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Biological activities of four *Parmotrema* species of Malaysian origin and their chemical constituents

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ABSTRACT

The present study was carried out to evaluate the antibacterial and antioxidant potential of acetone and methanol extracts of lichen (*Parmotrema praesorediosum*, *P. rampoddense*, *P. tinctorum* and *P. reticulatum*) and isolated chemical constituents which are praesorediosic acid, protocetraric acid, usnic acid, α–collatolic acid, β-alectoronic acid, atranorin and chloroatranorin. The antibacterial activity was evaluated using broth dilution method. Acetone extracts (except for *P. reticulatum*) showed good inhibitory activity against *S. aureus* and *B. subtilis* with MIC values ranging from 500–125 µg/mL, whereas, no activity was observed for the methanol extracts. Extracts exhibited zero inhibitory activity against *E. coli*. The antioxidant ability was measured using a DPPH free radical scavenging activity assay. Only methanol extract of *P. praesorediosum* exhibited more than 50% scavenging activity. Among the isolates, usnic acid exhibited the strongest antibacterial activity against *S. aureus* and *B. subtilis* with MIC value 7.81 µg/mL. Praesorediosic acid and protocetraric acid isolates exclusively inhibited *E. coli* at concentration 125 µg/mL and displayed results exceeding 50% scavenging activity (57.57% and 63.97%, respectively). Hitherto, it is the first evaluation of antibacterial activity on lichens of Malaysian origin and to our knowledge; the first reported study on the biological activity of praesorediosic acid and *Parmotrema rampoddense*.

INTRODUCTION

Lichen is a symbiotic organism between 'mycobiont' and 'photobiont' where 'mycobiont' is a fungus and 'photobiont' is an algae or cyanobacteria (Mason and Hale, 1983). Since the composition of the fungus is unique and it controls the symbiosis process of the lichen, lichen is regarded as a kind of fungi. Lichen and moss form the dominant organisms in the ecosystem that includes more than 10% of world land habitats, especially on higher grounds (Nash and Egan, 1988). Like all other living organisms, lichen also needs nutrients and energy to grow. Required nutrients are obtained from the air in the environment that includes dust, water and other substrates which are found on the habitats it is grown on. Energy derived from algae and cyanobacteria are responsible for carrying out the photosynthesis process. The symbiotic process of lichens can withstand extreme environmental conditions. Despite the competition with other plants, lichen has the potential of a life-span of several thousand years (Denton and Karlen, 1973; Hale, 1984). Lichen of genus *Parmotrema* is a member of the family Parmeliaceae which is the largest family of lichen in the world and is currently estimated to comprise more than 1000 species in 60 or more genera. Several decades ago, the number of genera in the family *Parmeliaceae* increased significantly, partly due to the narrow generic concept (Hale, 1984). The lichens in this family are characterized by the development of a foliose growth (Buaruang *et al*., 2009). Lichens have been used in traditional medicines for centuries and it still holds a great interest as alternative treatments in various parts of the world (Ronkovic *et al*., 2007; Richardson, 1991).

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Lichens and its secondary metabolites have been reported to have many biological activities such as antimicrobial, antiviral, antiprotozoal, enzyme inhibitory, insecticidal, antitermite, cytotoxic, antioxidant, wound healing, antiherbivore, analgesic and anti-inflammatory (Bombuwala *et al*., 1999; Kumar *et al*., 2010; Mitrovic *et al*., 2011; Barreto *et al*., 2013). Lichens represent a variety of new bioactives and most of which still remain to be characterized and discovered of its potential, mainly due to their natural occurrence in low concentration (Mitrovic *et al*., 2011). In the present study, antibacterial and antioxidant activity of four *Parmotrema* species (*Parmotrema praesorediosum*, *P. rampoddense*, *P. tinctorum* and *P. reticulatum*) and its isolated compounds were evaluated.

MATERIALS AND METHODS

Sample collection

P. praesorediosum was collected from Bukit Fraser, Pahang, Malaysia (3°46'N, 111°43'E, 1300m above sea level) and *P. rampoddense*, *P. reticulatum* and *P. tinctorum* were collected from Bukit Larut, Perak, Malaysia (4°50'N, 100°48'E, 1035m above sea level). Voucher specimens of these lichens have been deposited at the School of Chemical Sciences and Food Technology, Universiti Kebangsaan Malaysia.

Extraction

Lichen samples are cleaned and air-dried to remove dirt and moisture. Following this, the dried lichen materials were pulverized to obtain powder of 40 mesh size. Powdered lichen samples (250g each) were subjected to soxhlet extraction sequentially using two solvents, acetone and methanol. The extracts were then filtered through Whatman No. 1 filter paper, concentrated *in-vacuo* and air-dried under fume hood to obtain dry extracts which were stored at 4°C prior to use.

Bacterial strains and media

Lichen extracts were tested against Gram positive bacteria: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 19659); and Gram negative bacteria: *Escherichia coli* (ATCC 25922) obtained from American Type Culture Collection, USA. Bacterial cultures were maintained in Mueller-Hinton (MH) agar (Oxoid Ltd., Hampshire, England) and stored at 4ºC.

Antibacterial assay

Minimum inhibitory zone **(**MIC) of the lichen samples were determined using the 96 well plate following method as described by Eloff (Eloff, 1998) with slight modification. A stock solution of 40 mg/mL was prepared by dissolving 4 mg of extract in 100 µL of DMSO (dimethyl sulfoxide). The working solution of 1000 µg/mL was prepared by dissolving 25 µL of the stock solution in 975 µL of Mueller Hinton broth. The working solution was serially diluted two-fold using Mueller Hinton broth as diluent. Each well was inoculated with 100 µL of bacterial suspension containing 1.5×10^8 CFU/mL (equivalent 0.5 McFarland turbidity). The percentage of solvent was kept at approximately 1% throughout the assay. Final concentration of extracts and positive control ranged from 500-7.81 µg/mL. The 96 well plates were incubated at 37 ºC for 18 hours. Chloramphenicol served as positive control and DMSO served as negative control. Bacterial growth was evaluated by addition of 50 µL of 0.2 mg/mL freshly prepared INT (*para*-iodonitrotetrazolium) aqueous solution into each wells. The microplates were then covered and incubated for a further 30 minutes and were monitored for possible color changes. Wells that had color changes to purple were interpreted as presence of bacterial growth. MIC is defined as the lowest extract concentration that inhibits bacterial growth, whereby it is indicated by no changes in color. All experiments were performed in triplicate.

Antioxidant assay

Total phenolic content

Total phenolic content (TPC) was estimated by the Folin–Ciocalteu method with slight modification (Singleton and Rossi, 1965). Stock solutions of 1 mg/mL for extracts and gallic acid were prepared in DMSO and distilled water respectively. A series of gallic acid standards were prepared by diluting the stock solution with distilled water (0.01–0.5 mg/mL). A volume of 250 µL of gallic acid solution from each concentration was added into individual test tubes followed by addition of 1 mL of distilled water and 250 µL of Folin–Ciocalteu phenol reagent. The samples were then thoroughly shaken and allowed to incubate for 6 minutes at room temperature (22 ºC-24 ºC). Then, 2.5 mL of 0.332 g/mL sodium carbonate aqueous solution, Na_2CO_3, were added and allowed to incubate further for 2hours in a dark room at room temperature. After incubation, 200 µL was transferred from each test tube into a 96 well plate and absorbance was measured at 760 nm using microplate reader. A linear regression curve (standard curve) was generated using gallic acid (0–500 µg/mL). The results were expressed as gallic acid equivalents (GAE)/g dry weight of lichen extract. All samples were analysed in triplicates.

Total flavonoid content

The total flavonoid content (TFC) of the extracts was determined using colorimetric method as described by Sakanaka (Sakanaka *et al*., 2005). 125 µL of extract or standard solution (quercertin) at 1 mg/mL were added to individual test tube and then diluted with 625 µL of distilled water. Later, 37.5µL of 5% sodium nitrite solution, $NaNO_2$, were added and incubated for 6 minutes. After incubation, 75µL of 10% aluminium chloride solution, $AlCl_3$, were added and the mixtures were allowed to incubate for another 5 minutes. Then, 250 µL of 1M sodium hydroxide was added and the total volume was made up to 1.25 mL with distilled water. The solution was mixed well and 200µL of each solution was aliquot into a 96 well plate and absorbance was measured at 510 nm using microplate reader. Quercetin (0.05–0.001 mg/mL) was used for calibration of a standard curve. The

results were expressed as quercetin equivalent (QE)/g dry weight of lichen extract. The experiments were performed in triplicates.

1, 1-diphenyl-2-picryl-hydrazyl (DPPH)

A quantitative estimation of free radicals scavenging activity was measured using DPPH as described by Shimada (Shimada *et al*., 1992). The assay was carried out by adding 20 µL of extract (1 mg/mL) and standard into a 96 well plate followed by 180 µL of 0.2 mM of DPPH solution in each well. As for blank sample, 180µL of methanol was added with 20 µL of sample. Control contained methanolic DPPH solution. The plate was then covered and incubated in the dark for 20 minutes. The values were measured spectrophotometrically at 510 nm using a microplate reader and results were expressed as percentage of free radical scavenging using the equation below:

$$\text{Free radical scavenging activity (\%)} = ([A_0 - (A_1 - A_2)] / A_0) \times 100$$

Where, A_0 is the absorbance of the control sample, A_1 is the absorbance of the tested extracts and A_2 is the absorbance of blank sample. Trolox was used as the standard. A calibration curve was constructed using Trolox with concentration ranging from 0.5–0.02 mg/mL. The assay was carried out in triplicates. IC_{50} value of the extract was determined using the SPSS software.

Isolation of chemical constituent from lichen extract

Liquid-liquid partition

2g of bioactive extracts (*P. praesorediosum and P. rampoddense*) were subjected to liquid-liquid partition using *n*-hexane and methanol at a ratio of 1:1. The extracts were dissolved in 100mL of methanol and partitioned with 100mL *n*-hexane. The procedure was repeated thrice. The fractions obtained were dried using the rotory evaporator.

Thin-layer chromatography (TLC)

The bioactive fractions were dissolved in an appropriate solvent before spotting it onto the TLC plate. Solvent systems used were modified from Culberson (Culberson, 1972) to give broader Rf values. There were 4 solvents systems used; (A) benzene-dioxane-acetic acid (180:45:5, 230 mL), (B) hexane-diethyl ether-formic acid (130:80:20, 230 mL), (C) toluene-acetic acid (200:30, 230 mL) and (G) toluene-ethyl acetate-formic acid (139:83:8, 230 mL). The spots were identified under long and short ultraviolet light before the TLC was sprayed with 10% sulphuric acid and heated at 110°C.

Column chromatography

Fractions that showed good separation on the TLC plate was subjected to column chromatography. The solvent systems used were the same as the one used for TLC analysis, which gave a good separation. 0.5g of fraction was dissolved in 10mL of solvent system to avoid solubility problems inside the column. The sub-fractions were collected according to the bands formed in the column and air-dried before being subjected to TLC.

Preparative high performance liquid chromatography (Prep-HPLC)

The column used for separation was a Phenomenex Hypersil 5µ C18 column (150 x 21.2 mm) and the mobile phases were methanol (A) and 1% orthophosphoric acid (B). The fractions were eluted using gradient elution with a flow rate of 4mL/min. The run started with 100% A and was raised to 50% B within 5 minutes, then to 40% B within a further 10 minutes, followed by 20% B within the next 30 minutes. 1000µg/mL of sample with the injection volume of 500µL was injected and compound peaks were detected using Photodiode Array Detector at three different wavelengths (222nm, 230nm and 280nm). The fractions were then collected according to the retention time.

Identification of isolated chemical constituents

An analytical HPLC method adapted from Din (Din *et al*., 2010) was used to evaluate the purity of the isolated compounds. A Phenomenex Hypersil 3µ C18 column (250 by 4.6 mm) with a flow rate of 1mL/min was used. Two solvent systems: 1% aqueous orthophosphoric acid (A) and methanol (B) at the ratio of 7:3 were used as mobile phases. The gradient system started with 100% A and was raised to 58% B within 15 minutes, then to 100% B within the next 16 minutes, followed by isocratic elution in 100% B for a further 10 min. The compounds were then subjected to mass spectrometry (LC-MS ToF) and 1D NMR FT-NMR 600MHz Cryo (Fourier Transform Nuclear Magnetic Resonance 600MHz Cryoprobe) spectrometry.

Atranorin, P1

Figure 1: Prisms, (0.84g); ^{1}H NMR (600MHz, $CDCl_3$) δ: 2.10 (3H, s, Me-8'), 2.55 (3H, s, Me-9), 2.70 (3H, s, Me-9'), 3.99 (3H, s, 7'-COOMe), 6.41 (1H, s, H-5), 6.52 (1H, s, H-5'), 10.37 (1H, s, CHO-8), 11.94 (1H, s, HO-2'), 12.51 (1H, s, HO-2), 12.56 (1H, s, HO-4); ^{13}C NMR (600MHz, $CDCl_3$) δ: 9.39 (C-9'), 24.06 (C-8'), 25.62 (C-9), 52.38 (C-7'-COOMe), 102.84 (C-1), 108.55 (C-3), 110.26 (C-3'), 112.87 (C-5), 116.03 (C-5'), 116.80 (C-1'), 139.90 (C-6'), 151.99 (C-4'), 152.47 (C-6), 162.89 (C-2'), 167.50 (C-4), 169.11 (C-2), 169.72 (C-7), 172.23 (C-7'), 193.88 (C-8). HPLC retention time: 28.95 min.

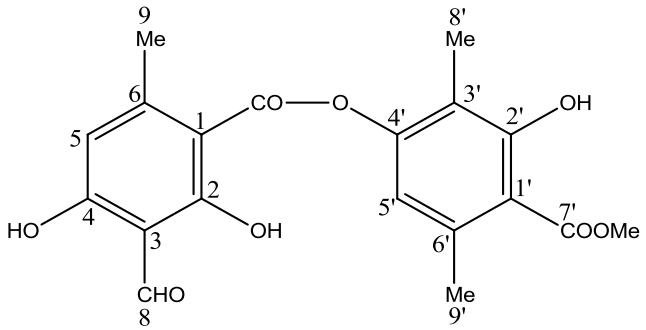

Fig. 1: Structure of atranorin, P1

Chloroatranorin, R5

Figure 2: Prisms, (0.24g); ^{1}H NMR (600MHz, CDCl3) δ: 2.10 (3H, s, Me-9), 2.56 (3H, s, Me-8'), 2.87 (3H, s, Me-9'), 4.06 (3H, s, 7'-COOMe), 6.57 (1H, s, H-5'), 10.38 (1H, s, CHO-8), 12.05, 12.42, 12.60 (3×1H, 3×s, 3×-HO-); ^{13}C NMR (600MHz, CDCl3) δ: 9.39 (C-8'), 21.08 (C-9), 23.96 (C-9'), 52.28 (C-7'-COOMe), 108.85 (C-1), 110.67 (C-3'), 112.87 (C-3), 115.82 (C-5'), 115.99 (C-5'), 116.80 (C-1'), 139.90 (C-6'), 149.09 (C-6), 152.07 (C-4'), 162.89 (C-2'), 163.49 (C-4), 166.20 (C-2), 169.11 (C-7), 172.23 (C-7'), 193.88 (C-8). HPLC retention time: 28.93 min.

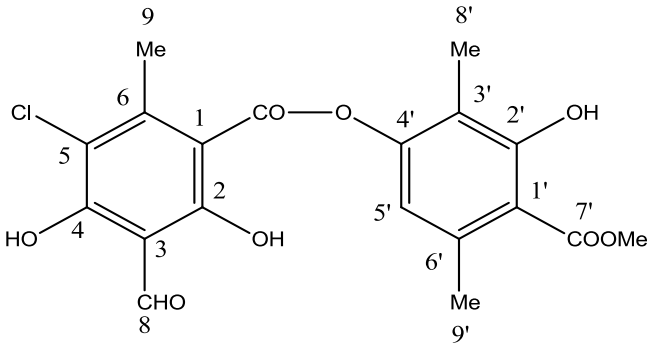

Fig. 2: Structure of chloroatranorin, R5

Usnic acid, R6

Figure 3: Yellow needles, (0.02g); ^{1}H NMR (600MHz, CDCl3) δ : 1.77 (3H, s, Me-13), 2.10 (3H, s, Me-16), 2.55 (3H, s, Me-15), 2.67 (3H, s, Me-18), 5.99 (1H, s, H-4), 11.05 (1H, s, C-10-OH), 13.33 (1H, s, C-8-OH); ^{13}C NMR (600MHz, CDCl3) δ: 7.57 (C-16), 27.94 (C-13), 31.32 (C-18), 32.14 (C-15), 59.09 (C-12), 98.36 (C-4), 101.54 (C-5), 103.97 (C-9), 105.24 (C-11), 109.34 (C-7), 155.22 (C-3), 157.52 (C-8), 163.89 (C-10), 179.40 (C-2), 191.73 (C-6), 198.07 (C-1), 200.37 (C-14), 201.81 (C-17). HPLC retention time: 28.73 min.

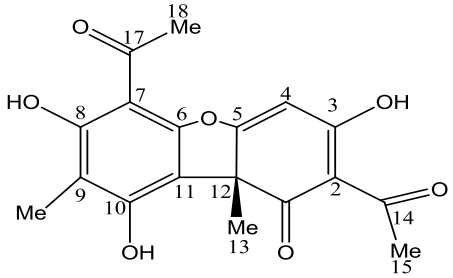

Fig. 3: Structure of usnic acid, R6

α–Collatolic acid, 2.16

Figure 4: Prisms, (0.003g); ^{1}H NMR (600MHz, acetone) δ: 0.91 (3H, s, Me-7'''), 0.91 (3H, s, Me-7''), 1.31 (4H, s, -CH2-6'',-CH2-6'''), 1.43 (4H, s, -CH2-5'',-CH2-5'''), 1.60 (2H, s, -CH2-4'''), 1.68 (2H, s, -CH2-4''), 2.52 (2H, s, -CH2-3''), 3.11,3.50 (2×1H, 2×s, -CH2-1'''), 3.76 (3H, s, MeO-4), 3.93 (2H, s, -CH2-1''), 6.53 (1H, d, H-5), 6.64 (1H, d, H-3), 6.88 (1H, s, H-3'), 11.03 (1H, s, HO-2'). HPLC retention time: 31.59 min.

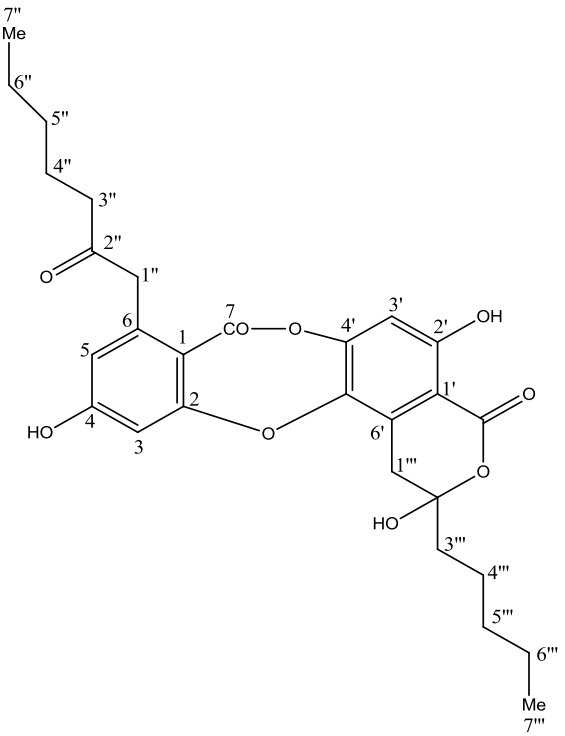

Fig. 4: Structure of α-collatolic acid, 2.16

β-Alectoronic acid, 3.16

Figure 5: Crystals, (0.002g); ^{1}H NMR (600MHz, acetone) δ: 0.88, 0.91 (2×3H, 2×t, 2×-Me), 1.31-1.40 (5H, s, 5×-CH2-), 1.76 (2H, s, -CH2-3''), 2.52 (2H, s, -CH2-3'''), 3.08 (2H, s, -CH2-1'''), 6.20, 6.60 (2×1H, 2×t, H-3, H-5), 6.38 (1H, s, H-1''), 6.50 (1H, s, H-3'). HPLC retention time: 33.40 min.

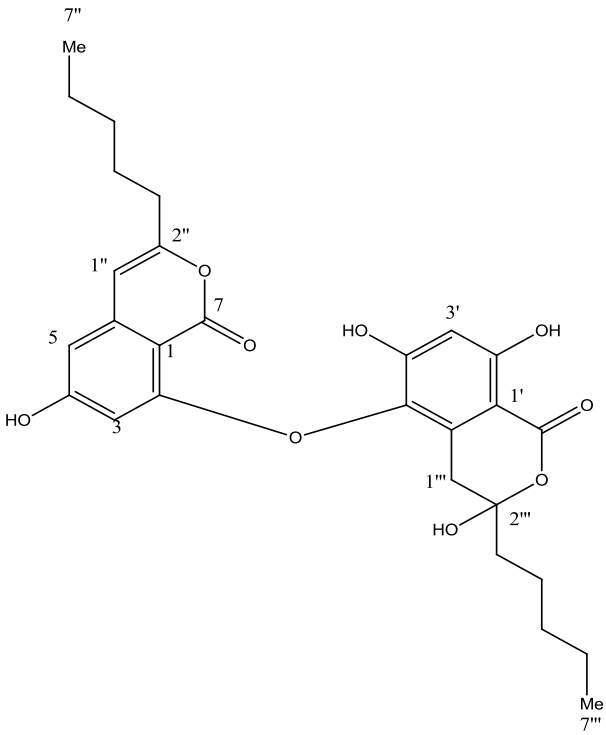

Fig. 5: Structure of β-alectoronic acid, 3.16

Praesorediosic acid, 5.1

Figure 6: Microcrystals, (0.002g); ^{1}H NMR (600MHz, acetone) δ: 1.30 [24H, bs, -$(CH2)_{12}$-], 1.62 (2H, s, -CH2-), 2.22 (3H, d, Me-5), 2.28 (2H, d, -CH2-20), 5.17 (1H, s, H-4). HPLC retention time: 24.03 min.

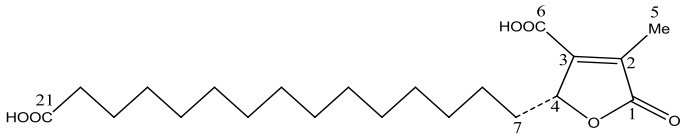

Fig. 6: Structure of praesorediosic acid, 5.1

Protocetraric acid, 9.1

Figure 7: Crystals, (0.002g); ^{1}H NMR (600MHz, acetone) δ: 2.47, 2.70 (2×3H, 2×s, 2×-Me), 4.79 (2H, s, -CH2-8'), 6.75 (1H, s, H-5), 10.73 (1H, s, -CHO). HPLC retention time: 24.37 min.

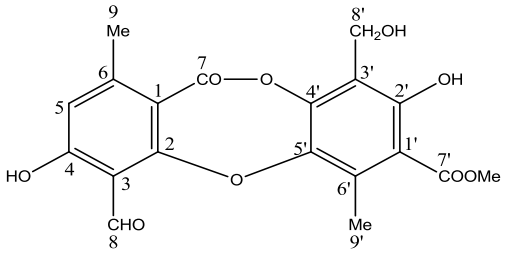

Fig. 7: Structure of protocetraric acid, 9.1

Bioassay of isolated chemical constituents

The isolated compounds were screened for antibacterial and antioxidant activities at 500µg/mL according to the methods described in section 2.4 and 2.5.3. The active chemical constituents were then tested for their minimal inhibitory concentration.

RESULTS AND DISCUSSION

For over 100 years, scientists have been investigating secondary chemicals produced by lichens. Lichens synthesize a variety of unique secondary metabolites, which are known to have multiple biological activities (Gayathri and Swamy, 2012). In Malaysia, distributions of lichens are found to be more diverse in mountainous areas (Din *et al*., 1998). In the present study, antibacterial and antioxidant activities of four *Parmotrema* species and their isolated chemical constituents were evaluated. Antibacterial activity of extracts tested at 500µg/mL is summarized in **Table 1**. The acetone extracts of tested lichen species except, *P. reticulatum*, displayed promising inhibitory activity against Gram positive bacteria. However, none of the extracts had inhibitory activity against *E. coli* (Gram negative bacteria). Therefore, the bioactive extracts of these three lichen species were further evaluated to determine their Minimum Inhibitory Concentration (MIC) as shown is **Table 2**. Test extracts displayed dose dependent inhibitory activity against the two tested Gram positive bacteria, *S. aureus* and *B. subtilis*. Acetone extract of *P. praesorediosum* showed highest inhibition against the test bacteria. Chloramphenicol and vancomycin which was used as the reference antibiotic displayed highest inhibitory efficacy compared to the lichen extracts. Methanol which was used as negative control did not exhibit any inhibition on test bacteria.

Table 1: Antibacterial potential of *Parmotrema* extracts at 500µg/mL

Test Bacteria	***P. p***		***P. r***		***P. reti***		***P. t***		**Chloramphenicol**	**Vancomycin**
	A	**M**	**A**	**M**	**A**	**M**	**A**	**M**		
S. aureus	+	-	+	-	-	-	+	-	+	+
B. subtilis	+	-	+	-	-	-	+	-	+	+
E. coli	-	-	-	-	-	-	-	-	+	+

P. p: *P. praesorediosum*, ***P. r***: *P. rampoddense*, ***P. reti***: *P. reticulatum*, ***P. t***: *P. tinctorum*, **A**: acetone extract, **M**: methanol extract, (+): had inhibitory against tested bacteria, (-): had no inhibitory against tested bacteria.

Table 2: MIC of bioactive extracts against the gram positive bacteria (µg/mL)

Test bacteria	***P. p***	***P. r***	***P. t***	**Chloramphenicol**	**Vancomycin**
	A	**A**	**A**		
S. aureus	250	500	500	31.25	7.81
B.subtilis	125	125	250	15.63	7.81

P. p: *P. praesorediosum*, ***P. r***: *P. rampoddense*, ***P. t***: *P. tinctorum*, **A**: acetone extract

In a recent study, Vivek (Vivek *et al*., 2014) reported extracts of *P. tinctorum* and *P. praesorediosum* collected in India displayed concentration dependent inhibition against test bacteria (*S. aureus* and *E. coli*) using disc diffusion method. Whereas, Sinha and Biswas (Sinha and Biswas, 2011) reported that acetone and methanol extracts of *P. reticulatum*, collected from Sikkim (India), had moderate antibacterial potential with 10 mm zone inhibition against *S. aureus* and 30 mm zone inhibition against *E. coli*, respectively. This could be due to certain adaptations and modification that could take place for survival of the species in a given geographical location and climatic conditions (Honegger, 1993). The production of harmful free radicals which exceeds the antioxidant defense capacity of the body results in the increase of oxidative stress. The production of these reactive oxygen species is partly due to environmental factors such as pollution, temperature, drought, excessive light intensities and nutritional limitation (Arora *et al*., 2002; Marxen *et al*., 2007). These highly reactive free radicals are known to be responsible for some human diseases like cancer and cardiovascular diseases (Jacob and Burri, 1996). Hence there is a need for an external source of antioxidant to fight the effects of the reactive free radicals, and lichens have shown to possess promising antioxidant activity (Jayaprakasha and Rao, 2000; Kekuda *et al*., 2009; Kekuda *et al*., 2011). The total phenolic, total flavonoid and free radical scavenging (DPPH)

activity of acetone and methanol extracts of four *Parmotrema* species at 500µg/mL are as shown in **Table 3**. Acetone extracts of *P. rampoddense* and *P. praesorediosum* displayed highest total phenolic content and total flavonoid content, respectively. Methanol extract of *P. praesorediosum* showed highest scavenging potential, whereas the rest of the extracts showed less than 50% scavenging activity. However, the result shows no correlation between the total phenolic and total flavonoid content with percentage scavenging of DPPH free radicals. Since only methanol extract of *P. praesorediosum* showed good scavenging activity, it was further tested to determine the IC_{50}. The percentage scavenging activity of *P. praesorediosum* methanol extract compared to a standard (Trolox) is shown in **Table 4**. The results suggest that the scavenging potential was found to increase with increasing concentration of the extract. However, the radical scavenging potency of *P. praesorediosum* methanol extract (IC_{50} 233.48 µg/mL) was lower than that of trolox (IC_{50} 156.54 µg/mL).

Table 3: Free radical scavenging activity, total phenolic content (TPC) and total flavonoid content (TFC) of *Parmotrema* extracts at 500µg/mL.

	Extracts	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	DPPH (% scavenging)
A	***P. praesorediosum***	109.28 ± 1.13	443.86 ± 0.43	16.99 ± 1.06
	P.rampoddense	215.72 ± 1.78	41.14 ± 1.13	11.7 ± 0.31
	P.reticulatum	76.92 ± 0.79	144.25 ± 1.87	44.44 ± 1.24
	P.tinctorum	191.70 ± 1.60	22.81 ± 0.12	16.86 ± 2.42
M	***P.praesorediosum***	81.11 ± 1.31	140.03 ± 1.64	84.48 ± 0.21
	P.rampoddense	138.03 ± 1.45	24.45 ± 2.05	31.68 ± 1.30
	P.reticulatum	41.97 ± 1.32	134.92 ± 1.56	8.51 ± 1.67
	P.tinctorum	204.31 ± 2.34	43.79 ± 1.16	25.83 ± 1.06
Trolox				98.19 ± 0.66

A: acetone extract, **M**: methanol extract, **GAE**: gallic acid equivalent, **QE**: quercertin equivalent.
Standard curve (R^2) for TPC = 0.99
Standard curve (R^2) for TFC = 0.991

Table 4: DPPH radical scavenging activity of *P. praesorediosum* methanolic extracts.

Concentration (µg/mL)	% scavenging	
	P. praesorediosum	**Standard (Trolox)**
500	79.41	95.76
250	45.94	57.88
125	27.14	23.71
62.50	15.39	18.65
31.25	10.59	15.97
15.63	8.65	14.96
7.81	9.17	16.90
3.91	9.49	16.83

In this study, the methanol extract of *P. praesorediosum* displayed highest percentage scavenging of DPPH free radicals which correlates with the findings reported by Vivek and his colleagues (Vivek *et al*., 2014). Whereas, Sharma and Kalikoty (Sharma and Kalikoty, 2012), reported moderate DPPH scavenging activity of methanol and ethanol extracts of *P. reticulatum* (10.1% and 16.5% inhibition at 25 µg/mL, respectively) which is not in agreement with the present study whereby the acetone and methanol extracts of *P. reticulatum* displayed low activity. Ghate (Ghate *et al*., 2013) reported that 70% methanolic extract of *P. reticulatum* showed considerable radical scavenging activity (20% inhibition at 80 µg/mL). In this study, *P. tinctorum*, both acetone and methanol extract displayed low percentage scavenging activity, although Kekuda (Kekuda *et al*., 2009) observed a high dose dependent scavenging activity of DPPH free radicals in the lichen *P. pseudotinctorum* (90.74% radical scavenging at 0.5 mg/mL). However, the difference in activity of previous studies with the present one is likely due to the geographical influence. This study showed no correlation between the total phenolic or total flavonoid content, with the free radical scavenging potential of the lichen extracts. This observed finding is in agreement with those reported by Stanley (Stanley *et al*., 2011). However, Vivek (Vivek *et al*., 2014) observed direct correlation between the phenolic content and the antioxidant activity, which has been supported by other studies (Tilak *et al*., 2004; Coruh *et al*., 2007; Rekha *et al*., 2012; Poornima *et al*., 2012). Antibacterial results of the extracts are more promising compared to the antioxidant results. Hence, isolation of the compounds was based on the antibacterial potential of the extracts. As the acetone extract of *P. praesorediosum* and *P. rampoddense* showed high antibacterial potential, these two extracts were subjected to isolation, a total of 7 compounds were isolated using preparative HPLC, characterized and identified using NMR, mass spectrometry and analytical HPLC.Praesorediosic acid and protocetraric acid were isolated from *P. praesorediosum*, while usnic acid, α–collatolic acid and β-alectoronic acid were isolated from *P. rampoddense*. Atranorin and chloroatranorin were isolated from both the species. Usnic acid exhibited the highest antibacterial potential with MIC value of 7.81µg/mL against both Gram positive bacteria which is comparable to the standard, while α–collatolic acid and β-alectoronic acid showed the lowest inhibition against *S. aureus* at 500µg/mL and moderate inhibition against *B. subtilis* at 125µg/mL. Praesorediosic acid and protocetraric acid are the only two isolates that inhibited *E.coli* at 125µg/mL. Similarly, these isolates exhibited more than 50% scavenging of DPPH free radicals. Results are summarized in **Table 5**.

Table 5: Minimal Inhibitory Concentration (MIC) and DPPH radical scavenging of the isolated compounds.

Compounds	MIC (µg/mL)			% DPPH Scavenging at 100µg/mL
	S. aureus	***B. subtilis***	***E. coli***	
Atranorin	62.50	15.63	-	34.26 ± 0.25
Chloroatranorin	62.50	15.63	-	28.15 ± 0.29
Usnic Acid	7.81	7.81	-	42.58 ± 0.14
α–Collatolic acid	500	125	-	20.00 ± 0.29
β-Alectoronic acid	500	125	-	23.62 ± 0.16
Praesorediosic acid	250	125	125	57.57 ± 0.48
Protocetraric acid	250	125	125	63.98 ± 0.16
Vancomycin	7.81	7.81	7.81	
Trolox				98.24 ± 0.02

-: no activity against tested bacteria.

Secondary metabolites from lichens have been reported to possess unique biological activities. Usnic acid is a well-known lichen metabolite with dibenzofuran structure which exhibits high antibacterial potential previously reported by Tay (Tay *et al*., 2004), in which the lowest inhibitory concentration against *S.*

aureus was at 3.1 µg/62.5 µL and *B. subtilis* at 0.78 µg/62.5 µL. In another study, Rankovic (Rankovic *et al*., 2014) reported that usnic acid exhibited high antimicrobial activity against *S. aureus*, *B. subtilis* and *E.coli* at concentration 125 µg/mL, 0.8 µg/mL and 250 µg/mL respectively, followed by atranorin and chloroatranorin. It is also reported that the antibacterial activity of the compounds were stronger than the antifungal activity. However, in the present study, it is observed that both atranorin and chloroatranorin exhibited similar antibacterial potential. Thadhani (Thadhani *et al*., 2012) observed moderate inhibitory activity by atranorin against *B. subtilis* and *E. coli* at 100µg/mL, while Yilmaz (Yilmaz *et al*., 2004) observed strong antibacterial activity by atranorin (15.6 µg/mL for *B. subtilis* and 500 µg/mL for *S. aureus*) isolated from the lichen *Cladonia foliacea* using disc diffusion method. Chloroatranorin is found to exhibit moderate activity against *S. aureus*, *B. subtilis* and *E. coli* at 156 µg/25 µL (Turk *et al*., 2006). Verma (Verma *et al*., 2011)reported promising bactericidal activity of atranorin, β-alectoronic acid, α-collatolic acid, protocetraric acid and other secondary metabolites. This finding strongly supports the results obtained in the present study. However, antibacterial results obtained in this study are efficacious and differ significantly compared to other reported studies. This could be attributed to the difference in method used to evaluate the antibacterial potential. Disc diffusion method is considered as qualitative and has disadvantages whereby it is unable to generate accurate MIC value and difficult to examine the susceptibility of fastidious and slow-growing bacteria (Wilkins and Thiel, 1973). Therefore, MIC (broth dilution) test is a more reliable and quantitative method to test against anaerobic bacteria. In addition, disc diffusion method is not a suitable method for natural antimicrobial compounds because they scarcely soluble or insoluble in water and their hydrophobic nature prevent uniform diffusion through the media (Klancnic *et al*., 2010). As for the antioxidant potential of tested isolates, praesorediosic acid and protocetraric acid are the only compounds observed to exhibit more than 50% scavenging activity of DPPH free radicals. However, Manojlović (Manojlovic *et al*., 2012) reported protocetraric acid to exhibit strong antioxidant activity (IC_{50} 119.10 µg/mL). This current finding shows that the antioxidant potential of usnic acid, atranorin and chloroatranorin evaluated is low and it is similar to the results reported by Thadhani (Thadhani *et al*., 2011). The DPPH free radical scavenging potential is evaluated by the hydrogen donating potency of compounds; therefore, usnic acid, atranorin and chloroatranorin have few or no labile hydrogen atoms to bind with the DPPH ions to exhibit good free radical scavenging activity. Millot (Millot *et al*., 2008) observed that α-collatolic acid and β-collatolic acid (IC_{50} 463 and 122 µM, respectively) displayed better superoxide anion scavenging activity than quercetin(IC_{50} 754 µM), which suggests that collatolic acid derivatives have strong antioxidant potential. Research has shown that the derivative of alectoronic acid (α-alectoronic acid) is known to possess promising antioxidant activity (Millot *et al*., 2008; Ravaglia *et al*., 2014). However, in this study, β-alectoronic acid displayed low percentage scavenging activity. Hitherto, there is no report on biological activity of praesorediosic acid.

CONCLUSION

Lichens chemical constituents exhibit a huge array of biological activities and most of them have ecological benefits. The properties of lichen substances make them a promising and possible pharmaceutical agent (Molnar and Farkas, 2010). This is the first reported study on biological activities of these lichens found in Malaysia, except for the antioxidant evaluation of *P. tinctorum* which has been reported previously. Among the isolated chemical constituents in this present study, β-alectoronic acid which is a derivative of alectoronic acid and praesorediosic acid are the first to be evaluated for their biological potentials. According to the results obtained, usnic acid and atranorin has high potential as an antibacterial agent, whereas, praesorediosic acid and protocetraric acid has moderate potential as an antioxidant agent. Data from this study suggest that the isolated compounds can be a potential lead for research and development of active agents against infectious diseases and free radical induced oxidative stress.

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