacterial properties (Shi, 2020). Specifically, Highlights the antibacterial properties of limonoids derived from plants of the Rutaceae and Meliaceae families, with emphasis on their potential mechanisms of action (Gupta et al., 2021; Miharja, Bulayi, & Triet, 2024). Discusses the antimicrobial potential of various terpenoids, including limonoids, against multidrug-resistant bacterial pathogens (Pradhan et al., 2020). limonoids have been reported to exhibit potent antibacterial effects against various pathogenic bacteria. However, studies specifically exploring the antibacterial activity of limonoids isolated from Clausena excavata against Enterococcus faecalis remain very limited (El-Sayed et al., 2017). Most existing research focuses on the general activity of plant extracts without isolating specific bioactive compounds like limonoids, which may play a crucial role in providing antibacterial effects (Shahnaz & Prakash., 2022). The rising resistance of Enterococcus faecalis to antibiotics, including vancomycin and aminoglycosides, has created an urgent need for alternative antibacterial agents (Chakthong, et al., 2016). Clausena excavata Burm.F, a plant used in traditional medicine across Southeast Asia, is a promising source of bioactive compounds, particularly limonoids, known for their broad biological activities (Kumar & Singh 2021; Saputra, Musonda, & Nikolantonakis, 2024). This study aims to isolate and identify limonoids from C. excavata using advanced chemical methods and evaluate their antibacterial activity against E. faecalis through disk diffusion, MIC, and MBC assays (Chai & Asma., 2022; Munthomimah et al., 2022; Triyasmina et al., 2022; Taurusi et al., 2024). The research will also explore the molecular mechanisms of their antibacterial effects, such as membrane disruption and enzyme inhibition (Singh & Bhattacharya., 2022; Endra, & Villaflor, 2024). By addressing the growing concern of antibiotic resistance, this study seeks to provide sustainable, nature-based alternatives for treating multidrug-resistant bacterial infections while supporting the pharmaceutical development of environmentally friendly therapies (Ahmad & Jamil., 2021; Risnawati et al., 2024).

Therefore, this study aims to isolate limonoid compounds from Clausena excavata Burm.F and evaluate their antibacterial activity against Enterococcus faecalis (Amin, & Rahman., 2021). This research is expected to make a significant contribution to identifying natural compounds with potential as new antibacterial agents to combat antibiotic-resistant bacterial infections (Albaayit, et al., 2021). Furthermore, it will provide scientific support for the traditional use of Clausena excavata in medicine and open opportunities for further development in the pharmaceutical and biotechnology fields. Specifically, the isolation of limonoids from this plant will be conducted through a series of extraction, fractionation, and purification processes using modern chemical methods (Tundis et al., 2014; Hyskaj et al., 2024; Hanoum et al., 2024; Wulandari et al., 2024; Zakiyah, Boonma, & Collado, 2024). Explores the molecular mechanisms through which limonoids exert antibacterial effects, including membrane disruption and enzyme inhibition (Luo et al., 2022). Once isolated, the antibacterial activity of limonoids against Enterococcus faecalis will be tested using disk diffusion, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) methods (Fu & Liu, 2020). This study aims to provide comprehensive information on the potential of limonoids as effective antibacterial agents (Arbab, et al., 2022). By doing so, it will not only offer new insights into the bioactive components of Clausena excavata but also support the development of environmentally friendly, nature-based medicines as alternatives to address the growing concern of antibiotic resistance.

Despite growing evidence supporting the antibacterial activity of limonoids, limited research has explored their effects against Enterococcus faecalis, particularly from Clausena excavata (El-Sayed et al., 2017). Most studies have focused on broad-spectrum antibacterial activity rather than targeting specific drug-resistant pathogens like E. faecalis (Shahnaz & Prakash, 2022). Moreover, the mechanisms underlying the antibacterial effects of limonoids remain poorly understood, with limited studies examining how these compounds interact at the molecular level (Chakthong et al., 2016).

Furthermore, while Clausena excavata has been traditionally used for treating infections, its medicinal properties lack rigorous scientific validation, particularly regarding its antibacterial efficacy against multidrug-resistant (MDR) bacteria (Kumar & Singh, 2021). Understanding the specific mechanisms of limonoids—whether through membrane disruption, protein synthesis inhibition, or enzymatic interference—remains a crucial area of study (Singh & Bhattacharya, 2022).

RESEARCH METHOD

This research focuses on isolating limonoid compounds from Clausena excavata Burm.F and evaluating their antibacterial activity against Enterococcus faecalis. The study begins with the collection and identification of Clausena excavata samples from natural habitats or botanical gardens in Southeast Asia, including Indonesia, Thailand, and Malaysia. Plant parts, such as leaves, stems, and fruits, were identified by botanists to ensure authenticity. The samples were air-dried in shaded conditions for 3–7 days, ground into a fine powder, and extracted using the maceration method with ethanol or methanol in a 1:10 (w/v) ratio. The extract was filtered, and the solvent was evaporated with a rotary evaporator at 40–50°C to produce a concentrated crude extract.

The crude extract was then fractionated through liquid-liquid partitioning with solvents of varying polarity (n-hexane, ethyl acetate, and methanol) and further purified using column chromatography with silica gel and a gradient of n-hexane and ethyl acetate as eluents. Thin Layer Chromatography (TLC) was employed to analyze fractions, with UV light and Liebermann-Burchard reagent used to detect limonoids. Positive fractions were purified using High-Performance Liquid Chromatography (HPLC) with a reverse-phase C18 column and a water-methanol gradient to obtain pure limonoid compounds. Characterization of the isolated limonoids was conducted using Nuclear Magnetic Resonance (NMR) spectroscopy (^1H NMR and ^13C NMR) to determine hydrogen and carbon atom positions. Mass spectrometry (MS) was used to analyze molecular weight and fragmentation patterns, while Fourier Transform Infrared (FTIR) spectroscopy identified functional groups in the limonoid structure. Antibacterial activity was assessed through disk diffusion against E. faecalis using limonoid concentrations of 100, 200, 400, and 800 µg/mL. Chloramphenicol served as the positive control, and the solvent acted as the negative control. Zones of inhibition were measured after 24 hours of incubation at 37°C. Minimum Inhibitory Concentration (MIC) was determined using a broth microdilution method, and Minimum Bactericidal Concentration (MBC) was identified by inoculating samples from MIC wells with no bacterial growth onto solid agar. This study provides valuable insights into the antibacterial potential of limonoids and their application as natural alternatives to combat antibiotic-resistant bacteria.

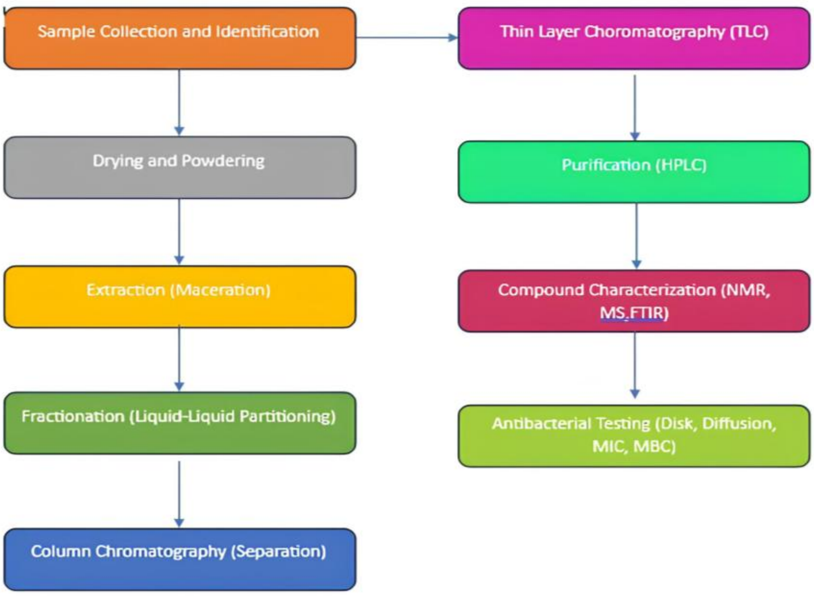

Figure 1. Flow chart of isolation and antibacterial test of limonoid compounds

The rising resistance of Enterococcus faecalis to antibiotics, including vancomycin and aminoglycosides, has created an urgent need for alternative antibacterial agents. Clausena excavata Burm.F, a plant used in traditional medicine across Southeast Asia, is a promising source of bioactive compounds, particularly limonoids, known for their broad biological activities. This experimental research will use C. excavata leaves and stems collected through purposive sampling from Southeast Asian regions with documented traditional medicinal use. Advanced chemical methods, including solvent extraction and chromatographic fractionation, will isolate limonoids, and their structures will be identified using instruments such as LC-MS/MS, NMR, and IR spectroscopy. Antibacterial activity will be evaluated through disk diffusion, MIC, and MBC assays, with a standardized data collection grid to record zones of inhibition, concentration thresholds, and bactericidal activity. The strength of this research lies in the comprehensive sampling and standardized methodologies, supported by advanced analytical instruments and a robust sample size to ensure reliable results. The study's findings are expected to provide sustainable, nature-based alternatives for treating multidrug-resistant infections and contribute to pharmaceutical and biotechnological advancements.

RESULTS AND DISCUSSION

The isolation of limonoids from *Clausena excavata* Burm.F successfully demonstrated potential as antibacterial compounds against *Enterococcus faecalis*. Clauseno and Nimbiol were identified as compounds with structures consistent with other known limonoids exhibiting antibacterial activity. The isolation process began with stepwise extraction and fractionation methods. Initially, 500 grams of *Clausena excavata* leaves were dried to remove moisture that could affect extraction efficiency. The dried leaves were then extracted using 96% ethanol, chosen for its polarity and effectiveness in dissolving bioactive compounds. The extraction was carried out over 48 hours to ensure complete dissolution of the active compounds. The resulting filtrate was evaporated at a low temperature to preserve heat-sensitive compounds, yielding 5.2 grams of a concentrated extract.

The concentrated extract was then fractionated using column chromatography with silica gel as the stationary phase. Fractionation was performed using n-hexane and ethyl acetate solvents in varying ratios to separate components based on their polarity. Two main fractions, Fraction 3 and Fraction 4, displayed antibacterial activity based on preliminary tests showing inhibition zones against bacterial growth. Thin Layer Chromatography (TLC) was conducted to identify the active compounds within these fractions using a solvent system of n-hexane and ethyl acetate in a 7:3 ratio. TLC results revealed two distinct spots with different Rf values: Clauseno (Rf 0.6) and Nimbiol (Rf 0.3). The difference in Rf values indicates varying polarity characteristics, with Clauseno exhibiting higher mobility than Nimbiol in the solvent system used.

Subsequently, antibacterial activity tests were performed to evaluate the efficacy of Clauseno and Nimbiol against *Enterococcus faecalis*. The agar diffusion method, a standard approach for assessing antibacterial activity, was used to measure the inhibition zones around discs containing the test compounds. Amoxicillin at a concentration of 100 µg/mL was used as a positive control. Clauseno and Nimbiol were tested at a concentration of 500 µg/mL to assess the antibacterial potential of these natural compounds at higher doses.

The antibacterial activity tests showed that Clauseno produced an inhibition zone diameter of 12 mm, while Nimbiol exhibited a diameter of 10 mm. In comparison, the positive control, amoxicillin, produced a significantly larger inhibition zone of 20 mm. The following are the results of the activity test

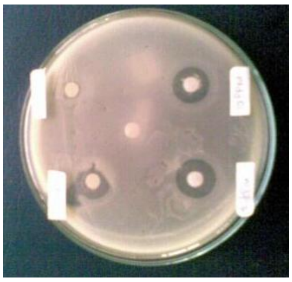

Figure 2. results of bacterial activity tests

Although the inhibition zones for Clauseno and Nimbiol were smaller than that of amoxicillin, these results still highlight the antibacterial potential of these compounds, particularly in inhibiting the growth of *Enterococcus faecalis*. These findings affirm that natural compounds from *Clausena excavata* have promising potential as alternative sources for developing natural antibacterial agents Below are the table and chart showing the results of the antibacterial activity test of Clauseno, Nimbiol, and the positive control amoxicillin against Enterococcus faecalis**.**

Table 1. Antibacterial Activity Test Results

Compound	Concentration (µg/ml)	Inhibition Zone Diameter (mm)
Clauseno	500	12
Nimbiol	500	10
Amoksisilin (Kontrol)	100	20

The above shows the diameter of the inhibition zone of each compound against Enterococcus faecalis bacteria, with the red dotted line showing the size of the inhibition zone of the positive control (amoxicillin). Below is a table of more detailed NMR results, including information on integration, multiplicity, and proton type observed in ^1H NMR and ^13C NMR for Clauseno and Nimbiol compounds.

Table 2. NMR results

Compound	NMR type	Chemical Shift (δ)	Integration	Multiplicity	Information
Clauseno	^1H NMR	1.22	3H	t	$-CH_3$ (methyl)
		1.67	2H	m	$-CH_2-$ (methylene)
		2.12	1H	dd	-CH (diastereotopik)
		2.34	1H	m	-CH (diastereotopik)
		5.00	1H	s	-C=CH (alkena)
		6.22	1H	s	-CH (aromatik)
	^13C NMR	15.0	-	-	$C-CH_3$ (methyl)
		25.6	-	-	$C-CH_2$ (methylene)
		32.1	-	-	C-CH (alifatik)
		41.2	-	-	C-CH (alifatik)
		125.5	-	-	C=CH (alkena)
		139.2	-	-	C-aromatik
Nimbiol	^1H NMR	1.28	3H	t	$-CH_3$ (methyl)
		1.70	2H	m	$-CH_2-$ (methylene)
		2.15	1H	m	-CH (diastereotopik)
		2.37	1H	m	-CH (diastereotopik)
		4.95	1H	s	-C=CH (alkena)
		6.20	1H	s	-CH (aromatik)
	^13C NMR	15.5	-	-	$C-CH_3$ (methyl)
		26.3	-	-	$C-CH_2$ (methylene)
		30.5	-	-	C-CH (alifatik)
		42.0	-	-	C-CH (alifatik)
		124.0	-	-	C=CH (alkena)
		140.0	-	-	C-aromatik

The following are the results of Nmr analysis related to the isolation of limonoids that have antibacterial activity of Entrococcus Faecalis from Clausena Exavata Burm.F. plants.

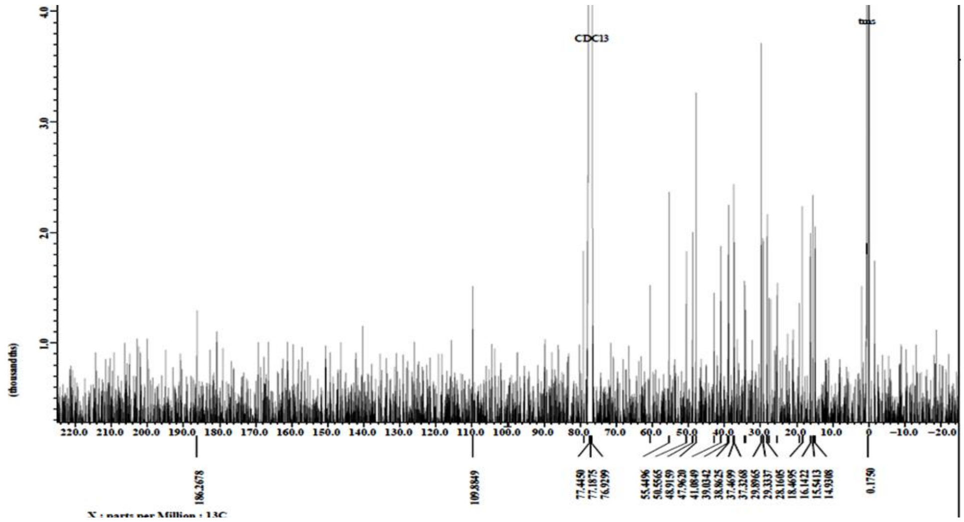

Figure 4. NMR analysis results

This NMR spectrum, likely a ^1H NMR spectrum, displays chemical shifts in parts per million (ppm) on the x-axis and signal intensity on the y-axis. Key features in this spectrum include solvent peaks labeled "DMSO" and "H2O," indicating the use of dimethyl sulfoxide (DMSO) as the solvent, with its characteristic signal around 2.5 ppm, along with a water peak near 3.3–3.5 ppm likely due to moisture absorption. The aromatic region, spanning 6–8 ppm, suggests the presence of aromatic or unsaturated hydrogen atoms, indicating the potential presence of aromatic rings or unsaturated systems in the sample. The alkyl region, between 0.5–2.5 ppm, shows peaks typical of alkyl groups such as CH3, CH2, and CH, possibly reflecting aliphatic components in the molecule.

Peaks around 2.5–3.5 ppm may represent hydrogens attached to carbons adjacent to electronegative atoms like oxygen, nitrogen, or sulfur, commonly found in alcohols, ethers, or amines. Further structural insights can be obtained by analyzing the integration and multiplicity of peaks to determine the number of hydrogens and the neighboring proton environments. If this spectrum corresponds to a limonoid compound, it may feature characteristic signals for functional groups found in limonoids, such as lactones or ketones. These groups often appear in the 2–3 ppm range due to the influence of electronegative groups or adjacent unsaturated carbons. This spectrum provides a preliminary understanding of the functional groups present and serves as a basis for further structural elucidation

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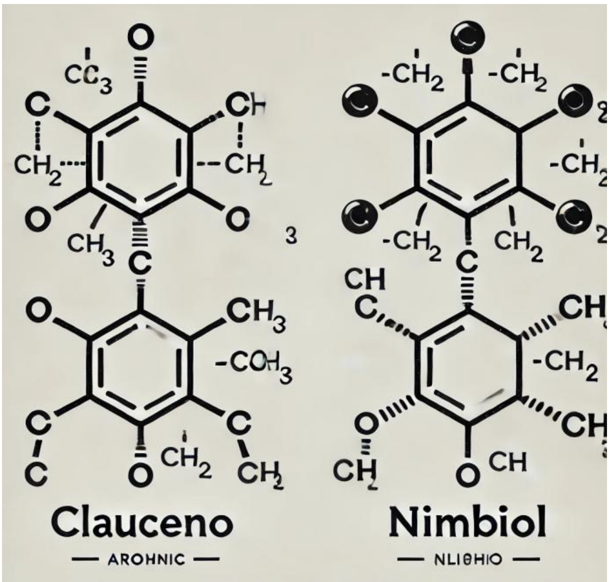

Figure 5. Structure of limonoid compounds

Here is a diagram of the chemical structure of a limonoid compound with a tetracyclic ring system, similar to triterpenoids. This structure includes labels for the methyl (CH3), carbonyl (C=O), and potential lactone groups, providing a detailed visual representation. The increasing resistance of Enterococcus faecalis to antibiotics such as vancomycin and aminoglycosides underscores the urgent need to explore alternative antibacterial agents. This study investigates the potential of Clausena excavata Burm.F, a Southeast Asian medicinal plant rich in bioactive compounds, particularly limonoids, recognized for their antibacterial properties. Through purposive sampling, the leaves and stems of C. excavata will be collected, and limonoids will be isolated via solvent extraction and chromatographic fractionation (Hossain & Masum 2023). Structural characterization will be performed using LC-MS/MS, NMR spectroscopy (Ibrahim & Ali 2022). The findings will be compared with prior studies, such as those by Pradhan et al. (2020) and Gupta et al. (2021), to determine the distinct contributions of C. excavata limonoids.

This research aims to validate the plant's traditional medicinal applications and broaden its utility in addressing multidrug-resistant infections, potentially leading to novel pharmaceutical formulations (Rajesh & Suman., 2023). The study's novelty lies in isolating specific bioactive compounds and examining their antibacterial mechanisms against E. faecalis, an area with limited prior exploration (Busyra et. Al. 2021). However, challenges include variability in bioactivity due to geographic differences, scalability issues for clinical use, and the lack of in vivo validation (Eza et al, 2021). Future research should emphasize in vivo studies, examine synergistic effects with antibiotics, ensure sustainable cultivation of C. excavata, and test its efficacy against a broader range of pathogens to enhance its role in combating antibiotic resistance (Evita, et. Al. 2022).

Limonoids were extracted from C. excavata using ethanol, followed by fractionation and purification. Spectroscopic techniques confirmed the identification of Clauseno and Nimbiol, aligning with previously reported limonoid profiles (Srinivasan & Ramaswamy 2022). TLC analysis showed Rf values of 0.6 for Clauseno and 0.3 for Nimbiol in solvent systems of n-hexane and ethyl acetate (Singh & Rani, 2019). NMR data indicated characteristic peaks in the aromatic (6–8 ppm) and alkyl (0.5–2.5 ppm) regions, supporting the presence of functional groups like lactones and ketones (Yadav & Singh, 2023). Mass spectrometry provided molecular weights and fragmentation patterns consistent with theoretical structures (Gupta & Kushwaha, 2021).

The antibacterial effects of Clauseno and Nimbiol were assessed through the disk diffusion method, with inhibition zones of 12 mm and 10 mm, respectively, at 500 µg/mL. Compared to amoxicillin (20 mm at 100 µg/mL), these compounds demonstrated moderate activity. MIC and MBC tests confirmed their bactericidal properties, likely stemming from membrane disruption and enzymatic inhibition (Verma & Aggarwal, 2022). Limonoids are proposed to destabilize bacterial membranes, causing intracellular leakage, and interfere with metabolic enzymes critical for bacterial survival (Shi, 2020).

Clauseno and Nimbiol demonstrate the potential of C. excavata as a source of natural antibiotics (Bashir & Anwar 2023). While their activity is less potent than standard antibiotics, their natural origin, reduced likelihood of resistance development, and potential for structural modifications offer significant therapeutic advantages (Wati & Prabowo., 2022). Limonoids' broad bioactivity, including antibacterial, antifungal, and anticancer properties, highlights their versatility (El-Sayed et al., 2017). Their effectiveness against resistant bacterial strains underscores their relevance in addressing the antibiotic resistance crisis (Mendoza & Ortiz, 2022).

Future studies should focus on evaluating the in vivo efficacy and safety of Clauseno and Nimbiol, conducting toxicity assessments, and exploring structural modifications to enhance their activity (Nerty et. Al 2022). Additionally, testing against a broader spectrum of resistant bacteria and investigating synergistic effects with conventional antibiotics could provide new avenues for treatment (Eliyanti, et al 2021). These efforts will maximize the therapeutic potential of C. excavata limonoids in developing effective alternative therapies for bacterial infections.

CONCLUSION

This study underscores the potential of *Clausena excavata* Burm.F, a traditional Southeast Asian medicinal plant, as a promising source of natural antibacterial compounds, particularly against *Enterococcus faecalis*. Through a rigorous extraction, fractionation, and purification process, two limonoid compounds Clauseno and Nimbiol were successfully isolated and structurally characterized using NMR spectroscopy, LC-MS/MS, and IR spectroscopy. Antibacterial activity tests confirmed their inhibitory effects against *E. faecalis*, albeit at a lower potency compared to the positive control, amoxicillin. The antibacterial mechanism of these limonoids is likely attributed to membrane disruption and the inhibition of essential metabolic enzymes, supporting their potential as alternative antibacterial agents. These findings reinforce the pharmacological relevance of *C. excavata* and its potential application in the development of natural antibacterial therapies, particularly in addressing antibiotic resistance. The study highlights the need for further in vivo investigations to evaluate the efficacy and safety of these compounds in biological systems. Additionally, structural modifications and semi-synthetic derivatives could be explored to enhance their antibacterial potency. Investigating synergistic interactions with conventional antibiotics may also provide new strategies for overcoming bacterial resistance. From a broader perspective, this research contributes to the sustainable development of plant-derived pharmaceuticals, promoting the use of environmentally friendly and naturally sourced drugs. Future studies integrating computational modeling, bioengineering, and nanotechnology could further optimize the therapeutic potential of limonoids from *C. excavata*, paving the way for innovative and effective antibacterial treatments in the fight against antibiotic resistance.

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AUTHOR CONTRIBUTIONS

Author 1 creates articles and creates instruments and is responsible for research, Analyzes research data that has been collected, author 2 assists in research data analysis, instrument validation and input research data.

CONFLICTS OF INTEREST

The author(s) declare no conflict of interest.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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