Phytochemical profiling and antibacterial activities of extracts from five species of Sumatran lichen genus *Stereocaulon*

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ABSTRACT

In continuing the inventory and investigation of Sumatra lichen, a collection of five species from the genus *Stereocaulon* has been carried out. In metabolite profiling analysis, the four main compounds, i.e. atranorin, MOC, lobaric acid, and stictic acid, were rapidly isolated as marker compounds by chromatography methods from *S. halei* and *S. montagneanum*. Then, the extracts from each species were analyzed using HPTLC densitometry measured at five wavelengths of 220, 254, 280 320, and 365 nm. Atranorin and MOC are two compounds found in all five *Stereocaulon* species tested. Subsequently, atranorin quantification was done by densitometric scanning at 254 nm from the ethyl acetate extract of each *Stereocaulon* species. The highest atranorin concentration was detected from *S. graminosum* (325,498 mg/g extract) and equivalent to 0.065 mg/g dried whole thallus, while the lowest was from *S. verruculigerum* (23,356 mg/g extract) and 0.023 mg/g dried whole thallus. Furthermore, all extracts and main compounds resulting from the isolation were evaluated for their antibacterial activity by the microdilution method. Ethyl acetate and acetone extracts from *S. massartianum* (1) showed the highest antibacterial activity against *E. faecalis* (MIC = 1.25 mg/mL).

Keywords: Atranorin , HPTLC-densitometry , Lichen , Metabolite profiling , *Stereocaulon*.

1 INTRODUCTION

The genus *Stereocaulon* Hoffm. (Stereocaulaceae, Lecanorales, Ascomycota) is an interesting genus from lichen which is found throughout the world. The morphology of the *Stereocaulon* genus consists of the crustose type primary thallus and fruticose type secondary thallus. The primary thallus in most species of *Stereocaulon* is disappeared at a very early stage of development. In the secondary thallus, there are several important parts such as pseudopodetia which show persistent phyllocladia (or phyllocladioid branchlets), apothecia as a sexual organ that contains spores, and in most species cephalodia which contain cyanobacteria (Nostoc, Rhizonema or Stigonema)[1].

Although the *Stereocaulon* genus is grow scattered throughout the world, its scientific study is still limited, thus, it needs to be deeply researched to uncover its potential. Some famous lichenologists have described this genus, such as Nylander in his Synopsis Lichenum[2], Riddle[3], Magnusson[4], Dodge[5], Johnson[6], Duvigneaud[7], Lamb[8-10], Boehkout[11], and McCarthy[12]. Also, secondary metabolite profiles of the genus *Stereocaulon* play an essential role in the identification of lichen as Duvigneaud[7] and Lamb[9,10] have done using microchemistry. Until now, several thorough phytochemical studies of Stereocaulon species have been carried out[13,14], and among the 40 species studied, the depsides, depsidone, dibenzofurans, diphenyl ethers groups were isolated for the most

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representative secondary metabolites for lichens[1].

In Sumatran lichen exploration, we have carried out several phytochemical studies on some genera such as Cetrelia and Usnea[15,16] and focused more on the genus *Stereocaulon*, such as *S. halei, S. montagneanum,* and *S. graminosum*[17-19]. In the surveys conducted in the period 2015-2016 in the mountains and highlands of West Sumatra and Jambi province, Indonesia, we collected five species of the genus *Stereocaulon*. This paper described a phytochemical approach with rapid isolation of the main depside compound (atranorin, **1**), monoaromatic compound (Methyl-β-orcinol carboxylate, MOC, **2**) and depsidones (stictic acid (**3**) and lobaric acid, **4**) from *S. halei* and *S. montagneanum* (Figure 1). These compounds were used as marker compounds in the metabolite profiling analysis with the HPTLC-densitometry. We also conducted the determination of atranorin concentration with the densitometry approach at 254 nm of the extracts, and this was done for the first time. Then, an antibacterial evaluation was carried out for each extract and isolated compound against four pathogenic bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 12228, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853) with the microdilution method.

2 *MATERIALS AND METHODS*

2.1 Lichen material

Five lichen species were collected in the highlands and mountains in the regions of West Sumatra and Jambi province, Indonesia. *S. halei* and *S. massartianum* were collected in different locations and altitudes, so the number of the sample analyzed was seven samples. Some ecological parameters are recorded in Table 1. We confirmed our identification by sending all samples to Harrie Sipman (Berlin Museum), and specimen vouchers were deposited at the Biota Sumatran Laboratory, Andalas University, West Sumatra (Indonesia) with the reference numbers cited in Table 1.

2.2 Instrumentations

Some equipment was used for the characterization of compounds. The melting point was measured on a Fisher Melting Point apparatus. UV spectra were performed on a Shimadzu 1700 spectrophotometer. FTIR spectra were run on a Perkin Elmer FTIR spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded at 500 and 125 MHz, respectively, on a Jeol 500 MHz NMR spectrometer using $CDCl_3$ and DMSO-*d6* as solvents. The HPTLC plate was densitometrically scanned with CAMAG TLC scanner 4. Data acquisition and processing were recorded using the software winCATS version 1.4.7.

2.3 Reagents and materials

All the chemicals and reagents used in this study were analytical grade and were purchased from Merck. Chromatographic separation was performed using vacuum liquid chromatography on silica gel (Merck 0.063-0.200 mm). HPTLC aluminium sheets 20x20 cm, Merck KGaA, Darmstadt Germany, Cat.no.1.05548, were eluted using two standard solvent systems[20] that is toluene/acetic acid (85:15) (C); toluene/EtOAc/formic acid (139:83:8) (G). Visualization of plates was carried out under UV light (254 and 365 nm) and then sprayed with anisaldehyde $H2SO_4$ (v/v) reagent followed by heating at 110ºC.

2.4 Isolation marker compounds

The Compound **1**, **2**, **3** and **4** as marker compounds were isolated from *S. halei* (collected from Mount Singgalang) and *S. montagneanum* (collected from Sirukam, Solok) based on previous work. These two species were chosen based on their sufficient biomass. Air-dried whole thallus powder (500 g) from *S. halei* were macerated successively with ethyl acetate, acetone, and methanol (2x1 L). The obtained ethyl acetate extract was filtered and evaporated to half, and afterward, it was left for 24 hours at room temperature to precipitate crude crystalline. The crude crystal was recrystallized with ethyl acetate repeatedly and 6.3 g compound **1** (1.26% w / w) was obtained. The ethyl acetate filtrate (5 g) was chromatographed on a vacuum liquid chromatography

silica gel column (100 g) eluted with a step gradient polarity solvents consisted of n-hexane–EtOAc (100:0→0:100) as the mobile phase, and lastly washed with methanol. The eight subfractions acquired (E1-E8) were monitored by TLC and subfraction E4 and E6 indicate the spot estimated as compound **2** and **4**, respectively. Both subfractions were chromatographed over Sephadex LH 20 column chromatography to yield compound **2** (150 mg) from subfraction E4 and Compound **4** (90 mg) from subfraction E6.

Meanwhile, 500 g air-dried whole thallus powder from *S. montagneanum* were extracted by step gradient polarity starting from ethyl acetate, acetone, and methanol (2x500 ml). The acetone liquors are concentrated to half and allowed to stand for 24 hours at room temperature. The resulting precipitation was then filtered and washed with acetone:methanol (1:1) and obtained 300 mg compound **3**. The physical data, IR, melting point, ^{1}H and ^{13}C NMR data of all isolated compounds were analyzed.

2.5 Preparation of lichen extracts and standard solution for metabolite profiling

Approximately 50 g of seven air-dried whole thallus of *Stereocaulon* species were macerated successively with ethyl acetate, acetone, and methanol (2 times × 250 ml), and each extract was concentrated *in vacuo*. Preparation of each extract solution (EtOAc, Acetone, and methanol extracts) was carried out by weighing each extract accurately and dissolved with an appropriate solvent to obtain 10 mg/ml concentration of each sample.

The marker compounds (**1, 2, 3** and **4**) that were isolated before were weighed precisely as much as 10 mg each and dissolved in ethyl acetate and acetone with the volume was made up to 10 ml in a volumetric flask.

A volume of 2 µl of marker compounds and each extract solution were applied to the chromatographic plate (20 x 10 cm) using CAMAG Nanomat 4 and was developed at room temperature in flat bottom chamber (20 x 20 cm) with solvent system toluene:EtOAc:formic acid (139:83:8). After development, HPTLC plates were heated at 105ºC and scanned using the scanner at 5 wavelengths 220, 254, 280, 310, and 365 nm. Spot identification was based on its Rf value, the maximal wavelength, and visual evaluation after spraying anisaldehyde sulfuric reagent.

2.6 Preparation of lichen extracts and standard solution of atranorin (1) for quantitative analysis

As well as the sample preparation for the metabolite profiling, the quantitative analysis sample preparation was done by weighing 50 g of each air-dried whole thallus of **Stereocaulon** species and then was extracted by 250 ml of ethyl acetate solvent for two days, filtered and evaporated in vacuo. Afterward, each dried extract was weighed 10 mg and dissolved with ethyl acetate-acetone (ratio 3: 2) to the limit marking on a 10 ml volumetric flask.

Atranorin (**1**) standard solution is made by weighing 10 mg atranorin dissolved in 10 ml acetone which will then be diluted to make a calibration curve.

Preparation of calibration solutions was done by weighing 10 mg of atranorin (**1**) dissolved in 10 mL of acetone; solutions with concentrations of 250, 200, 150, 100, 50 and 25 µg/mL were prepared by dilutions of the stock solution. Atranorin (**1**) calibration curves are calculated by plotting the area of the concentration from the standard solution.

Atranorin (**1**) as standard solution and 7 *Stereocaulon* extracts were accurately taken as much as 5 µl each and applied to HPTLC plates (in triplicates) using CAMAG Nanomat 4. They were developed at room temperature in a flat bottom chamber (20x20 cm) with solvent system toluene:EtOAc:formic acid (139:83:8). After development, HPTLC plates were dried at 105ºC and scanned densitometrically at 254 nm.

2.7 Antibacterial assay

Antibacterial assays have been carried out on four pathogenic bacteria, which are *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 12228, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. The method used in this assays is a modified microdilution method[21,22] along with p-

iodonitrophenyltetrazolium violet (INT, Merck) as an indicator of bacterial cell viability[23]. Each extract was dissolved with DMSO (10% final volume) and diluted with Mueller Hinton Broth (OXOID) media to a final concentration of 10; 5; 2.5; 1.25; 0.625 and 0.3125 mg / ml. Meanwhile, the isolated compound and chloramphenicol (as a positive control, Merck) were prepared at the final concentration of 5; 2.5; 1.25; 0.625; 0.3125 and 0.15625 mg / ml. A total of 100 µl of each extract concentration and pure isolate were tripled in a microplate well, as well as sterility control (media + DMSO) and growth control (media + DMSO + bacteria). Each microplate well was inoculated with 5 µl bacterial suspension (106 cfu / ml) and incubated at 36 ° C for 18 hours, followed by the addition of 20 µl p-iodonitrotetrazolium (INT) in distilled water (0.5 mg/ml) and reincubated for another 30 minutes. INT is a compound that easily reducted by the presence of the enzyme dehydrogenase in bacteria becoming formazan, which can give a purple color. The change in color from yellow to purple indicates that there are still bacteria in the well.

3 *RESULTS AND DISCUSSION*

3.1 Physical and Spectroscopic Data of Isolation compounds

Atranorin (**1**); colorless needles (EtOAc); mp 196-197˚C; (undepressed in admixture with reference compound) IR νmax (cm−1): 2933, 2360, 1654; UV (Acetonitrile) λmax (log ε): 215 nm (4.2), 251 nm (4.4); ^{1}H NMR (500 MHz, DMSO-*d6*) δH(ppm) 2.03 (s, 3H Me-8'), 2.34 (s, 3H,CH_3-9'), 2.39(s, 3H, CH_3-9), 3.89 (s, 3H,COOMe), 6.42 (s, 1H, H-5'), 6.64 (s, 1H, H-5), 10.21 (s, 1H, -CHO) and 10.52 (s, 1H, OH);^{13}C NMR (125 MHz, DMSO-*d6*): δC(ppm) 107.95 (C-1), 163.54 (C-2), 109.08 (C-3), 161.61 (C-4), 115.78 (C-5), 151.45 (C-6), 164.66 (C-7), 194.03(C-8), 21.27 (C-9), 116.39 (C-1'), 157.43 (C-2'), 110.83 (C-3'), 149.13(C-4'), 115.79 (C-5'), 136.63 (C-6'), 169.83 (C-7'), 21.16 (C-8'), 9.43 (C-9'). Rf 0.86 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

Methyl-β-orcinol carboxylate, MOC (**2**); colorless needles ($CHCl_3$); mp 140-141˚C; (undepressed in admixture with reference compound); IR νmax (cm−1): 3399, 2927, 1728; UV (MeOH) λmax (log ε): 219 nm (3.9), 269 nm(4.15), 303 nm (3.7); ^{1}H NMR (500 MHz, $CDCl_3$) δH(ppm) 2.16 (s, 3H, CH_3-9), 2.45 (s, 3H, CH_3-8), 3.94 (s, 3H, -OCH_3), 6.12 (s, 1H, H-5), 12.13 (s, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$) δC(ppm) 105.33 (C-1), 158.06 (C-2), 108.54 (C-3), 163.24 (C-4), 110.60 (C-5), 140.24 (C-6), 172.69 (C-7), 7.73 (C-8), 24.20 (C-9), 51.93 (OCH_3-10). Rf 0.69 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

Stictic acid (**3**); white amorphous solid; (undepressed in admixture with reference compound); IR νmax (cm−1): 3421.47, 2906.22, 1918, 1692.95; UV (MeOH) λmax (log ε): 242.8 nm (3.85) and 309.4 nm (3.27); ^{1}H NMR (500 MHz, DMSO-*d6*) δH (ppm) 2.18 (s, 3H, CH_3-8'), 2,48 (s, 1H, CH_3-9) 3,89 (s, 3H, -OCH_3-4), 6.60 (s, 1H, H-9'), 7.10 (s, 1H, H-5), 8.27 (s, 1H, OH), 10,44 (s, 1H, CHO-8); ^{13}C NMR (125 MHz, DMSO-*d6*) δC(ppm) 113.25 (C-1), 163.34 (C-2), 114.53 (C-3), 162.65 (C-4), 112.97 (C-5), 151.18 (C-6), 161.00 (C-7), 186.93 (C-8), 21.72 (C-9), 109.30 (C-1'), 152.00(-), 129.43 (C-3'), 148.10 (C-4'), 137.66 (C-5'), 135.91 (C-6'), 166.52 (C-7'), 9.83 (C-8'), 95.41 (C-9'), 56.95 (OCH_3-4). Rf 0.30 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

Lobaric acid (**4**); colorless needles ($CHCl_3$); mp 196-197^{0} C; (undepressed in admixture with reference compound); IR νmax (cm−1): 3164.4, 2960.5, 1720.11, 1662.45; UV (MeOH) λmax (log ε): 213 nm (4.00), 262 nm (3.33) and 293 nm (4.2); ^{1}H NMR (500 MHz, DMSO-*d6*) δH(ppm) 0.87 (t, 6H, CH_3-5", CH_3-5"'), 1.31 (m, 4H, CH_2-4", CH_2-4"'), 1.33 (m, 2H, CH_2-3"'), 1.54 (m, 2H, CH_2-2"'), 2.83 (t, J=7.25, 2H,CH_2-2"), 2.86 (t, J=1.00, 2H, CH_2-1"'), 3.89 (s, 3H, OCH_3), 6.98 (s, 1H, H-3'), 6.93 (d, J=2.4, 1H, H-3), 7.03 (d, J=2.4, 1H, H-5); ^{13}C NMR (125 MHz, DMSO-*d6*) δC(ppm) 111.64 (C-1), 162.80 (C-2), 105.83 (C-3), 164.19 (C-4), 111.06 (C-5), 148.58 (C-6), 152.69 (C-7), 203.31 (C-1''), 39.66 (C-2''), 31.34 (C-3''), 21.54 (C-4''), 13.93 (C-5"), 111.64 (C-1'), 162.15 (C-2'), 106.24 (C-3'), 144.33 (C-4'), 140.64 (C-5'), 133.99 (C-

6'), 168.27 (C-7'), 27.30 (C-1‘''), 30.47 (C-2'''), 31.34 (C-3'''), 21.99 (C-4'''), 13.92 (C-5'''), 56.64 (OCH_3-4). Rf 0.56 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

3.2 Phytochemical investigation and HPTLC (High-Performance Thin Layer Chromatography) metabolite profiling

In this study, five species were collected from six different places are divided into seven samples (Table 1). *S. halei* (1 and 2) and *S. massartianum* (1 and 2) were collected in different locations and altitudes. *Stereocaulon* is a fruticose-type lichen that lives in high altitudes 1200 m ASL with low temperature and is usually found growing on the rock surface exposed by sunlight[24].

Table 1. Location and habitat of 5 Stereocaulon species collected.

Species	No. collection	Coordinate	Altitude	Locality	Habitat
S. montagneanum Lamb	Mon_ FIS-8	0°55'21.1"S 100°47'56.6"E	1300 m	Sirukam, Solok West Sumatra	on the rock, highland
S. halei Lamb (1)	Hal_ FS-13	0°23'24.0"S 100°19'50.9"E	2700 m	Mount Singgalang, West Sumatra	on the rock, slope mountain
S. halei Lamb (2)	FBS-006	0°21'31.5"S 100°17'36.6"E	1350 m	Malalak, West Sumatra	on the rock, highland
S. massartianum Hue (1)	SDA_ Fis-015/DA	1°05'59.4"S 100°45'34.1"E	1400 m	Danau di Atas, West Sumatra	on the rock, highland
S. massartianum Hue (2)	GN7_Fis-016/G7	1°42'06.0"S 101°24'41.0"E	2005 m	Danau Gunung Tujuh, Jambi	on the rock, mountain
S. verruculigerum Hue var. subfurfurascens Lamb	FTS-004	0°58'41.9"S 100°40'45.8"E	1300 m	Mount Talang, West Sumatra	on the rock, lower mountain forest
S. graminosum Schaer	Gra_FS-14	0°23'24.0"S 100°19'50.9"E	2700 m	Mount Singgalang, West Sumatra	on the rock, slope mountain

Compound **1**, **2**, **3**, and **4** (Figure 1). As marker compounds were isolated from *S. halei* (collected from Mount Singgalang) and *S. montagneanum* (collected from Sirukam, Solok) based on previous work[17,18]. The physicochemical properties and all spectral data from isolated compounds were suitable with those previously reported in the literature. (SM S1-S8)

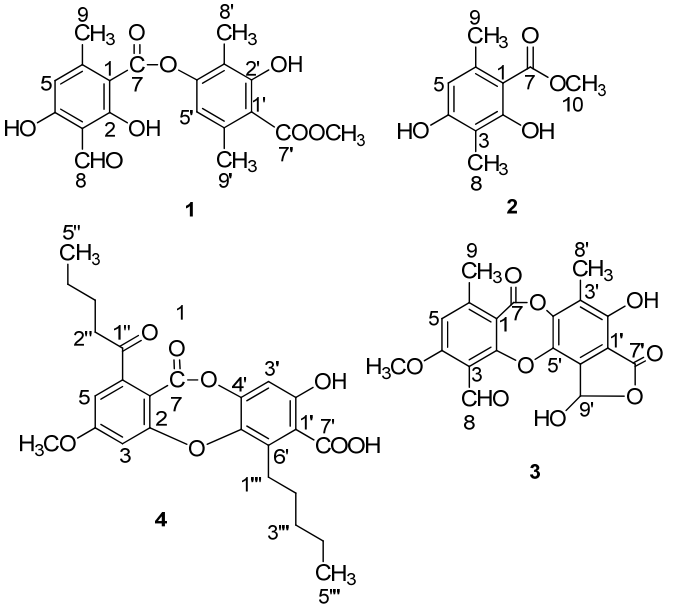
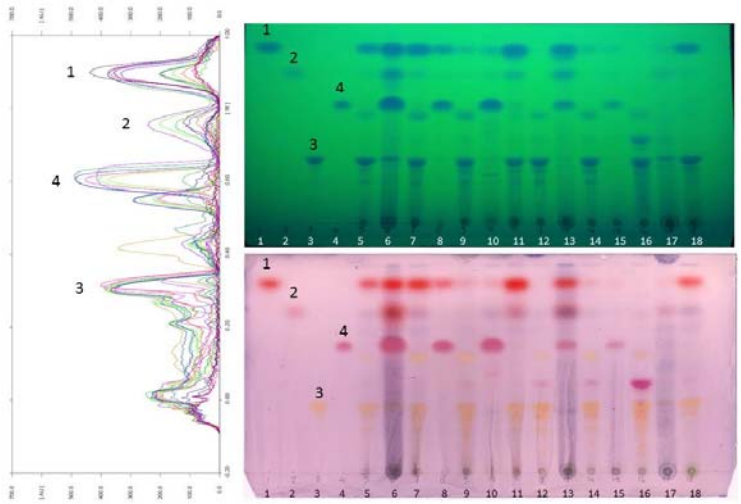


Figure 1. Isolated compounds from *S. halei* and *S. montagneanum*

HPTLC-densitometry metabolite profiling was carried out on different extracts of seven species of *Stereocaulon*. The extraction process was done sequentially by increasing polarity ranging from semipolar to polar (ethyl acetate, acetone, and methanol) to avoid decomposition or degradation of a depside as a major constituent of the genus *Stereocaulon*[25,26]. Identification of the compounds was realized for four major compounds (atranorin, MOC, lobaric acid, and stictic acid), and this study focused on detecting the similar compounds between the five species. Atranorin (track1; Rf = 0.80, λmax 220, 254 and 280 nm) was detected in all five species of *Stereocaulon* especially in EtOAc extracts (Table 2) (Figure 2 and SM Figure 9-11). Whereas in all acetone extracts, the atranorin spot began to thin out (except for *S.halei* (1) and *S.graminosum*, track 13 and 18 respectively, still look strong) even in methanol extracts the spot were not visible at all (Figure 3 and SM Figure 12-14). Atranorin, a β-orcinol depside, is the most common and widely found compound in almost all *Stereocaulon* species[9,10]. MOC (track 2; Rf = 0.69, λmax 220, 254, and 280 nm). It is only seen in EtOAc and MeOH extracts, so it is estimated that this monoaromatic compound is a precursor to depside biogenesis[27-29] and the result of atranorin depside degradation undergoes alcoholysis[30,31]. Lobaric acid (track 4; Rf = 0.56, λmax 220, 254 and 280 nm) was seen in both *S. halei* 1 and 2 (track 6, 8, 13 and 15) and *S. verruculigerum* (track 10) in EtOAc and acetone extracts. Furthermore, *S. montagneanum* (track 5 and 12), *S. massartianum* 1 and 2 (track 7, 9, 14 and 16), and *S. graminosum* (track 11 and 18) were detected to contain stictic acid (track 3; Rf = 0.30, λmax 220, 254 and 280 nm) in EtOAc and acetone extracts, respectively. Man-Rong described three chemotypes that have been found on *S. massartianum*[32]: the first one contains stictic and norstictic acids, the second one lobaric acid only, and the third one lobaric, stictic and norstictic acids. Lobaric and stictic acids are prenyl and furano-type depsidones, respectively, commonly discovered in the *Stereocaulon* genus[1]. Three types of prenyl-depsidones have been reported, namely lobaric acid, colensoic acid, and norlobaric acid, which were isolated from *S. colensoi* and *S. paschale*[33,34]. Stictic acid has been found in many lichen families in the Parmeliaceae, Pertusariaceae, and Usneaceae families[18,35].

As previously reported, generally, stictic acid in lichen experiences a chemical phenomenon known as stictic acid chemosindrome. This phenomenon shows that stictic acid can experience various degrees of oxidation of functional groups such as CHO, COOH, CH_2OH or OH and methylation of phenolic groups such as OCH_3, OH[18,36,37].



1= atranorin
2= MOC
3= Stictic acid
4= lobaric acid
19= MeOH extr. of *S. montagneanum*
20= MeOH extr. of S. halei (1)
21= MeOH extr. of *S. massartianum (1)*
22= MeOH extr. of *S. halei (2)*
23= MeOH extr. of *S. massartianum (2)*
24= MeOH extr. of *S. verruculigerum*
25= MeOH extr. of *S. graminosum*

Figure 2. The densitogram of marker compounds and extracts (EtOAc and Acetone) of genus *Stereocaulon* (left) scanned at 245 nm. The HPTLC profile in UV light at 254 nm developed with toluene:EtOAc:formic acid (139:83:8) (top right) and visualized with anisaldehyde sulfuric acid (bottom right)

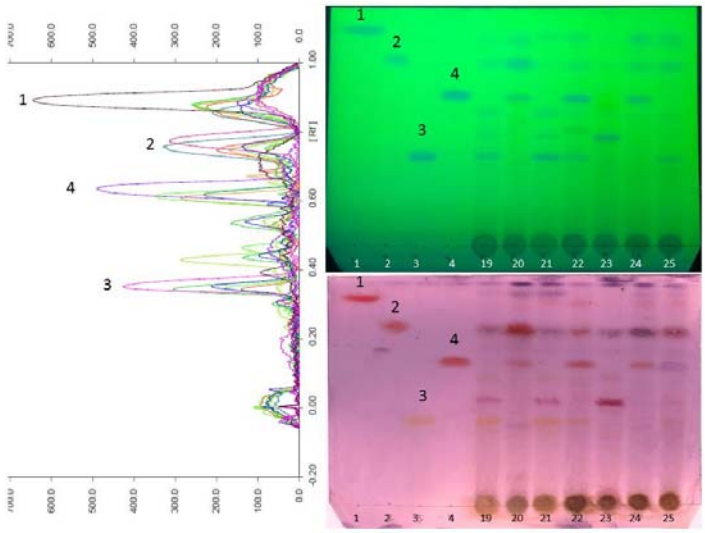


Figure 3. The densitogram of marker compounds and MeOH extracts of genus *Stereocaulon* (left) scanned at 245 nm. The HPTLC profile in UV light at 254 nm developed with toluene:EtOAc:formic acid (139:83:8) (top right) and visualized with anisaldehyde sulfuric acid (bottom right)

Table 2. Metabolites detected with HPTLC-UV in genus *Stereocaulon*

no	R_f	λmax (nm)	Compounds				Extracts of *Stereocaulon* species						
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	0.11	280, 310					+		+		+		+
2	0.20	220, 254					+		+		+	+	+
3	0.24											+	
4	0.30	220, 254, 280			+		+	-	+		+	-	+
5	0.39	254, 280, 320						+					
6	0.40	280, 320								+			
7	0.45											+	
8	0.49	220, 254							+		+		
9	0.51	220, 254					+					+	+
10	0.56	220, 254, 280				+		+	-	+			+
11	0.66	320					+						
12	0.69	220, 254, 280		+			+	+	+	-	-	+	+
13	0.80	220, 254, 280	+				+	+	+	+	+	+	+
			1	**2**	**3**	**4**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
1	0.08	280, 310					+						
2	0.11	220, 254							+				
3	0.20	220, 254					+						
4	0.27	220, 254						+	+			+	+
5	0.30	220, 254, 280			+		+	-			+	+	+
6	0.39	220, 254					+	+		+	+	+	
7	0.40	220, 254							+				+
8	0,49	220, 254					+		+		+	+	+
9	0.54	220, 254, 280						+		+			
10	0.56	220, 254				+						+	+
11	0.69	220, 254, 280		+			-	+	-	-	-	+	+
12	0.77	220, 254, 280						+					
13	0.80	220, 254, 280	+						+	+	+	+	+
			1	**2**	**3**	**4**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
1	0.30	220, 254			+		+		+				+
2	0.32	220, 254					+		+	+		+	+
3	0.38	220, 254, 280									+		
4	0.40	220, 254							+				
5	0.42	220, 254					+			+			+
6	0.51	220, 254					+		+	+	+		+
7	0.56	220, 254, 280				+							
8	0.58	220, 254					+	+		+			

9	0.64	220, 254									+	+		
10	0.69	220, 254		+			+	+	-	+	+	+	+	
11	0.80	220, 254, 280, 320, 365	+				+	+	+	+		+	+	

1 = atranorin, **2**= MOC, **3** = stictic acid, **4** = lobaric acid, **5**= EtOAc extr. of *S. montagneanum,* **6**= EtOAc extr. of *S. halei* (1), **7**= EtOAc extr. of *S. massartianum* (1), **8**= EtOAc extr. of *S. halei* (2), **9**= EtOAc extr. of *S. massartianum* (2), **10**= EtOAc extr. of *S. verruculigerum,* **11**= EtOAc extr. of *S. graminosum,* **12**= Acetone extr. of *S. montagneanum,* **13**= Acetone extr. of *S. halei* (1), **14**= Acetone extr. of *S. massartianum* (1), **15**= Acetone extr. of *S. halei* (2), **16**= Acetone extr. of *S. massartianum* (2), **17**= Acetone extr. of *S. verruculigerum,* **18**= Acetone extr. of *S. graminosum,* **19**= MeOH extr. of *S. montagneanum,* **20**= MeOH extr. of *S. halei* (1), **21**= MeOH extr. of *S. massartianum* (1), **22**= MeOH extr. of *S. halei* (2), **23**= MeOH extr. of *S. massartianum* (2), **24**= MeOH extr. of *S. verruculigerum* **25**= MeOH extr. of *S. graminosum*

3.3 Quantification of atranorin (1) in 7 extracts genus Stereocaulon using HPTLC

To date, there have been no reports of atranorin (**1**) quantification from the extracts of the *Stereocaulon* genus. In this measurement, we used a standard lichen eluent (toluene:EtOAc:formic acid (139:83:8))[20] with an atranorin Rf value of 0.87 detected at a wavelength of 254 nm. Based on the correlation between the concentration of atranorin standard (x) and peak area (y), the equation y = 34.167x + 1270.5 (r = 0.9963)(SM figure 15) was obtained. The highest atranorin content was detected from *S. graminosum* with atranorin value 325,498 mg/g extract and equivalent to 0.065 mg/g dried whole thallus while the lowest was from *S. verruculigerum* with 23,356 mg/g extract and 0.023 mg/g dried whole thallus (Table 3).

This quantification process is needed to determine specifically the content of atranorin in *Stereocaulon* and the correlation with its species and habitat. This is important due to atranorin is not only found in the genus *Stereocaulon* but generally is found in most lichen groups such as Cladoniaceae, Lecanoraceae, Parmeliaceae dan Stereocaulaceae[38]. Furthermore, atranorin is reported to have various pharmacological activities including anti-inflammatory[39], wound healing agent[40], antioxidant[35], and sunscreen substances[41].

Table 3. Calculation of atranorin concentration from extracts and dried whole thallus genus Stereocaulon

No	**Species**	**total extract (g)**	**Atranorin concentration**	
			per 1 gram extract (mg)	**per 1 gram dried-whole thallus (mg)**
1	*S. montagneanum* Lamb	2.118	176.174	0.035
2	*S. halei* Lamb (1)	2.706	270.534	0.054
3	*S. halei* Lamb (2)	2.647	94.974	0.019
4	*S. massartianum* Hue (1)	1.220	165.109	0.033
5	*S. massartianum* Hue (2)	1.233	32.002	0.032
6	*S. verruculigerum* Hue *var. subfurfurascens* Lamb	0.798	23.356	0.023
7	*S. graminosum* Schaer	1.029	325.498	0.065
Conc. 10mg/10 ml				

3.4 Antibacterial activity

Antibacterial test of pure extracts and isolates from the genus *Stereocaulon* lichen has been carried out using the liquid microdilution method, by determining the Minimum Inhibition Concentration (MIC) of extracts and pure isolates. This method was chosen because the samples and reagents used are relatively small but have a high level of sensitivity and provide quantitative results[21,22]. The test results showed all extracts in the genus *Stereocaulon* lichen had antibacterial activity against test bacteria but had different MIC values. Ethyl acetate extracts from *S. halei*, *S. massartianum*, and *S. graminosum* and acetone extract from *S. massartianum* showed the highest antibacterial activity against test bacteria with a range of MIC = 0.1-0.6 mg/mL. Furthermore, from the four isolated compounds tested, lobaric acid and MOC showed potential antibacterial activity against the four test bacteria with MIC values = 1.5-3 mg/mL.

The results of this test were in line with several other studies that reported a good potential of the genus *Stereocaulon* extract against pathogenic bacteria. Crude acetone extract of *S. massartianum* collected in the Philippines provides a large diameter of inhibition (19 mm) against *S. aureus* and *B. subtilis*[42]. Other studies show that an ethanolic extract from *S. foliolosum* inhibits the growth of M. tuberculosis H37Rv with MIC 500 mg/mL which is compared to rifampicin and isoniazid (MIC value 0.25 and 0.1 mg/mL, respectively)[43].

Table 4. Antibacterial activity from extracts and isolated compounds of genus *Stereocaulon*

Test materials	**MIC (mg/mL)**			
	SA	**PA**	**EC**	**EF**
S. montagneanum				
EtOAc extr.	10	5	2.5	2.5
Acetone extr.	5	2.5	2.5	5
MeOH extr.	10	2.5	5	5
***S. halei* (1)**				
EtOAc extr.	2.5	0.3	0.6	0.6
Acetone extr.	5	5	10	2.5
MeOH extr.	2.5	2.5	10	5
***S. massartianum* (1)**				
EtOAc extr.	2.5	2.5	5	0.1
Acetone extr.	2.5	2.5	5	0.1
MeOH extr.	2.5	5	5	5
***S. halei* (2)**				
EtOAc extr.	2.5	5	5	5
Acetone extr.	5	5	10	2.5
MeOH extr.	2.5	2.5	10	5
***S. massartianum* (2)**				
EtOAc extr.	2.5	2.5	5	2.5
Acetone extr.	2.5	2.5	5	2.5
MeOH extr.	2.5	5	5	5
S. verruculigerum				
EtOAc extr.	2.5	5	5	5
Acetone extr.	5	2.5	10	2.5
MeOH extr.	5	2.5	5	5
S. graminosum				

EtOAc extr.	0.6	0.6	0.6	0.6
Acetone extr.	1.2	2.5	2.5	0.6
MeOH extr.	2.5	2.5	2.5	0.3
Atranorin (1)	12	6	6	6
MOC (2)	3	3	3	1.5
Stictic acid (3)	-	-	-	-
Lobaric Acid (4)	3	1.5	1.5	1.5
Chloramphenicol	0.6	0.3	0.6	0.6

All values presented are Mean ± SD of triplicate readings

4 CONCLUSIONS

Metabolite profiling from 5 species showed that there was an atranorin compound in all test samples while stictic acid and lobaric acid were not. This appears that atranorin can be a marker compound for the genus *Stereocaulon*. The content of atranorin in each species also varies and is influenced by habitat and growing conditions. Based on the results of the quantification test, *S. graminosum* has the highest content of all samples. The result of two samples from the same species but of different habitats showed significant differences as well. This could be seen in the *S. halei* samples collected from a place with an altitude of 2700 m having more atranorin content than those growing at an altitude of 1350 m.

Furthermore, antibacterial tests on 4 pathogenic bacteria showed that all extract inhibited the growth of the tested bacteria. The ethyl acetate extract of *S. halei, S. massartianum, S. graminosum*, and acetone extract from *S. massartianum* have the highest antibacterial activity with a range of MIC = 0.1-0.6 mg/mL. These three species also exhibit stronger and specific antibacterial activity against *E. feacalis*. On the other hand, isolated compounds such as lobaric acid and MOC have MIC values = 1.5-3 mg/mL, whereas atranorin itself does not show satisfactory values compared with chloramphenicol.

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التوصيف الكيميائي النباتي والأنشطة المضادة للبكتيريا للخلاصات من خمسة أنواع من حزاز سومطرة من جنس ستيروكولون *Stereocaulon*

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ملخص

في الجرد والتحقيق المستمر في حزاز سومطرة، تم جمع خمسة أنواع من جنس ستيروكولون. من خلال تحليل التوصيف الأيضي، تم عزل المركبات الرئيسية الأربعة الموجودة في هذه الأنواع (مثل الأترانورين، ميثيل–β – أورسينول كربوكسيلات (**MOC**)، حمض اللوباريك، وحمض الستيكتيك) بسرعة كمركبات علامة باستخدام طرق كروماتوجرافيا تم تطويرها لـ س. حالي **S. halei** و س. مونتاجنيوم ***S. montagneanum***. تم تحليل الخلاصات من كل نوع باستخدام كروماتوجرافيا الطبقة الرقيقة عالية الأداء - (**HPTLC**) قياس الكثافة بخمسة أطوال موجية (220، 254، 280، 320، و 365 نانومتر). اترانورين و دوران الانقلاب الطولي **MOC** هما مركبان موجودان في جميع أنواع ستيروكولون الخمسة المختبرة. بعد ذلك، تم إجراء تقدير كمية الأترانورين عن طريق مسح قياس الكثافة عند 254 نانومتر من خلاصة أسيتات الإيثيل لكل نوع من أنواع ستيروكولون. تم الكشف عن أعلى تركيز للأترانورين في س. جراميوسوم ***S. graminosum*** (325.498 مجم / جم خلاصة) ما يعادل 0.065 مجم / جرام من الثاليوس الكامل المجفف، بينما كان أقل تركيز من س. فيروكوليجيروم ***S. verruculigerum*** (23.356 مجم / جرام خلاصة) و 0.023 مجم / جرام مجفف كامل ثالوس. إضافة إلى ذلك، تم تقييم جميع الخلاصات والمركبات الرئيسية الناتجة عن العزلة من حيث نشاطها المضاد للبكتيريا باستخدام طريقة التخفيف الدقيق. أظهرت خلاصات إيثيل أسيتات والأسيتون من س. ماسارتيانوم ***S. massartianum*** أعلى نشاط مضاد للجراثيم ضد هـ فيساليس **E. faecalis** (مكس **MIC** = 0.3125 مجم / جرام).

الكلمات الدالة: اترانورين • قياس كثافة–**HPTLC** • حزاز • تحديد خصائص المستقلبات • ستريكولون ***Stereocaulon*** .

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