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Antimicrobial activity and metabolite profiling of a methanolic extract from *Stereum rugosum*

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ABSTRACT: Although Indonesia is recognized as a global biodiversity hotspot, studies on macrofungi and their bioactive metabolites remain limited. This study evaluated the antimicrobial potential of macrofungi collected from Mount Halimun Salak National Park (MHSNP), Indonesia, focusing on species with strong antibacterial activity and their chemical profiles. Of the five species examined, *Stereum rugosum* exhibited the most potent effect. Its methanolic extract selectively inhibited Gram-positive bacteria, particularly *Staphylococcus aureus* and *Streptococcus mutans*, with minimum inhibitory concentrations (MIC) of 6.67 mg mL^{-1} and 13.33 mg mL^{-1}, and minimum bactericidal concentrations (MBC) of 13.33 mg mL^{-1} and 26.67 mg mL^{-1}, respectively. No activity was observed against Gram-negative bacteria or *Candida albicans*. Gas chromatography–mass spectrometry (GC-MS) analysis identified 22 compounds, primarily 2,5-cyclohexadiene-1,4-dione derivatives (24.22%), quinoline (17.07%), and methyl oleate (5.22%), which have all been associated with antimicrobial properties. This study is the first to report on the antimicrobial potential and chemical composition of *S. rugosum* from MHSNP. The selective inhibition of Gram-positive pathogens, especially *S. aureus*, establishes *S. rugosum* as a promising source of bioactive metabolites with potential pharmaceutical applications.

Keywords: Basidiomycota fungi; antibacterial potential; gram-positive bacteria; secondary metabolites; natural products; mount Halimun Salak National Park.

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Introduction

Indonesia is a tropical country rich in natural resources and biodiversity, including fungi (Diliarosta et al., 2020; Sun et al., 2024). Fungi are non-photosynthetic organisms that are classified into eight phyla, 12 subphyla, and 46 classes. Molecular studies suggest that fungi evolved from zoospores, with some groups previously classified as Zygomycetes now recognized as paraphyletic, while Ascomycota and Basidiomycota form a monophyletic clade within the subkingdom Dikarya. Fungi are generally categorized based on size: macroscopic fungi form multicellular fruiting bodies, while microscopic fungi do not (Rahi & Malik, 2016; Spatafora et al., 2017).

Historical research shows a significant increase in the number of identified fungal species. Bisby and Ainsworth (1943) reported over 100,000 species, which later increased to 250,000 species according to Martin (1951). Hawksworth (1991) estimated the number of species to be 1.5 million, while further research by Hawksworth & Luecking (2017) estimated the total potential diversity of fungi to range between 11.7 and 13.2 million species. All of this information, referencing earlier studies, is found in the article by Wu et al. (2019), which suggests that global fungal diversity ranges between 2.2 and 3.8 million species. These data indicate the vast potential for fungal exploration across various ecosystems in the world.

Asia is one of the regions that has contributed greatly to the documentation of fungal diversity. According to Hyde et al. (2024), China has reported over 15,600 fungal taxa, while Thailand has recorded more than 5,000 species. Indonesia is also recognized as a hotspot of tropical diversity, though research is limited. According to the National Research and Innovation Agency report on the status of Indonesia's biodiversity, there is potential for tens of thousands of fungal species. However, as of 2017, only 2,273 species had been taxonomically identified (Retnowati et al., 2019). This gap underscores the need for in-depth research on fungal diversity in various Indonesian ecosystems to strengthen the country's position as a center of tropical biodiversity.

In addition to their ecological value, fungi also have great potential in the fields of health and industry due to their production of bioactive metabolites. Various studies have shown that fungi produce compounds with nutritional and therapeutic properties, with potential to be used as functional foods, medications, or nutraceutical products (Martinez-Medina et al., 2021). Some bioactive compounds have demonstrated

immunomodulatory and hepatoprotective activities (Chopra et al., 2021). For example, *Armillaria mellea* contains mannitol and tocol, both of which have nutraceutical value, while *Ganoderma lucidum* produces ganoderenic acid, which exhibits hepatoprotective activity (Kostić et al., 2017; Zhao et al., 2019).

Based on this background, this study aimed to explore the antibacterial potential of macroscopic fungi found in Mount Halimun Salak National Park (MHSNP), Indonesia. By isolating and analyzing the produced bioactive compounds, this study is expected to contribute to the scientific understanding of tropical fungal biodiversity while opening up opportunities for its use in health-related fields.

Material and methods

Sampling and identification of basidiomycota fungi

Macroscopic Basidiomycota fungi were collected from MHSNP, West Java, Indonesia, from April to May of 2019, across forested areas. The collected specimens were cleaned, air-dried, and examined morphologically at the Microbiology and Genetics Laboratory at the Faculty of Biology at Universitas Nasional in Jakarta using standard microscopic procedures. Each sample was dried, powdered, and extracted by maceration in ethanol (1:5 w v^{-1}) for antimicrobial screening. Based on the results, the most active fungus was further extracted using methanol under the same conditions. The extracts were then filtered through Whatman No. 1 filter paper (Cytiva, China) and concentrated at 40–45°C using a rotary evaporator (Heidolph Instruments, Germany). Crude methanol extracts were stored at 4°C until further testing.

Preparation of test microorganisms

Antimicrobial assays were conducted against the following four bacterial strains: *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 6539, *Staphylococcus aureus* ATCC 25923, and *Streptococcus mutans* ATCC 45175, and one fungal strain, *Candida albicans* ATCC 10231. Bacteria were cultured in Nutrient Broth (NB) at 37°C for 24 hours, and the fungi were cultured in Potato Dextrose Broth (PDB) at 37°C for 48 hours. The turbidity of the microbial suspensions was adjusted to the 0.5 McFarland standard (~1.5×10^8 CFU mL^{-1}) using 0.9% NaCl. All procedures were performed aseptically in a Laminar Air Flow cabinet (Biobase, China).

Antimicrobial assays

Preliminary antimicrobial screening was carried out using the Kirby-Bauer disk diffusion method and the Gibex assay, following CLSI guidelines (Weinstein et al., 2020), with minor modifications. Sterile paper disks impregnated with fungal extracts were placed on Mueller-Hinton agar (MHA, for bacteria) or Potato Dextrose Agar (PDA, for fungi), and incubated at 37°C for 24–48 hours. Chloramphenicol (30 µg) and ketoconazole (30 µg) served as positive controls, and solvent-only disks served as negative controls. Inhibition zones were measured in millimeters.

The most active extract was further evaluated using microdilution and modified diffusion methods (disk and well diffusion) at concentrations ranging from 40 to 320 mg mL^{-1}. Antimicrobial activity was confirmed by adding MTT reagent (0.5 mg mL^{-1}), and colorimetric changes were recorded after incubation.

Determination of minimum inhibitory and bactericidal concentrations

The Minimum Inhibitory Concentration (MIC) was determined by broth microdilution in 96-well plates at four concentrations: 26.67, 13.33, 6.67, and 3.33 mg mL^{-1}. Wells contained growth medium, microbial suspension, and extract, with blanks (solvent only) and positive controls (chloramphenicol or ketoconazole). After incubation at 37°C (24 hours for bacteria, 48 hours for fungi), MIC was defined as the lowest concentration without visible turbidity. To determine the Minimum Bactericidal/Fungicidal Concentration (MBC/MFC), 100 µL aliquots from MIC wells were inoculated into fresh Mueller-Hinton Broth and incubated under the same conditions. The absence of growth indicated bactericidal/fungicidal activity (Chikezie, 2017).

GC-MS analysis

The methanol extract of *S. rugosum*, the most active species, was analyzed using a GC-MS system (Agilent 7890/5975, Agilent Technologies, USA) equipped with an HP Ultra 2 column (30 m × 0.20 mm × 0.11 µm). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The oven program ranged from 80°C (initial) to 280°C, and the total run time was 26 min. The operating parameters included an injector at 250°C, an ion

source at 230°C, an interface at 280°C, and a quadrupole at 140°C, with electron impact ionization at 70 eV. Compounds were identified by comparing mass spectra with the NIST library data using Balitro.Mp software.

Statistical analysis

All assays were performed in triplicate. Data were analyzed using GraphPad Prism 10. Differences among the treatment groups were evaluated using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical significance was accepted at $p < 0.05$.

Results and discussion

Preliminary screening

Five macrofungal species were collected from MHSNP, namely *Microporus affinis*, *Stereum rugosum*, *Clavaria* sp. nov., *Inonotus obliquus*, and *Polyporus* sp. The crude ethanol extracts from these species were screened for antimicrobial activity against *E. coli*, *S. aureus*, and *C. albicans* using the Gibex and disk diffusion methods.

The Gibex assay (Table 1) revealed that none of the extracts inhibited *E. coli*. In contrast, four species (*M. affinis*, *S. rugosum*, *Clavaria* sp. nov., and *I. obliquus*) displayed inhibitory activity against *S. aureus*. All five species produced colorimetric changes against *C. albicans*, although turbidity analysis revealed weak inhibition, suggesting limited antifungal potential. These findings align with the well-documented resistance of Gram-negative bacteria, such as *E. coli*, whose outer membrane and lipopolysaccharide barrier hinder the penetration of many natural products (Gauba & Rahman, 2023).

Quantitative assessment using the disk diffusion method (Table 1) confirmed these observations. Extracts from *M. affinis*, *S. rugosum*, and *Clavaria* sp. nov. produced inhibition zones of 6.8–11.9 mm against *S. aureus* and *E. coli*. In contrast, *I. obliquus* and *Polyporus* sp. exhibited no activity. Among them, *S. rugosum* exhibited the strongest activity, with an inhibition zone exceeding 11.9 mm against *S. aureus*, indicating promising antibacterial potential. Similar activity has been reported in other *Stereum* species, such as *S. ostrea* and *S. rameale*, which produce metabolites with selective antibacterial effects, particularly against Gram-positive bacteria (Tian et al., 2020).

Taken together, these preliminary results suggest that the macrofungi of MHSNP exhibit selective antibacterial activity, with *S. rugosum* demonstrating the greatest potential. Therefore, this species was selected for further evaluation of its antimicrobial activity and chemical profiling.

Table 1. Preliminary antimicrobial screening of fungal extracts using Gibex and disk diffusion methods.

Fungal species	*Escherichia coli* (Gibex / Zone mm)	*Staphylococcus aureus* (Gibex / Zone mm)	*Candida albicans* (Gibex / Zone mm)
Microporus affinis	– / 7.3	+ / 6.8	± / 6.0
Stereum rugosum	± / 9.7	+ / 11.9	± / 6.0
Clavaria sp.	± / 7.4	+ / 7.3	± / 6.0
Inonotus obliquus	– / 6.0	+ / 6.0	± / 6.0
Polyporus sp.	± / 6.0	± / 6.0	± / 6.0
Positive control	+ / 27.2	+ / 25.4	+ / 28.1
Negative control	– / 6.0	– / 6.0	– / 6.0

"+" = clear inhibition (Gibex); "±" = weak/partial inhibition; "–" = no inhibition.

Antimicrobial activity of *Stereum rugosum*

After preliminary screening, *S. rugosum* was chosen for additional antimicrobial testing against five test microorganisms (*E. coli*, *S. typhi*, *S. aureus*, *S. mutans*, and *C. albicans*) using disk and well diffusion assays. The results (Tables 2 and 3; Figures 1 and 2) showed that the extract exhibited selective antibacterial activity, particularly against *S. aureus* and *S. mutans* strains. The inhibition zones were concentration-dependent; the largest zones were observed at 320 mg mL^{-1} (up to 9.9 mm for *S. aureus* in the disk diffusion assay and 11.8 mm for *S. mutans* in the well diffusion assay). No significant inhibition was detected against *E. coli*, *S. typhi*, or *C. albicans* at the tested concentrations.

Table 2. Antimicrobial Activity of *Stereum rugosum* Methanol Extract (Disk Diffusion).

Concentration (mg mL^{-1})	Inhibition Zone (mm)				
	Test Microbial Species				
	Escherichia coli	*Salmonella typhi*	*Staphylococcus aureus*	*Streptococcus mutans*	*Candida albicans*
320	6.0	6.0	9.9	6.0	6.0

160	6.0	6.0	8.9	6.0	6.0
80	6.0	6.0	7.7	6.0	6.0
40	6.0	6.0	7.2	6.0	6.0
Positive Control	25.3	27.1	26.6	22.7	29.5
Negative Control	6.0	6.0	6.0	6.0	6.0

Table 3. Antimicrobial Activity of *Stereum rugosum* Methanol Extract (Well Diffusion).

Concentration (mg mL^{-1})	Inhibition Zone (mm)				
	Test Microbial Species				
	Escherichia coli	*Salmonella typhi*	*Staphylococcus aureus*	*Streptococcus mutans*	*Candida albicans*
320	6.0	6.0	9.3	10.5	6.0
160	6.0	6.0	8.5	11.8	6.0
80	6.0	6.0	7.5	10.6	6.0
40	6.0	6.0	7.1	11.6	6.0
Positive Control	17.3	28.1	16.9	22.6	20.7
Negative Control	6.0	6.0	6.0	6.0	6.0

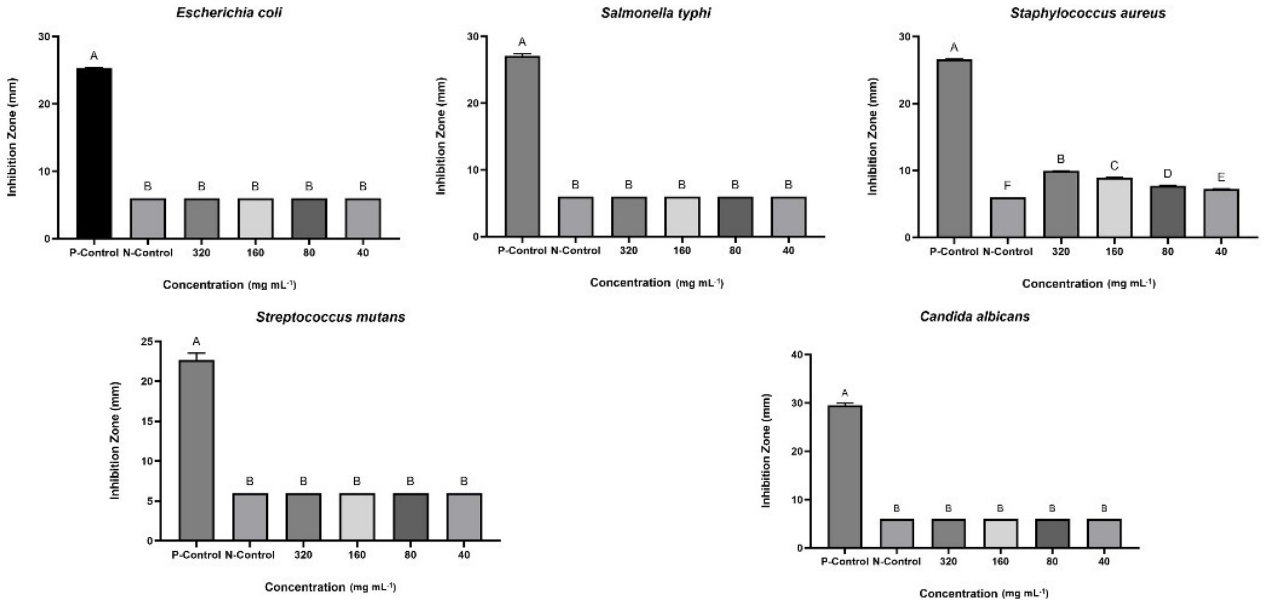

Figure 1. Inhibition Zones of *Stereum rugosum* methanol extract determined by the disk diffusion method.

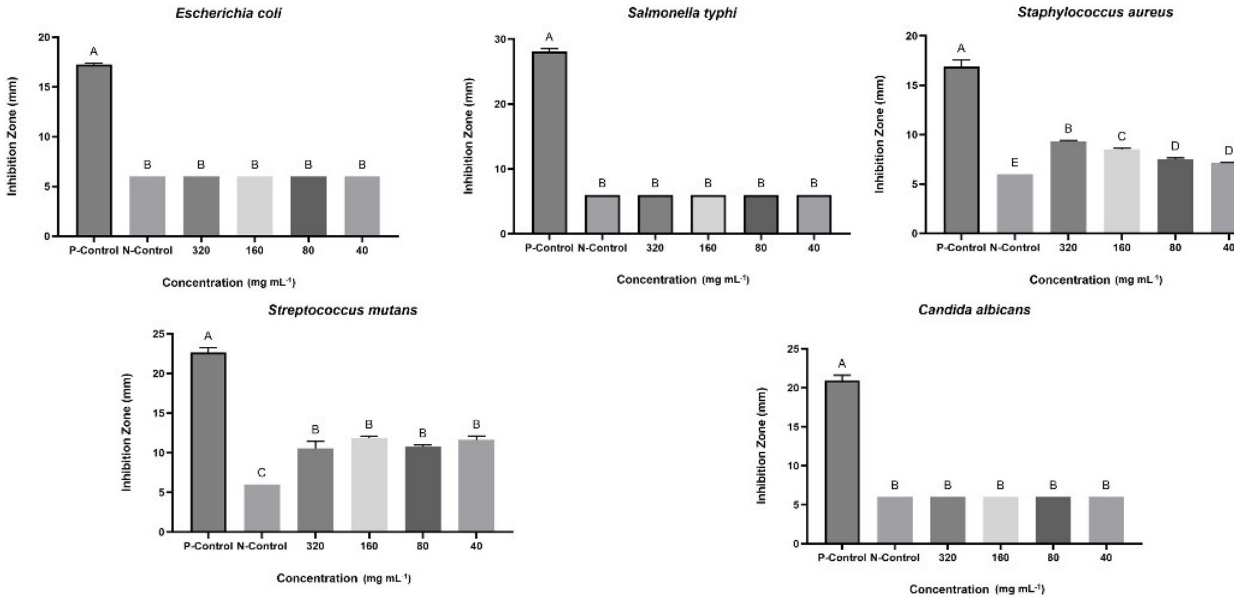

Figure 2. Inhibition Zones of *Stereum rugosum* methanol extract determined by the well diffusion method

Interestingly, inhibition of *S. mutans* was observed only in the well diffusion assay but not in the disk diffusion assay. This discrepancy may be due to differences in compound diffusion through the agar medium. The well diffusion technique allows greater penetration of polar bioactive compounds into the agar, thereby enhancing their contact with microbial cells (Hossain, 2024).

Statistical analysis confirmed significant differences among the treatments (ANOVA, $p < 0.0001$), with Tukey's test evidencing that the positive control (chloramphenicol/ketoconazole) differed significantly from all the other treatments. Although the extract's effects were generally weaker than the positive control, its activity against *S. aureus* was significantly higher than the negative control at all concentrations, demonstrating measurable antibacterial potency.

The selective inhibition of Gram-positive bacteria aligns with the structural differences between Gram-positive and Gram-negative cell walls. Gram-positive bacteria, such as *S. aureus* and *S. mutans*, possess a thick peptidoglycan layer but lack an outer membrane, making them more accessible to lipophilic antimicrobial compounds. In contrast, Gram-negative bacteria, such as *E. coli* and *S. typhi*, are more resistant due to their outer membrane barrier enriched with lipopolysaccharides (Impey et al., 2020; Pasquina-Lemonche et al., 2020).

Overall, these findings reinforce the potential of *S. rugosum* as a selective antibacterial agent against Gram-positive pathogens, particularly *S. aureus*, which is a clinically relevant bacterium often associated with multidrug resistance.

MIC and MBC of *Stereum rugosum*

The antimicrobial potency of the methanol extract of *S. rugosum* was further quantified using a microdilution assay. The extract exhibited inhibitory activity only against Gram-positive bacteria, with MIC values of 6.67 mg mL^{-1} for *S. aureus* and 13.33 mg mL^{-1} for *S. mutans* (Table 4). No inhibitory effect was detected against *E. coli*, *S. typhi*, or *C. albicans* at the tested concentrations. This result confirms the selective antibacterial spectrum observed in the diffusion assays.

Table 4. MIC and MBC Values of *Stereum rugosum* Methanol Extract Against Selected Bacterial Strains.

Concentration (mg mL^{-1})	Test Results														
	Escherichia coli Turbid/Clear			*Salmonella typhi* Turbid/Clear			*Staphylococcus aureus* Turbid/Clear			*Streptococcus mutans* Turbid/Clear			*Candida albicans* Turbid/Clear		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
26.67	+	+	+	+	+	+	-/*	-/*	-/*	-/*	-/*	-/*	+	+	+
13.33	+	+	+	+	+	+	-/*	-/*	-/*	-/*	-/*	-/*	+	+	+
6.67	+	+	+	+	+	+	-/*	-/*	-/*	+	+	+	+	+	+
3.33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Positive Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Negative Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) → Turbid/ There is bacterial growth; (-) → Clear/ No bacterial growth; (*) → Continue to MBC.

The bactericidal effects were confirmed by MBC testing (Table 5). The MBC was 13.33 mg mL^{-1} for *S. aureus* and 26.67 mg mL^{-1} for *S. mutans*. These results indicate that the extract is more potent against *S. aureus* than *S. mutans*, consistent with earlier research showing that *S. aureus* is particularly susceptible to fungal metabolites (Liao et al., 2024). The present values suggest that *S. rugosum* has stronger antibacterial potential.

Table 5. MBC Assay Results of *Stereum rugosum* Methanol Extract Against Selected Pathogens.

Concentration (mg)	Test Results					
	Staphylococcus aureus			*Streptococcus mutans*		
	+/-			+/-		
	1	2	3	1	2	3
26.67	-	-	-	-	-	-
13.33	-	-	-	+	+	+
6.67	+	+	+	+	+	+
3.33	+	+	+	+	+	+

(+) → There is bacterial growth; (-) → No bacterial growth

The higher sensitivity of *S. aureus* compared to *S. mutans* may reflect differences in their cell wall composition and susceptibility to lipophilic compounds. Although both are Gram-positive, *S. aureus* has a peptidoglycan layer that may be more permeable to secondary metabolites. On the other hand, *S. mutans* has

additional exopolysaccharides that contribute to biofilm formation and reduce antimicrobial penetration (Nikolic & Mudgil, 2023; van de Lagemaat et al., 2022).

Taken together, these findings confirm that the methanol extract of *S. rugosum* exhibits bacteriostatic and bactericidal activity against Gram-positive bacteria, with the strongest effect on *S. aureus*. This selective activity further supports the potential of *S. rugosum* as a natural source of antibacterial agents that target clinically relevant Gram-positive pathogens.

GC-MS analysis and bioactive compound

The methanol extract of *S. rugosum* exhibited the strongest antibacterial activity in earlier assays and was therefore subjected to GC-MS analysis to identify its chemical constituents. The chromatogram revealed 22 compounds (Table 6; Figure 3), five of which were major constituents present at concentrations greater than 5%: 2,5-cyclohexadiene-1,4-dione, 2,5-dimethoxy-3,6-bis(octylamino) (24.22%), quinoline derivative (17.07%), acetic acid derivative (6.54%), JWH-018 (5.62%), and methyl oleate (5.22%).

2,5-cyclohexadiene-1,4-dione was the most abundant of these compounds (24.22%). This compound has been reported to possess antioxidant and antimicrobial properties, suggesting that it may contribute significantly to the observed antibacterial activity (Rahman et al., 2022). Quinoline derivatives (17.07%) are also of particular interest because they are a well-established pharmacophore in drug discovery. Quinoline-based compounds exhibit broad biological activities, including antibacterial, antifungal, antimalarial, and anticancer effects (Moynihan et al., 2022; Qureshi et al., 2022). Their presence in the extract supports the inhibitory activity against *S. aureus* and *S. mutans*, and indicates possible broader pharmacological potential.

Table 6. Identified Volatile Compounds in *Stereum rugosum* Methanol Extract Based on GC-MS Analysis.

No	RT	Quality	Compound	Content (%)
1	28.431	99	Hexadecanoic acid, methyl ester	2.09
2	29.575	99	9, 12-Octadecadienoic acid (Z,Z)-, methyl ester	1.74
3	29.603	97	9-Octadecenoic acid, methyl ester, (E)-	5.22
4	29.748	99	Methyl stearate	2.10
5	30.099	98	9,12-Octadecadienoic acid (Z,Z)-	1.11
6	32.706	99	Tetracosanoic acid, methyl ester	2.02
7	34.457	64	Cobaltocene, decamethyl-	1.67
8	34.512	50	Cyclopenta [d] 1,2,3,4,4a,9,10,10a-octahydroantharacene, 6-formyl-3-isopropyl-8,11,dimethoxy-	1.07
9	34.836	38	2-(4-Cycnophenyl)-5-dimethylaminomethylenaminopyrimidine	1.72
10	34.892	52	4H-1,2-Diazepine, 5-(4-chlorophenyl)-3,7-diphenyl-	2.20
11	35.788	51	Estra-1,3,5 (10)-trien-17-ol,imethoxy-acetate, (17.beta.)-	1.55
12	36.567	45	Colchiceinamide	1.27
13	37.091	35	Hyodeoxycholic acid	1.20
14	37.781	99	Ergosterol	2.92
15	40.035	60	Phosphonic acid, P,P'- [(2,5-dimethoxy-1,4-phenylene) bis (methylene)] bis-, tetraethyl ester	1.70
16	40.277	58	Ergost-22-en-3-ol, (3.alpha.,5.beta.,22E)-	3.70
17	42.559	41	2,5-cyclohexadiene-1,4-dione,2,5-dimethoxy-3,6-bis(octylamino)-	24.22
18	42.745	38	3-Hydroxy-6,2'4'-4-trimethoxyflavone, trifluoroacetate	2.72
19	43.731	25	Quinoline, 1-(4-chlorophenyl)-1,4-dihydro-2,3-diphenyl-	17.07
20	43.952	92	Acetic acid, 2,2'-[(2,2'3,3'-tetrahydro-3,3,3'3'-tetramethyl-1,1'-spirobi[1H-indene]-6,6'-diyl) bis (oxy] bis-	6.54
21	44.228	25	JWH-018	5.62
22	46.138	52	Isobenzofuro [5,6-b] benzofuran-8-carboxylic acid,1,3-dihydro-7,10-dimethoxy-9-methyl-1-1-oxo-,methyl ester	3.72

In addition, methyl oleate (5.22%) and other fatty acid esters have been reported to disrupt microbial membranes and enhance the susceptibility of Gram-positive bacteria. This could partly explain why the extract was more effective against Gram-positive than Gram-negative bacteria, since fatty acid derivatives are known to insert into lipid bilayers and destabilize bacterial membranes (Blanco-Cabra et al., 2019).

Interestingly, some minor compounds, such as ergosterol (2.92%), were also detected. Ergosterol is a key fungal sterol that has been associated with immunomodulatory and antioxidant activity (Sun et al., 2019). Although present in smaller amounts, these metabolites may contribute synergistically to the extract's overall bioactivity.

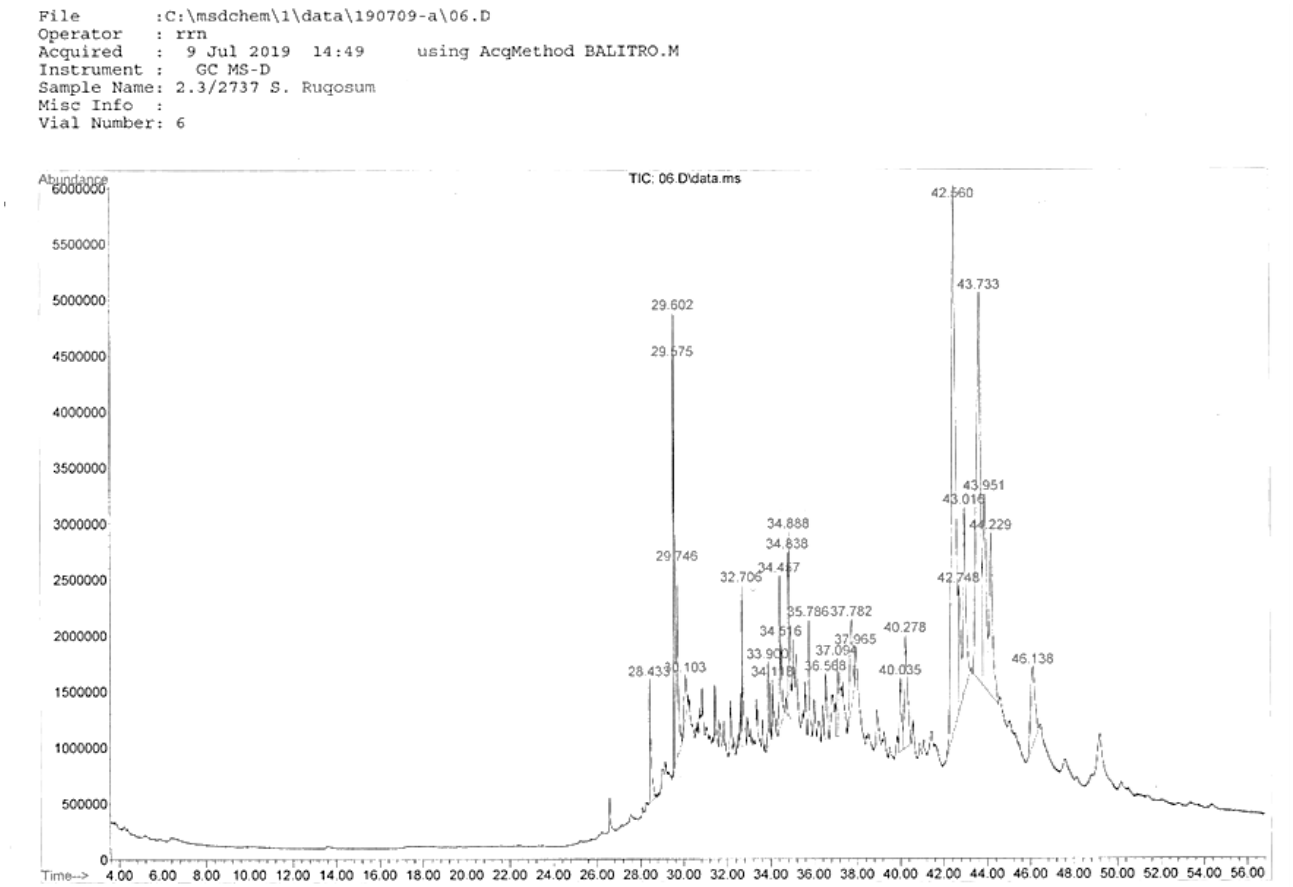

Figure 3. Chemical Profile of *Stereum rugosum* Methanol Extract Based on GC-MS Analysis.

Taken together, the GC-MS profile of *S. rugosum* reveals a variety of secondary metabolites with potential biological functions. The dominance of quinoline and cyclohexadiene derivatives strongly suggests that these compounds are the main contributors to the observed antibacterial activity against Gram-positive bacteria. However, further studies involving fractionation, purification, and structure–activity relationship (SAR) analysis are required to confirm the role of these compounds.

Overall, this study demonstrated that *S. rugosum* exhibited the most promising antibacterial activity among the five macrofungal species isolated from Mount Halimun Salak National Park. The extract selectively inhibited Gram-positive bacteria, particularly *S. aureus* and *S. mutans*, while showing little or no activity against Gram-negative bacteria and *C. albicans*. MIC and MBC assays confirmed the extract's stronger potency against *S. aureus*, indicating potential bactericidal activity at relatively low concentrations compared to other reported *Stereum* species. GC-MS analysis further revealed that the extract contains diverse secondary metabolites, with quinoline and cyclohexadiene derivatives identified as the dominant compounds likely responsible for the antibacterial activity. These findings highlight *S. rugosum* as a novel and valuable source of bioactive compounds from Indonesian tropical fungi. This contributes to our understanding of fungal biodiversity and the exploration of natural products with potential pharmaceutical applications.

Conclusion

This work provides the first evidence of the antimicrobial potential of *S. rugosum* from MHSNP, Indonesia. The methanolic extract was found to selectively inhibit Gram-positive bacteria, exhibiting significant effects against *S. aureus* and *S. mutans*. MIC and MBC assays confirmed the extract's potency, and GC-MS analysis revealed quinoline and cyclohexadiene derivatives as the dominant bioactive compounds. These findings support *S. rugosum* as a promising novel source of antibacterial metabolites with potential pharmaceutical applications.

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