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Antimicrobial and Phytochemistry study of *Liparis resupinata* Ridl. from Mount Gumitir, East Java, Indonesia

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Abstract: *Liparis resupinata* Ridl. is one of the abundant epiphytic orchids in the Mount Gumitir, Jember Regency with potency for phytopharmacy study. Our study focused on the observation of the species for their antimicrobial and metabolite profiles. This study used Gram-positive and Gram-negative bacteria, i.e., *S. aureus*, *S. typhi*, and *E. coli* with an agar diffusion method for antibacterial activity test. The metabolite profile was generated through GC-MS. The methanolic extract of the leaves of *L. resupinata* showed positive antibacterial activity. The GC-MS data analysis suggested the presence of antimicrobial substances e.g., (-)-loliolide, nonanoic acid, 1,2-ethanediamine, and hydroxy dimethyl furanone.

Keywords: Antimicrobial; *Liparis resupinata*; GC-MS; Metabolite; Orchids

1. Introduction

Plants have been used for medicinal treatment for 5,000 years and have become the main source for the development of modern pharmacy (Anand et al., 2019). The phytochemical properties that are often used in medicine are secondary metabolites, especially from the terpenoid, phenolic, flavonoid, alkaloid, and glycoside groups (Darojati et al., 2022; Velu et al., 2018). Although a high number of plant species had been utilized in traditional medicine, still a large number of wild plants were unexplored. Indonesia as one of the mega-biodiversity spots has a great potential medicinal resource with a total of 2,500 to 7,500 plant species recorded (Cahyaningsih et al., 2021; Erdelen et al., 1999).

The increase in microbe resistance to drugs or Multi-Drug Resistance (MDR) and the COVID-19 outbreak has encouraged more research in the exploration of new compounds (Lim et al., 2021; Mukherjee et al., 2022). This has a direction to develop research in isolation and identification of new phytochemical compounds to solve antimicrobial resistance (Vaou et al., 2021). Plant-based medicinal product contains rich phytochemical (Ramya, 2022) that require the identification of the bioactive compounds (Lautié et al., 2020) for the new medicine substance development. New drugs were discovered by identifying active compounds from natural products and screening based on their antioxidants, anti-inflammatories, antimicrobials, and anti-cancer activities (Olivia et al., 2021).

Our previous study reported the secondary metabolites of alkaloid and flavonoid content of wild orchids from the Mount Gumitir area (Setyati et al., 2022). *Liparis resupinata* Ridl. was one of the abundant orchids in the study area (Arum, 2023; Ulum et al., 2023) and was reported widespread in the tropical forest of South East Asia (Tetsana et al., 2014). In contrast, the phytochemical substance and the medicinal potency of *L. resupinata* were still unexplored. Genus Liparis consists of 320 species and is distributed from the tropic, sub-tropic, and temperate regions (Li et al., 2020). The medicinal potencies of the genus Liparis had been reported in traditional Chinese with pharmacological potencies as procoagulant, homeostasis, anti-inflammatory, and antimicrobial activity (Liang et al, 2019).

Plants produce medicinal compounds with various benefits from human health, however with a rich in compound varieties, making it difficult to identify the specific compound related to the medicinal benefit. The most popular method to separate and identify the phytochemical substance was with chromatographic technique, such as Gas chromatography coupled to mass spectrometry (GC-MS) which has been widely used in research on antimicrobial exploration from plants (Alonso et al., 2022). Vaou et al (2021) suggested the efficient method for antimicrobial exploration from plant extract should consider the plant extract with inhibition at low or moderate MIC (Minimum Inhibition Concentration) value. In this study, we aimed to investigate the medicinal potency of orchids *L. resupinata* based on the antimicrobial activity of leaf extract against gram-positive and negative pathogen bacteria and phytochemical characterization using GC-MS.

2. Material and Methods

2.1. Plant material

L. resupinata was collected from Mount Gumitir, Jember, East Java, Indonesia. The specimen was identified based on morphological structure by Lina Susanti Juswara from Biosystematics and Evolution Research Centre, BRIN, and based on molecular data of DNA barcode with three primers, i.e., *matK*, *rbcL*, and *ITS2* (Arum, 2023). A voucher specimen was planted in the Biology Department, Faculty of Mathematics and Sciences, The University of Jember, Indonesia (Ulum et al., 2023). Leaves extract was used for the antimicrobial assay and the metabolite analysis. Leaves (approximately 1 Kg) were cleaned by washing with tap water and were then air-dried at room temperature for 20 days. The dried sample was then pulverized with a commercial spice grinder.

2.2. Extraction

The leaf powder (60 gr) was macerated with methanol in a ratio of 1:5 for 3x24 hours and repeated once. The liquid sample was filtered with Wathman filter paper and the filtrate was concentrated with a rotary evaporator.

2.3. Antimicrobial assay

The antibacterial activity of the crude extract was performed with agar well diffusion methods (Balouiri et al., 2016) against a gram-positive bacterium i.e., *Staphylococcus aureus*, and two gram-negative bacteria i.e., *Salmonella typhi* and *Escherichia coli*. Before the antimicrobial test, all bacteria were subcultured and incubated until they were grown into the mid-logarithmic phase (Kim et al., 2017). For the positive control we used chloramphenicol 1% and the negative control was methanol absolute. The crude extract was assayed in a series of concentrations, i.e., 10 %, 20 %, 30 % 40 %, and 50 %.

Each bacteria culture was dissolved in 1 mL of nutrient agar (NA) media and poured into petri dishes then left to condense. A total of four wells were made with a cork borer (5 mm diameter). Each diffusion well was filled with 20 µL of crude extract and the control liquid. The assay was four plicates. The observation was conducted by measuring the diameter of the inhibition zone formed around the well. The antimicrobial activities were classified into three classes based on the diameter of the inhibition zone i.e. strong (> 20 mm), medium (5 – 20 mm), and weak (< 5 mm) (Putri, et.al., 2022).

2.4. Phytochemistry analysis based on GC-MC

The phytochemistry of methanol extract of *L. resupinata* was analyzed with Gas chromatography-mass spectrometry (GC-MC). GC-MS is a combination of two analytical techniques for the detection of compounds. Gass chromatography separates the volatile and semi-volatile compounds and mass spectrometry provides detailed structure information of the compound. GC-MS was carried out using QP2010 Plus Shimadzu, Japan. 1 µL of the sample was injected into the column. The setting of GC-MS was the temperature of the oven injectors 290 ^{0}C, MS detector 280 ^{0}C, column Rtx-50 (inner diameter 0.25 mm, length 30 m, thickness 0.25 µm), detector temperature at early stage 80 ^{0}C for 10 min, detector temperature at final stage 230 ^{0}C for 10 min, speed 5 ^{0}C/min. Helium was used as carrier gas at a flow of 3 mL/min. Identification of metabolite was conducted based on their mass spectra with the database of Wiley Library 9.

2.5. Statistical analysis

All statistical analyses were conducted with R (version 4.1.2 (2021-11-01) and R Studio (2021.09.1). Data handling and visualization were performed using the packages dplyr, and

rstatix. Differences between concentration on the inhibition diameter were explored with ANOVA and 95% Duncan test with DescTools.

3. Results and Discussion

3.1. Antimicrobial activity

The leaf extract of *Liparis resupinata* presented antimicrobial activities against both gram-positive and gram-negative bacteria (Figure 1). The positive and negative controls support the presence of antimicrobial substances in the methanolic extract. The widest inhibition zone was observed in chloramphenicol 1% as positive control. *S. aureus* was the most sensitive microbe that presented antimicrobial activity from the five gradual concentrations of methanolic extract. *S. typhi* was the second sensitive microbe to the *L. resupinata* extract as the inhibition zone were weaker at the low concentrations (10 % and 20 %). *E. coli* culture exhibited a slight inhibition activity by the antimicrobial substance. The methanolic extract of all concentrations imprinted a small inhibition zone (Table 2). Nevertheless, the antimicrobial activity of *L. resupinata* in this study was confirmed by the agar diffusion method as the therapeutic screening method (Selvarajan et al., 2022).

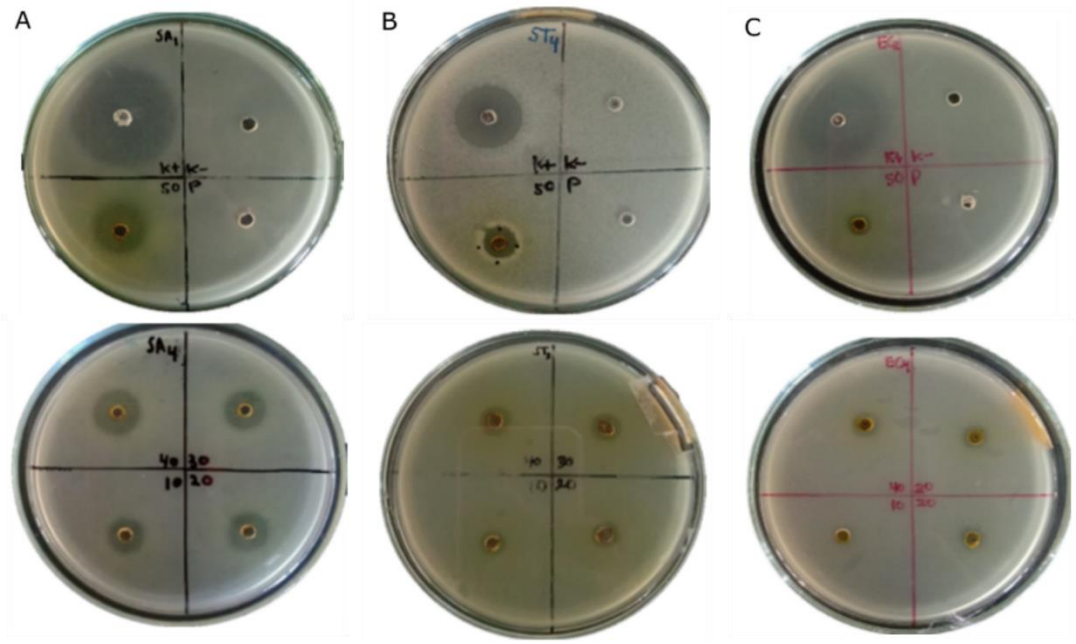

Figure 1. Inhibition zone of Methanolic extract *Liparis resupinata* against microbial tested: A) *S. aureus*, B) *S. typhi*, C) *E. coli*. K+ = Positive control (chloramphenicol); K- = Negative control (methanol) P = No liquid (empty); Number (50, 40, 30, 20, 10) = Concentration on methanolic extract in percent (%)

The size of the inhibition zone of methanolic extract in three microbe cultures is presented in Table 1. The high methanolic extract concentration had a stronger inhibition than the lower concentration. One-way ANOVA test confirmed the significant difference among the five concentrations from each pathogen. The difference in antimicrobial activities was marked by

the Duncan test (Table 1). The gram-positive bacteria, *S. aureus* were the most susceptible microorganism to all concentrations of *L. resupinata* extract. The sensitivity of this bacteria against the antibacterial substance of plant extract was reported (Farahmandfar et al., 2019). On the other hand, the gram-negative bacteria exhibited a gradual alternation of inhibition effect related to the antimicrobe content.

The diameter of the inhibition zone indicated the antimicrobial activity. The methanol extract of *L. resupinata* presented a medium antimicrobial activity against *S. aureus* and *S. typhi* culture tests. The lower concentration of *L. resupinata* extract with medium inhibition activity was at a concentration of 20 % against *S. aureus*. The low MIC of the extract against *S. aureus* indicated the high potential for antimicrobial development (Vaou et al., 2021). On the other hand, in *S. typhi* the medium antimicrobial activity was observed only at a higher concentration (40 % and 50 %). In contrast, the difference in inhibition size in *E. coli* was different among all concentrations albeit with a low antimicrobial activity. The phytochemical extract generally had a stronger inhibition effect against the gram-positive than the gram-negative. The difference in antimicrobial activities among gram-positive and gram-negative bacteria might related to the structure of outer membranes. The outer membrane of gram-negative bacteria is covered with a hydrophobic structure which protects the structure by inhibiting the binding of hydrophilic molecules such as phenolic compounds (Anand et al., 2019). Furthermore, plant extracts had antimicrobial substances in the non-polar form that made it difficult to spread for the agar diffuse test (Eloff, 2019) might influenced the antimicrobial activity of our study. The presence of an inhibition mark in this study shed light on the antimicrobial potency of *L. resupinata*.

Table 1. Comparison of *in vitro* antimcrobial activity of *L. resupinata* methanolic extract against three patogens. The data expressed the means ± SD of the diameter of inhibition zone (mm). The data were compared using One-way analysis of variance (ANOVA) among five concentrations and the positve control of each patogen, followed by Duncan test at level of sigificant 5 % ($p < 0.05$). The difference letters indicate the significant differences between the concentration of each pathogen.

Concentration (%)	**Inhibition zone (mm)**		
	S. aureus	***S. typhi***	***E. coli***
10	4.22 ± 0.37^{d}	1.40 ± 0.68^{d}	0.93 ± 0.13^{e}
20	5.41 ± 0.88^{cd}	3.12 ± 1.05^{c}	1.64 ± 0.29^{d}
30	6.33 ± 0.38^{bc}	4.41 ± 0.93^{b}	2.58 ± 0.37^{c}
40	7.99 ± 0.39^{ab}	5.03 ± 0.67^{ab}	3.24 ± 0.54^{b}
50	9.49 ± 2.27^{a}	5.71 ± 0.51^{a}	3.87 ± 0.30^{a}
Control (+)	26.17 ± 2.50	18.95 ± 1.46	21.68 ± 0.91
p-value of One-way ANOVA	0.00006	0.00001	0.0000002

S. aureus was one of the high-resistance pathogens due to its active transferable genetic element (Bitrus et al., 2018). *S. typhi* was the main typhoid fever and was studied among tropical

countries for its resistance (Rahman et al., 2020). *E. coli* was also reported as one of the most studied gut pathogens around the globe (Puvača & Frutos, 2021). Studying on exploration of new medicinal products is a common strategy to solve pathogenic spreading (Eloff, 2019). Plant extract is one of the popular medicine resources for antibacterial medicine (Nigussie et al., 2021; Ramasamy et al., 2022). The methanol extraction method with *Simarouba glauca* indicated a slightly stronger inhibition activity against the same pathogen in our study compared to the Ethanol (Ramasamy et al., 2022). Utilization of the local undiscovered wild orchids *L. resupinata* in this study opened further steps in the exploration of new medical resources.

3.2. Phytochemistry Profile

The chromatogram of GC-MS analysis of the methanolic leaf extract of *L. resupinata* presented the peaks that indicated the occurrence of different phytochemical compounds (Figure 2). Identification of bioactive compounds resulted from a total of 35 compounds based on the molecular weight. The compound name, percentage, and the retention timing of the chemical substance are shown in Table 2. The three major components (30% of the area) of the metabolite were 1,2,3,4-butanetetrol, (11,4%), tetradecanoic acid (10,3%), and 9-octadecenoic acid (Z)-(16,9%). Erythritol is a synonym of 1,2,3,4-butanetetrol, a polyol (sugar alcohol) compound functionally as an antioxidant and carbon-storage molecule within plants (Scanga et al., 2018). This compound was reported to have an antimicrobial activity against Group B streptococci (Hulbah, et al., 2021). Tetradecanoic acid and 9-octadecenoic acid are fatty acids with a potential for antimicrobial activity as was reported in Gajbhiye and Kapadnis (2021).

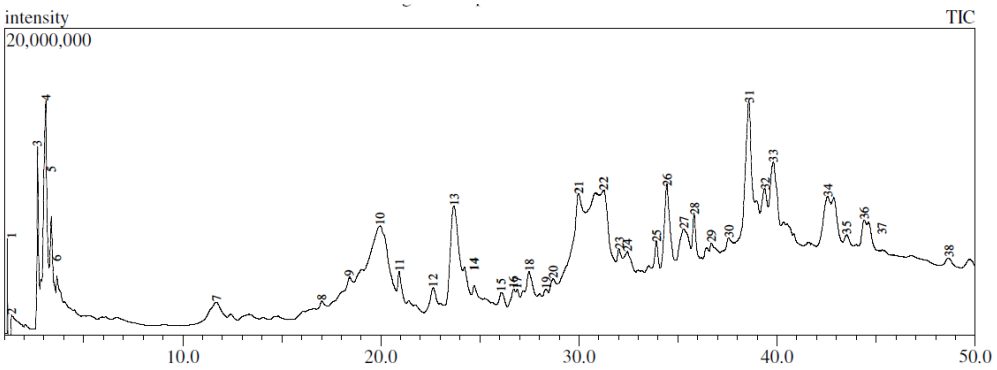

Figure 2. GC-MS chromatogram represents the separated phytochemical compound of *Liparis resupinata* methanolic leaf extract

The literature survey was also applied through the publication report (e.g. Swamy et al., 2017) to observe the antimicrobial reports of each detected phytochemicals in this study. Among the rest of the 35 phytochemicals detected from GC-MS in this study, a total of four bioactive compounds with antimicrobial activities were presented, i.e., (-)-loliolide, nonanoic acid, 1,2-ethanediamine, and hydroxy dimethyl furanone.

Table 2. Phytochemical compound identified in methanolic leaf extract of *Liparis resupinata*.

No	Retention time	Area (%)	Name
1	1.2	0.15	monoammonium salt (CAS) Ammonium carbamate
2	1.4	0.17	cyclobutanol (CAS) cyclobutyl hydroxide
3	2.7	1.47	methanamine, N,N-dimethyl- (CAS) trimethylamine
4	3.1	3.80	acetic acid (CAS) ethylic acid
5	3.4	1.50	2-propanone, 1-hydroxy- (CAS) Acetol
6	3.6	0.39	1,2-ethanediamine, N-ethyl-
7	11.7	0.72	hexanoic acid (CAS) n-hexanoic acid
8	17.0	0.11	hydroxy dimethyl furanone
9	18.4	0.72	ketoisophorone
10	20.0	6.97	1,2,3-propanetriol
11	20.9	0.61	N-acetylpyrrolidone
12	22.7	0.77	nonanoic acid
13	23.7	4.66	2,3-dihydro-benzofuran
14	24.7	0.73	phenol, 4-ethenyl-2-methoxy-
15	26.1	0.37	1H-indole
16	26.7	0.37	2-propenoic acid, 3-phenyl-, methyl ester
17	26.9	0.24	phenol, 2,6-dimethoxy-
18	27.5	1.34	pyrrolidine, 1-(1-pentenyl)-
19	28.3	0.33	3a,6-Methano-3aH-indene-4-d, octahydro-, (3a.alpha.,4.alpha.,6.alpha.,7a.beta.)-
20	28.7	0.74	trans Sabinene hydrate
21	30.0	7.41	9-octadecenoic acid (Z)-
22	31.3	11.36	1,2,3,4-butanetetrol, [S-(R*,R*)]-
23	32.0	1.20	2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-
24	32.5	2.15	trans-2-pinanol
25	33.9	2.67	2-Pentadecanone, 6,10,14-trimethyl-
26	34.5	3.98	Tetradecanoic acid
27	35.3	3.54	3-buten-2-one, 4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)- (CAS) 4-(4-hydroxy-2,2,6-trimethyl-7-oxa-bicyclo[4.
28	35.8	2.05	pentadecanoic acid, 14-methyl-, methyl ester (CAS) methyl 14-methyl-pentadecanoate
29	36.7	3.44	naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl- (cas) 1,2,3,4-tetrahydro-2,5,8-trimethylnaphthalene
30	37.6	2.62	9-octadecenoic acid (Z)-
31	38.6	10.29	tetradecanoic acid
32	39.4	3.18	(-)-Loliolide
33	39.8	5.17	phytol isomer
34	42.6	6.98	9-octadecenoic acid (Z)-
35	43.5	1.88	9,12,15-octadecatrienoic acid, methyl ester
36	44.4	4.38	1,16-hexadecanediol
37	45.3	1.02	hexadecanamide (CAS) amide 16
38	48.7	0.48	5-heptylpentan-5-olide

Loliolid, the synonym of (-)-loliolide, belongs to the terpenoid substance that was reported for antimicrobial activity (Grabarczyk et al., 2015) and for neurotherapeutic (Silva et

al., 2021). Nonanoic acid is a naturally occurring saturated fatty acid and was reported the inhibition activity to the growth of gram-positive bacteria (Sahin et al., 2006), reduction of microbe translocation, and improved antimicrobe activity (Wang et al., 2018). Ethylenediamine, the synonym of 1,2-Ethanediamine, is an ammonia group that was studied for the antimicrobe and was suggested for further pathogen therapeutic (Musa et al., 2010). Hydroxy dimethyl furanone is a heterocyclic organic compound and showed anti-biofilm properties that inhibited microbe development (Devadas et al., 2019).

Exploration of Orchids as medicinal herbs in Indonesia has been conducted e.g. in Lake Toba (Aswandi and Kholibrina, 2021), Papua (Utami et al., 2017), and middle Java (Nugroho et al., 2016). Some orchid species from the genus Liparis have been used in traditional Chinese for the treatment of anti-inflammatory, antibacterial activities, and hemostasis (Liang et al., 2019). The study of *L. nervosa* reported the alkaloid substance had strong antimicrobial activities against a total of 14 pathogenic bacteria (Dong et al., 2010) and the detection of essential oil-based GC-MS analysis (Zhao et al., 2023). With the potential of antimicrobe activities and high variation of the phytochemical substance of the *L. resupinata* in the present study, further observation on non-volatile compounds such as with LC-MS would be beneficial to enrich the data of bioactive compounds from these orchids.

4. Conclusion

The present study revealed the antimicrobial activity and the metabolite profile of *L. resupinata* leaf extract. The leaf methanolic extract of the sample had a medium antibacterial activity against gram-positive pathogens whereas in gram-negative bacteria the inhibition activity was weak. The phytochemical of this wild orchid comprising of antimicrobial against viz., Erythritol, Tetradecanoic acid, 9-octadecenoic, (-)-loliolide, nonanoic acid, 1,2-ethanediamine, and hydroxy dimethyl furanone.

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Conflict of Interest

All authors declared that there was no conflict of interest.

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