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TLC-Bioautographic and LC-MS/MS Detection of Antimicrobial Compounds from Four Semipolar Extracts of *Cladonia* Species

Friardi Ismed[1,2*] Widi Novella Desti[1] Nurwahidatul Arifa[1] Rustini Rustini[1] Deddi Prima Putra[1,2]

[1] *Faculty of Pharmacy, Andalas University, Padang 25163, Indonesia*
[2] *Laboratory of Biota Sumatera, Andalas University, Padang 25163, Indonesia*
[*]*Corresponding author. Email: friardi@phar.unand.ac.id*

ABSTRACT
To continue the study of Sumatran lichen, six species of the genus *Cladonia* were screened for phytochemicals and their antibacterial bioactivity. The procedure included sequential extraction (semi polar solvent), antibacterial activity tests using the agar diffusion method, and microdilution to determine the minimum inhibitory concentration (MIC) value of each extract. The study also involved the thin layer chromatography (TLC)-bioautography method and liquid chromatography-mass spectrometry (LC-MS/MS) analysis (in positive mode), which aimed to provide information about the composition and activity of individual components of the extracts that have an antibacterial effect. The test results indicated that all studied *Cladonia* lichen semipolar extracts had an antibacterial effect, while five of them had very strong bacterial growth inhibition activity (MIC range values 2.5 to 10 mg/mL). Additionally, the identities of compounds found in lichen with broad inhibition, which include the depside and depsidone groups, were confirmed by LC-MS/MS. Based on existing test data, *C. scabriuscula*, *C. crispata*, *C. rappii*, and *C. macilenta* are potential antibacterial candidates.

Keywords: *Cladonia, depside, depsidone, TLC-bioautography, LC-MS/MS.*

1. INTRODUCTION

Although the use of antibiotics as multipurpose drugs is common, some bacteria are resistant to these compounds. In 2018, the World Health Organization (WHO) issued a list of priority bacteria and noted the need for antibiotic agents that are effective against these organisms [1]. In 2019, surveillance conducted at hospitals in Indonesia noted that some bacteria had increased resistance to antibiotics, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (21%), carbapenem-resistant *Acinetobacter* (50%), and *Pseudomonas aeruginosa* (33%) [2]. As such, antibiotic resistance is a matter of concern.

Notably, the development of antibacterials derived from natural ingredients aims to overcome various problems caused by bacterial resistance. One such natural ingredient is lichen due to its ability to survive in extreme environments. Lichens represent a symbiotic group of organisms living in collaboration with fungi (mycobionts) and algae/cyanobacteria (photobionts). Lichens are distributed worldwide and include approximately 18,500 species [3]. They are also used as disinfectants, alternative treatments for wounds, and to stop bleeding, among other uses. These biological activities are inseparable from the chemical constituents of lichen. In 1996, approximately 800 compounds were obtained from lichen [4]—a number that has now increased to 1050 [5]. Several studies have shown that secondary metabolites from lichens have the potential to inhibit bacterial growth at much lower concentrations when compared to other sources of antibiotic therapy [6]. One particularly interesting lichen group is the genus *Cladonia* (Cladoniaceae), which includes a total of approximately 500 species worldwide [7]. To expand on studies of Indonesian lichen that previously explored the genera *Stereocaulon* and *Cetrali* [8], [9], several

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collections of *Cladonia* species were carried out. These included *C. scabriuscula* Delise (CS), *C. crispata* (Ach.) Flot (CC), *C. rappii* Evans (CR), and *C. macilenta* Hoffm (CM). In this paper, evaluations of the antibacterial activity of ethyl acetate of the lichen genus *Cladonia* against *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 12228, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were performed using the diffusion and dilution methods. Thereafter, thin layer chromatography (TLC)-bioautography and liquid chromatography-mass spectrometry (LC-MS/MS) analysis were conducted to determine the metabolite profiles and constituents providing antibacterial activity.

2. MATERIALS AND METHODS

2.1 Lichen Material

Four lichen species were harvested from the highlands and mountains of West Sumatra between October 2018 and July 2019 (Table 1). We confirmed our lichen identification by sending all samples to Harrie Sipman (Berlin Museum). Specimen vouchers were then deposited at the Biota Sumatran Laboratory, Andalas University, West Sumatra (Indonesia) with the reference numbers cited in Table 1.

2.2 Preparation of Crude Extracts

For each species, approximately 10 g of dried lichen was extracted by a semi polar solvent (ethyl acetate) (2 times × 100 mL). Each extract was concentrated in *vacuo*.

2.3 Agar Diffusion Method for Antibacterial Assay

The diffusion method for antibacterial testing was performed on ethyl acetate extracts from the four collected *Cladonia* species with concentrations of 200, 150, 100, 50, and 25 mg/mL dissolved in dimethyl sulfoxide (DMSO, Merck, Germany). Furthermore, 10 µL of each sample was pipetted into a disk (Macherey Nagel, dia. 6 mm). The disk was then placed on a solidified agar surface and incubated for 18–24 hours at 37°C. The bacteria used in the tests included *E. coli* ATCC 25922, *E. faecalis* ATCC 12228, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853, with a positive control for chloramphenicol (30 µg/disk, Merck) and a negative for DMSO. All experiments were performed in duplicate [10].

2.4 Extract Minimum Inhibitory Concentration (MIC) Value Determination

The microdilution method was used to determine the minimum inhibitory concentration (MIC) value. Each of the extracts and chloramphenicol (positive control) were dissolved in DMSO to obtain final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.1562 mg/mL, respectively. In addition to positive and negative controls, a total of 100 µL of each concentration was placed into 96 microplate wells. Each well was then inoculated with a bacterial suspension of 5 µL and incubated at 37°C. Tests were performed in triplicate. After 18 hours, 20 µL of iodonitrotetrazolium (INT, Sigma Aldrich) solution was added to each microplate well and incubated again for 30 minutes at 37°C. Bacterial growth was indicated by a color change in the wells, namely from pink to red. MIC values were determined by identifying the weakest concentration that did not exhibit bacterial growth [11].

2.5 Antibacterial Profiling by TLC–Bioautography Assay

The chemical profiling of lichen extracts typically employs the TLC system, which involves using standard solvent G (toluene: ethyl acetate: formic acid = 139: 83: 8), which was developed by Huneck [4]. A total of 10 µL of each extract from the five studied species of *Cladonia* (concentration 10 mg/ml) was spotted on a Si_{60} F_{254} analytical TLC plate (10 × 20 cm, aluminum-backed, Merck, Germany). The solvent (eluent G) was then added at room temperature and the Rf values of each stain were calculated. After being dried, the stain spots were viewed under a UV lamp (254 and 365 nm), sprayed with anisaldehyde-H_2SO_4 reagent (ANS, v/v), and heated at 110^{0}C.

Table 1. Location and altitude of the four collected *Cladonia* species

Species	Reference numbers	Coordinates	Altitude	Locality
C. scabriuscula Delise (CS)	FTS 009	0°58'41.9"S 100°40'45.8"E	1300 m	Mount Talang, West Sumatra
C. crispata (Ach.) Flot (CC)	FFHP 030	0°06'10.3"S 100°40'09.7"E	1000 m	Harau Valley
C. rappii Evans (CR)	FTS-006	0°58'41.9"S 100°40'45.8"E	1300 m	Mount Talang, West Sumatra
C. macilenta Hoffm (CM)	FTS 002	0°58'41.9"S 100°40'45.8"E	1300 m	Mount Talang, West Sumatra

The TLC-bioautography method was tested using 1 mL of bacterial suspension equalized with 0.5 McFarland standard mixed into 30 mL of Mueller-Hinton agar (MHA) that was homogenized and then allowed to solidify. Thereafter, the TLC plate developed with each extract affixed to the agar surface and was then incubated at 37ºC for 18–24 hours [12]. Next, the TLC plate was removed and sprayed evenly with INT dye (iodonitrotetrazolium, Sigma Aldrich, 4 mg/mL in alcohol) and allowed to stand for 30 minutes. Antibacterial activity was only considered potent when there was an inhibition area in the form of a white zone at the red TLC plate base.

2.6 LC-MS/MS Analysis

High-resolution mass spectrometry experiments were performed using an ultra-performance liquid chromatography (UPLC) unit (LC: ACQUITY UPLC® H-Class System, Waters, USA) and a mass spectrometer (Xevo G2-S QTof, Waters, USA). This involved using a C18 column (1.8 µm 2.1x100 mm, ACQUITY UPLC® HSS, Waters, USA) at temperatures of 50°C (column) and 25°C (room). The LC analysis used a mobile phase that was water + 5 mM ammonium formic (A) and acetonitrile + 0.05% formic acid (B), with a flow rate of 0.2 mL/min (step gradient) running for 23 minutes (see slide moving phase) and an injection volume of 5 µL (initially filtered through a 0.2 µm syringe filter). The mass spectrometry (MS) analysis was conducted using electrospray ionization (ESI) in positive mode with a mass range of 50–1200 m/z and source and desolvation temperatures of 100 and 350°C, respectively. Additionally, cone and desolvation gas flow rates of 0 L/hr and 793 L/hr were also used correspondingly, while the collision energy varied between 4 and 60 eV. Masslynx software version 4.1 was used for data acquisition and analysis as well as instrument control.

3. RESULTS AND DISCUSSION

The *Cladonia* (Cladoniaceae, Lecanoromycetes) is one of the largest and most diverse groups of lichen-forming fungi [13]. This genus is typically found in cold habitats and altitudes above 150 m [14], [15]. The information presented in table 1 shows that the four studied species were collected in upland areas at 1000–2000 m in elevation. For the extraction process, thallus powder was immersed in a semi polar solvent (ethyl acetate).

3.1 Antibacterial Activity

The antibacterial testing results indicate that several extracts provided a strong inhibition zone (above 12 mm) against bacterial growth. ECC showed a strong inhibitory diameter against *E. coli* (17.15 ± 0.494 mm) and *E. faecalis* (16.35 ± 0.424 mm) at a concentration of 50 mg/mL (Table 2). Furthermore, ECM strongly inhibited *E. coli* (15.20 ± 0.212 mm), *E. faecalis* (16.07 ± 0.035 mm), *P. aeruginosa* (16.12 ± 0.530 mm), and *S. aureus* (16.65 ± 0.141 mm) at the lowest test concentration (25 mg/mL).

Table 2. Antibacterial activity of *Cladonia* extracts against pathogen bacteria

Extracts conc. (mg/mL)	**Zone of Inhibition (mm)**			
	E. coli	***E. faecalis***	***P. aeruginosa***	***S. aureus***
ECS				
200	12.77 ± 0.176	15.82 ± 0.318	12.87 ± 0.742	13.35 ± 0.070
150	12.6 ± 0.500	14.57 ± 0.247	12.55 ± 0.353	13.05 ± 0.282
100	12.55 ± 0.356	14.12 ± 0.459	12.4 ± 0.424	12.9 ± 0.212
50	12.42 ± 0.106	12.37 ± 0.459	11.92 ± 0.742	12.42 ± 0.176
25	12.22 ± 0.247	12.1 ± 0.424	11.475 ± 0.176	12.25 ± 0.282
MIC (mg/mL)	5	5	5	5
ECC				
200	18.57 ± 0.035	18.00 ± 0.424	17.67 ± 0.318	16.8 ± 0.00
150	18.27 ± 0.106	17.82 ± 0.459	17.2 ± 0.636	16.77 ± 0.035
100	18.17 ± 0.176	17.65 ± 0.565	17.00 ± 0.565	16.55 ± 0.070
50	17.15 ± 0.494	16.35 ± 0.424	15.3 ± 0.282	15.55 ± 0.070
25	14.25 ± 0.212	13.97 ± 0.883	14.17 ± 0.671	12.45 ± 0.070
MIC (mg/mL)	5	5	5	5

ECR				
200	15.27 ± 0.318	16.05 ± 0.353	15.1 ± 0.494	13.6 ± 0.636
150	15.12 ± 0.459	15.5 ± 0.070	13.35 ± 0.212	13.27 ± 0.742
100	14.8 ± 0.353	15.22 ± 0.176	12.77 ± 0.035	13.1 ± 0.848
50	14.35 ± 0.707	14.75 ± 0.424	12.3 ± 0.282	12.97 ± 0.742
25	13.05 ± 0.636	14.15 ± 0.141	11.5 ± 0.353	12.45 ± 0.565
MIC (mg/mL)	5	5	5	5
ECM				
200	17.47 ± 0.035	19.25 ± 0.00	18.07 ± 0.671	18.72 ± 0.459
150	17.12 ± 0.388	18.9 ± 0.353	17.65 ± 0.565	17.72 ± 0.035
100	16.97 ± 0.388	18.6 ± 0.353	17.1 ± 0.565	17.32 ± 0.318
50	16.67 ± 0.106	18.1 ± 0.424	16.4 ± 0.424	17.12 ± 0.459
25	15.2 ± 0.212	16.07 ± 0.035	16.12 ± 0.530	16.65 ± 0.141
MIC (mg/mL)	2.5	2.5	2.5	2.5
Chloramphenicol	24.36 ± 0.565	24.44 ± 0.318	24.53± 0.424	24.41 ± 0.035
MIC (mg/mL)	0.1562	0.1562	0.1562	0.1562

± Standard error; ECS: ethyl acetate of *C. scabriuscula*; ECC: ethyl acetate of *C. crispata*; ECR: ethyl acetate of *C. rappii*; ECM: ethyl acetate of *C. macilenta*; positive control (chloramphenicol, 30 µg/disk).

3.2 Minimum Inhibitory Value of Genus Cladonia Extract

The dilution method was mostly used to determine the MIC of both pure compounds and extracts [16]. The MIC values of four extracts from the genus *Cladonia*, which indicate their ability to inhibit the growth of four pathogenic bacteria, are presented in Table 2. All studied extracts exhibited inhibition against the four tested bacteria. Notably, ethyl acetate of *C. macilenta* (ECM) showed strong potential as antibacterials with a MIC of 2.5 mg/ml. Meanwhile, ECS, ECC, and ECR had moderate activity at 5 mg/ml. Different types of extracts also affected antibacterial activity, while the role of solvents (and their polarity) in this process was very important to dissolving secondary metabolites. Certain examples, such as the semipolar extracts of *C. rangiformis* and *C. convolute*, were more active as antimicrobials than their methanol counterparts [17]. However, the methanol obtained from *C. furcata* was more active than its semipolar extract [18].

3.3 *TLC-Bioautography Assay*

Tests for antibacterial activity were also conducted using TLC-bioautography. This assay is very helpful in determining the spots responsible for antibacterial activity [19] when each studied extract is added onto a TLC plate (10 µL) and developed with the eluent toluene-EtOAc-formic acid (139: 83: 8, solvent G). After drying and observing the plate (with a UV lamp and ANS reagent), the pattern formed by each stain and the distribution of each extract showed good separation (Fig.1). There were three spots on the CS ethyl acetate extract bioautogram plate (Rf values: 0.84, 0.66, and 0.55) with a fairly large growth inhibition area observed for *E. faecalis* (Fig.1B). Also, one separation spot of CC ethyl acetate extract (Rf value: 0.73) inhibited the growth of *E. coli* (fig. 4E). At Rf 0.77, spots from the CR and CM ethyl acetate extracts inhibited the growth *of E. faecalis* (Fig.2C and 3D).

The TLC-bioautography technique is the most efficient method used to determine and isolate bioactive compounds. Thus, the antimicrobial bioactive compounds of chlorinated xanthones (along with usnic acid and depside groups) were successfully isolated from the bioautography-guided extract of lichen *C. incrassate* [20]. The same process was performed for *Usnea florida* and *Flavoparmelia caperata,* where the TLC-bioautographic protocol was used to detect compounds active against *S. aureus* [21].

3.4 LC-MS/MS Investigation of the bioactive extracts as antibacterials

The investigation process was continued with an LC-MS/MS scan to obtain initial information about the antibacterial extracts secondary metabolite components. Table 3 presents results from the scans of four *Cladonia* extracts (retention time (Tr), precursor ion mass (positive mode, m/z), ion fragments formed, and resulting formulas).

The CS ethyl acetate extract contained two depsides groups: divaricatic acid and atranorin with retention times (TRs) of 12.42 and 16.40 minutes, respectively (Fig.1). Additionally, this acid had a mass-to-charge ratio (m/z) of [M + H] + 388.2994 and the molecular formula $C_{21}H_{24}O_7$. This was followed by ion fragments with m/z values of 222.1115 ($C_{11}H_{10}O_5$), 193.0852 ($C_{11}H_{13}O_3$), 179.0645 ($C_{10}H_{11}O_3$), and 137.0529 ($C_7H_5O_3$), respectively (Fig.5). Atranorin was detected with an m/z of [M + H] + 373.0799 and the molecular formula $C_{19}H_{17}O_8$, while the ionic fragments formed had m/z values of 195.0864 ($C_9H_7O_5$), 146.9799 ($C_9H_6O_2$) and 123.0783 ($C_7H_7O_2$).

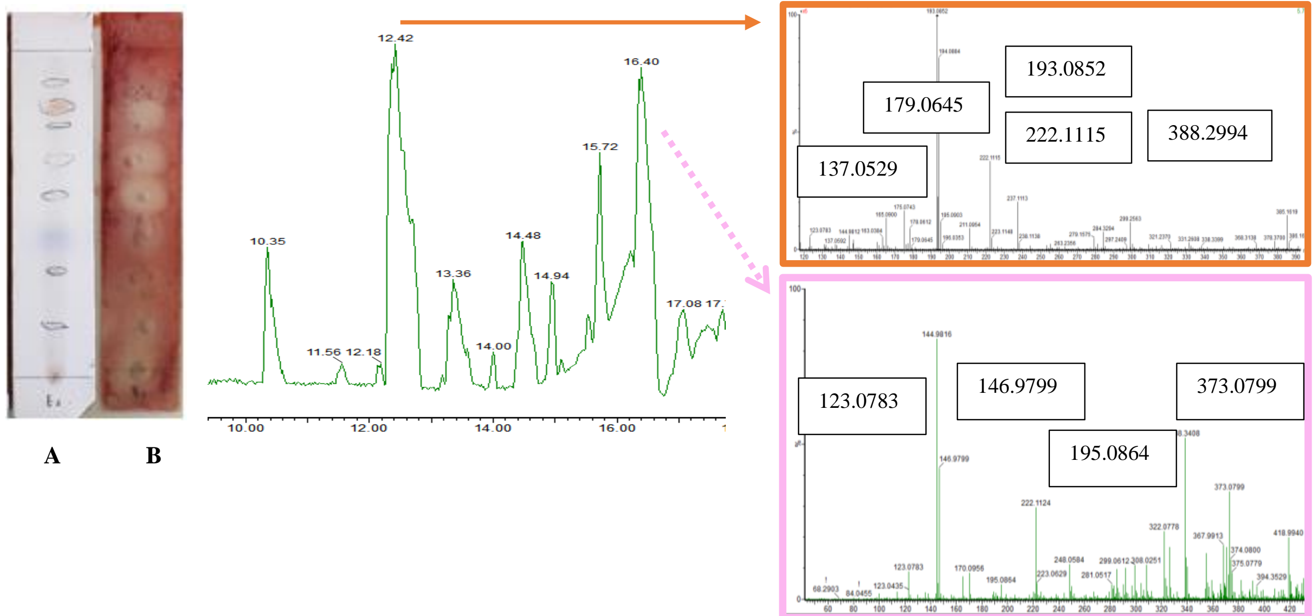

Figure 1. Thin layer chromatograms of CS ethyl acetate extract, bioautograms and LC-MS/MS spectra. (Applied volume 10 µL, mobile phase: toluene: ethyl acetate: formic acid = 139: 83: 8). (A) TLC chromatogram detection ANS reagent, (B) TLC-bioautogram of CS ethyl acetate extract against *E. faecalis.*

Table 3. Compounds identified by LC-MS/MS in extracts from four *Cladonia* species

Sample name	No.	Tr (min)	[M+H]+, m/z	Ion fragments, m/z	Formula	Tentative identification	Ref.
ECS	1	12.42	388.2994	- 222.1115 193.0852 179.0645 137.0529	$C_{21}H_{24}O_7$	Divaricatic acid	[22], [23]
	2	16.40	373.0799	- 195.0864 146.9799 123.0783	$C_{19}H_{17}O_8$	Atranorin	[23]
ECR	1	12.42	388.1680	- 222.1115 193.0852 179.0645 137.0529	$C_{21}H_{24}O_7$	Divaricatic acid	[22], [23]
ECM	1	14.48	343.3392	- 296.3308 222.1118 165.0900 146.9799 123.0440	$C_{18}H_{16}O_7$	Hypoprotocetraric acid	[24]

ECC	1	8.14	391.1034	- 227.0562 209.0456 182.9862	$C_{19}H_{18}O_9$	Squamatic acid	[25]
	2	15.74	443.3360	- 338.3424 223.0640	$C_{25}H_{32}O_7$	Perlatolic acid	[26]
	3	16.46	373.0787	- 195.0878 146.9804 123.0791	$C_{19}H_{17}O_8$	Atranorin	[23]
	4	17.73	360.0295	- 195.0876 165.0918	$C_{19}H_{20}O_7$	Barbatic acid	[25]

The CR ethyl acetate extract was detected divaricatic acid with an ion molecule m/z of 388.1680, followed by four ion molecules with m/z values of 222.1115, 193.0852, 179.0645, and 137.0529, respectively (Fig.2 and 5). The *C. rappii* lichen collected in Brazil was successfully isolated by rappidic acid (an o-orsellinic acid derivative) and usnic acid [27].

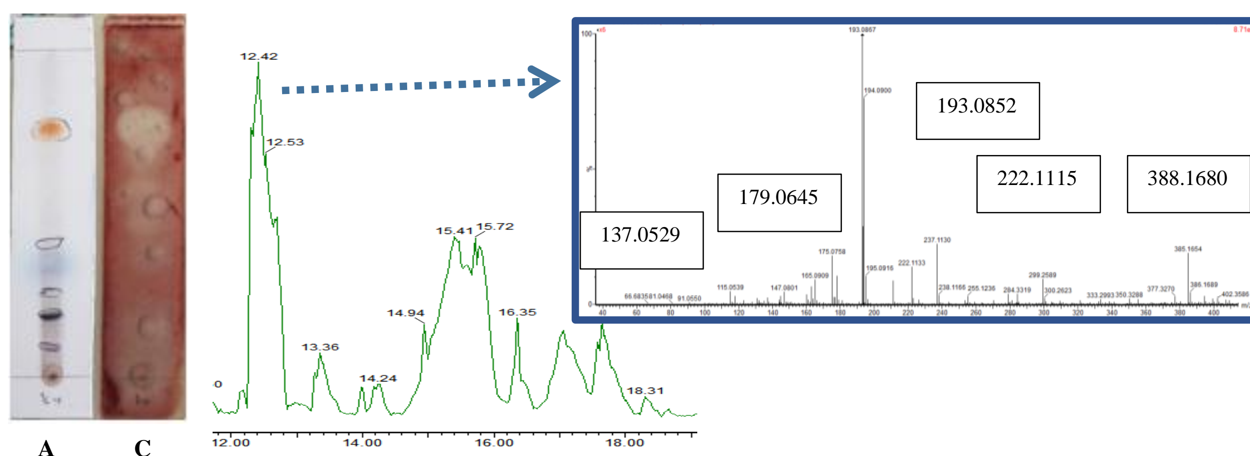

Figure 2. Thin layer chromatograms of CR ethyl acetate extract, bioautograms and LC-MS/MS spectra. (Applied volume 10 µL, mobile phase: toluene: ethyl acetate: formic acid = 139: 83: 8). (A) TLC chromatogram detection ANS reagent, (C) TLC-bioautogram of CR ethyl acetate extract against *E. faecalis.*

Unlike other extracts, CM ethyl acetate extract was detected by a hypoprotocetraric acid compound with an ion molecule m/z of 343.3392 (or ion fragment m/z values of 296.3308 ($C_{17}H_{13}O_5$), 222.1118 ($C_{10}H_6O_6$), 165.0900 ($C_9H_9O_3$), 146.9799 ($C_9H_6O_2$) and 123.0440 ($C_8H_{10}O_5$)) and the formula $C_{18}H_{16}O_7$ (Fig.3 and 5).

In CC ethyl acetate extract, the peak at 8.14 minutes had a molecular ion with an m/z of 391.1034 with molecular fractions that had m/z values of 227.0562, 209.0456 and 182.9862. This compound was identified as squamatic acid ($C_{19}H_{18}O_9$). Barbatic acid appeared at a peak of 17.73 minutes with an ion molecule m/z of 360.0295 ($C_{19}H_{20}O_7$), followed by two ion molecules with m/z values of 195.0876 and 165.0918, respectively. Two others were also detected, including perlatolic acid and atranorin with m/z values of [M + H] + 443.3360 and 373.0803, respectively (Fig.4 and 5).

The group of divaricatic acid compounds exhibited antibacterial activity against the bacteria *Enterobacter cloacae, Klebsiella pneumoniae, Bacillus mycoides, B. subtilis, S. aureus, S. epidermidis, Streptococcus mutans,* and *Enterococcus faecium* [22], [28]. The aforementioned compound and atranorin are often found in many lichen families such as *Cladoniaceae, Lecanoraceae, Parmeliaceae*, and *Streocaulaceae* [9], [29]. Moreover, atranorin has antibacterial activity in both gram-positive and gram-negative bacteria [30], [31].

A lichen acid group that often appears in the genus *Cladonia* is perlatolic acid [32]. This compound is a aliphatic chains side depside with antibacterial activity, especially for *S. aureus* and *E. coli* [32], [33]. Also, squamatic and barbatic acid—also from the depside group—is potent as an effective antimicrobial [34]. Two depsidone group compounds that were detected as active antibacterials were hypoprotocetraric (active against nine

bacteria) with MIC values ranging from 12.2 to 278.5 µg/mL [35]. Meanwhile, substictic acid (a derivative of the stictic acid group)—a depsidone—is rarely detected in lichen [36]

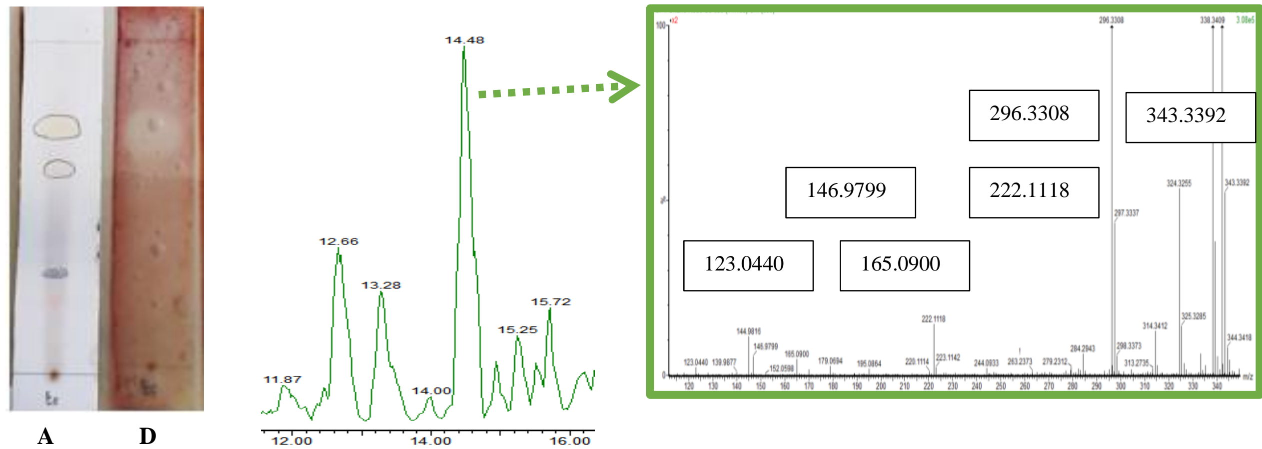

Figure 3. Thin layer chromatograms of CM ethyl acetate extract, bioautograms and LC-MS/MS spectra. (Applied volume 10 µL, mobile phase: toluene: ethyl acetate: formic acid = 139: 83: 8). (A) TLC chromatogram detection ANS reagent, (D) TLC–bioautogram of CM ethyl acetate extract against *E. faecalis*.

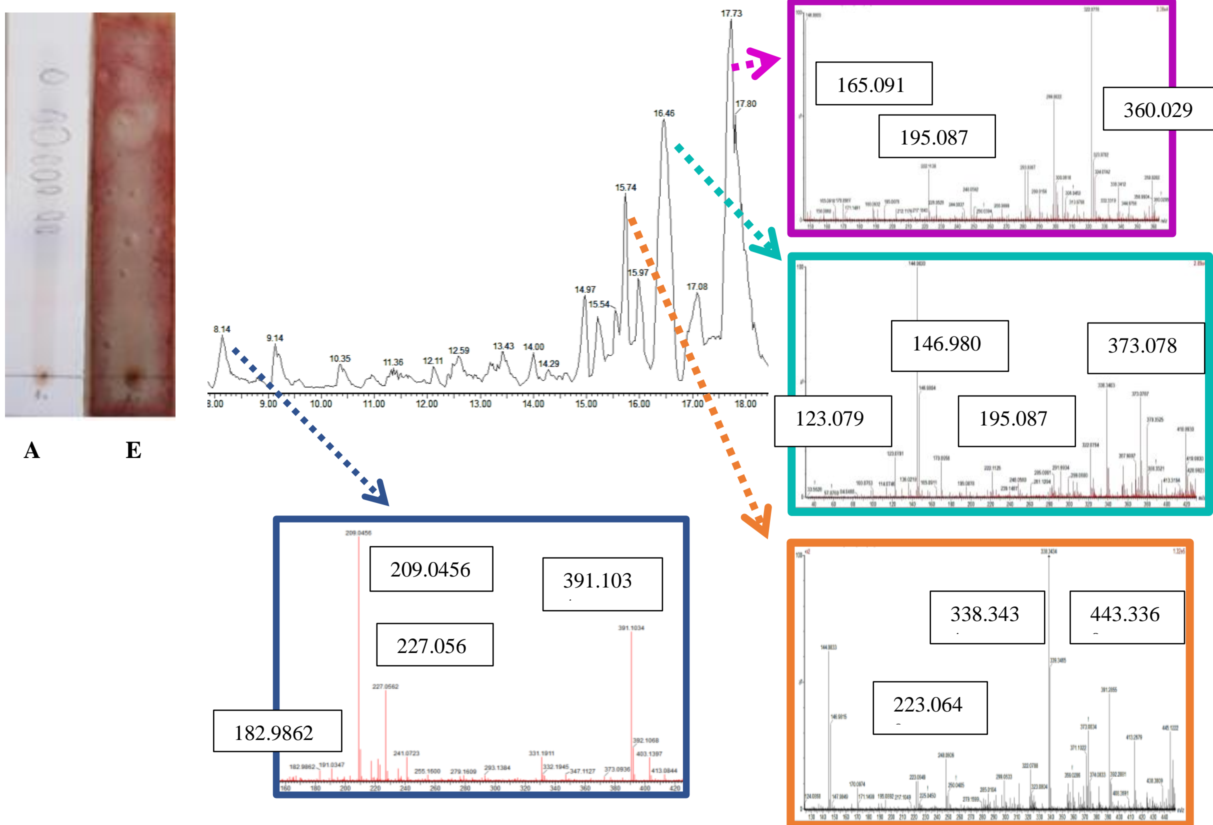

Figure 4. Thin layer chromatograms of CC ethyl acetate extract, bioautograms and LC-MS/MS spectra. (Applied volume 10 µL, mobile phase: toluene: ethyl acetate: formic acid = 139: 83: 8). (A) TLC chromatogram detection ANS reagent, (E) TLC-bioautogram of CC ethyl acetate extract against *E. coli*.

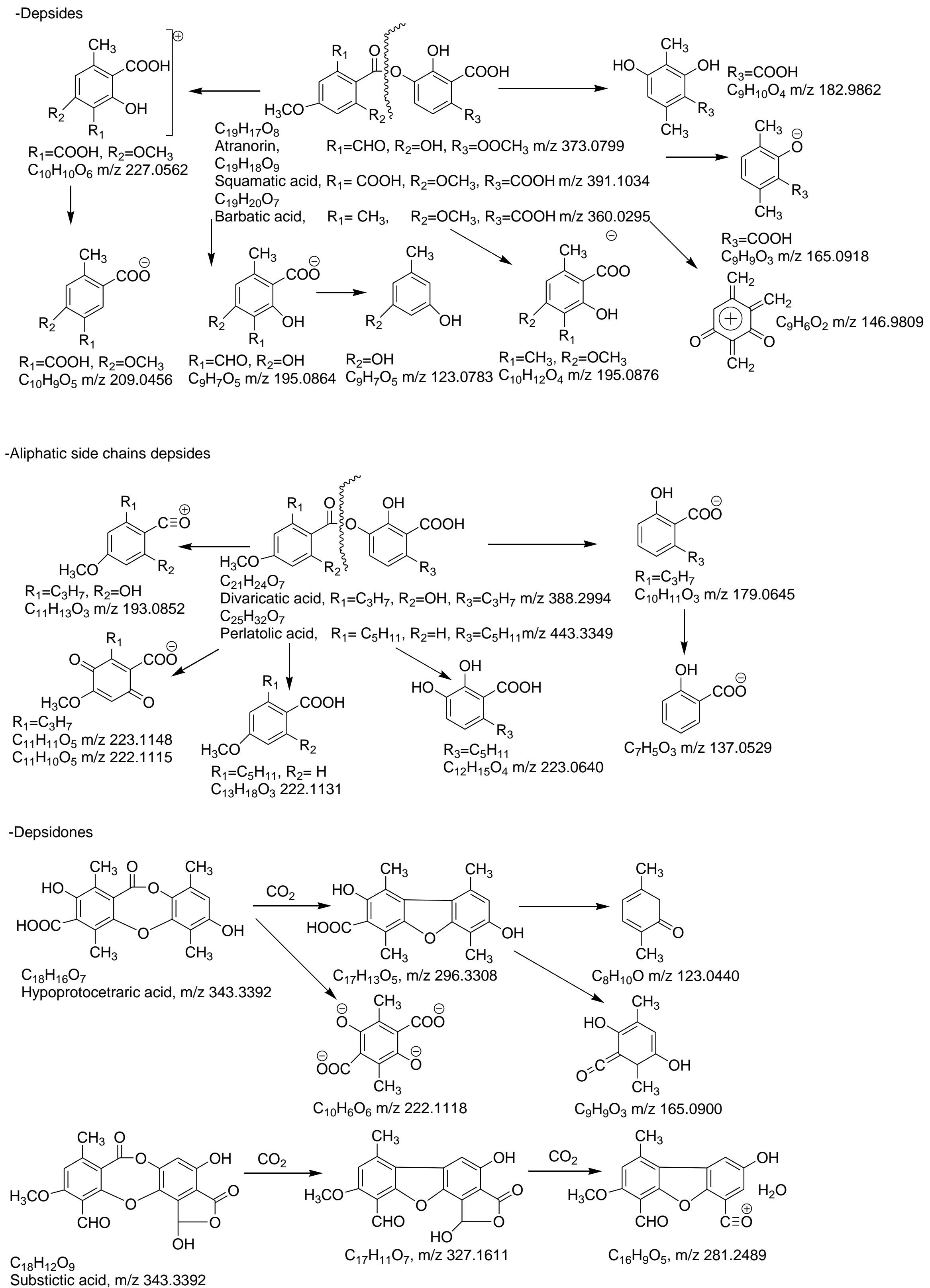

Figure 5. The proposed fragmentation pattern of depside, aliphatic chains depsides and depsidones

4. CONCLUSIONS

Six *Cladonia* species *(C. scabriuscula, C. crispata, C. rappii, C. macilenta, C. ochrochlora,* and *C. malayana)* exhibited antibacterial activity by inhibiting all tested pathogenic bacteria. Notably, *C. scabriuscula, C. crispata, C. rappii,* and *C. macilenta* have very strong activity when compared to other types. The bioactivity and phytochemical screening with TLC-bioautography of four extracts showed several responsible spots as components with antibacterial effects. With the support of LC-MS/MS, depside groups (atranorin, divaricatic, perlatolic, squamatic and barbatic acids), depsidones (hypoprotocetraric and substictic acids) were detected as compounds in the extracts. These were hypothesized to be antibacterials, as supported by existing literature. Further studies are required to isolate and identify undetected compounds, which are also believed to contribute to antibacterial activity.

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