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Antibacterial and Anticancer Activities of Nine Lichens of Indonesian Java Island

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Abstract: Lichen is a unique composite organism that arises from algae and fungi symbiotic relationship. There are 18,500 recorded lichen species worldwide but only limited number of global species has been tested for their biological activities. In particular, Indonesian lichens are rarely investigated. In this study, we collected and identified nine lichen species from six different locations in East Java Indonesia and screened their crude methanol extracts against gram-negative bacteria (*Pseudomonas aeruginosa*) and cancer cells (MCF7, Widr and Hela). While only the methanol extract of *Parmelia cetrata* Ach and *Parmelia dilatata* Vain inhibited *Pseudomonas aeruginosa*, most lichen extracts possessed moderate cytotoxicity. *Cladonia scabriuscula* methanol extract was cytotoxic against MCF7, Widr and Hela cell lines with IC_{50} value of 324, 324, 476 µg/mL, respectively. Moreover, methanol extract of *Physcia* cf. *millegrana* Degel indicated cytotoxicity against Hela cell line with IC_{50} value of 137 µg/mL. This study revealed anticancer potency of lichen of Java Island for the first time and further research is necessary for isolating the bioactive compounds.

Key words: Medicinal plant, Lichen, Java island, phytochemical, antimicrobial, anticancer.

Introduction

Indonesia is the largest archipelagic country in the world covered by tropical rain forest, seasonal forest, mountain vegetation, subalpine shrub vegetation, swamp and coastal vegetation. Located between Asian and Australian continent, Indonesia possess the second largest biodiversity in the world, with around 40,000 recorded endemic plant species [1]. The archipelago is also rich in lichen species and it has been part of Indonesian traditional medicine sources, especially the *Usnea* genus, which is locally used in treating inflammatory diseases. Lichen is a unique composite organism that arises from algae and fungi symbiotic relationships in which 99 % of fungal symbionts are ascomycetes [2]. Lichen is a slow-growing and complex symbiotic organism between fungus as the mycobiont, and algae or cyanobacteria as the photosynthetic partner or photobiont. The fungal partner is usually the dominant one but each part-

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ner benefit from their symbiotic association. While the mycobiont protect the photobiont from intense exposure to sunlight and also absorb the mineral nutrients, the photobiont synthesizes the organic nutrients needed in lichen [3].

Although lichens has small biomass and it is a slow growing organism, it covers 8 % of total land on Earth [4]. Lichens are reported from seas to mountains, from tropics to Antarctica and the polar Arctic region. It could grow in wide range of environment including rocks, dead trees, metal, trees, woods, tree barks, and also in the desert [5]. More than 18,500 lichen species are recorded worldwide and new species are being discovered every year [6,7]. These lichens produce secondary metabolites responsible for protection againts both biotic and abiotic stress including UV and oxidative stress, animal predation, microbial infections, and the plant competitors. The production of secondary metabolites is complex and depends on its environment and also metabolic pathways that comprises acetyl-polymalonyl, shikimic acid, and mevalonic acid pathways [3]. To date, about 1000 secondary metabolites including its specific secondary metabolites, the depside, depsidone, and dibenzofuran [8], have been characterised from the lichen sources [9]. These secondary metabolites possess various biological activities including anticancer, antimicrobial, arresting cell cycle, apoptosis, necrosis and inhibition of angiogenesis [9]. Some probable antimicrobial mechanisms of lichen substances are inhibition of cell wall, nucleic acid and protein synthesis, cell membranes alteration, and also inhibition of metabolism [3]. Overall, only few lichens have been studied for their chemical content and the biological activities and lots remain untapped especially the lichens from Java island. There are very limited reports on the taxonomical, chemical and bio-prospecting studies of Indonesian lichens [10]. In this study, we have collected and identified nine lichens from Java island of Indonesia and assessed them for their chemical content and biological activities including antibacterial and anticancer activities for the first time.

Materials and methods

Lichen collection and identification

Nine lichens were collected from Meru Betiri National Park (three samples under voucher code A1, A2, A3), Bondowoso district (four samples under voucher code B1, B2, B3, B4), Jember district (one sample under voucher code C1), and Pasuruhan district (one sample under voucher code D1) in Java Island - Indonesia (See map in Fig.1). Lichens were taxonomically identified by a Lichenologist, Mrs Ludmilla F Untari at Faculty of Biology, Gadjah Mada University-Indonesia and the samples with assigned voucher specimen numbers were deposited at the Faculty of Pharmacy, University of Jember.

Extraction and phytochemical screening methods

Lichen samples (see table 1 for mass weight details) were frozen with liquid nitrogen, pulverised/powdered and then extracted with methanol for 24 hours. The solution was filtered, and the supernatant was collected in the round bottom flask and then dried using rotary evaporator to produce crude methanol extract. The % yield of extract for each lichen species was calculated using the formula as: Percentage yield = (crude dried extract/dried sample) x 100 %. The crude methanol extracts were tested for the presence of five different classes of phytochemicals including alkaloid, terpenoid, flavonoid, and polyphenol using the methods described by us earlier [11]. Briefly, to see if the lichens contained alkaloid, we resuspended the cude extract in methanol and applied to thin layer chromatography (TLC) plate F_{254}. The TLC plate was developed with a solvent system of ethyl acetate: methanol:water (9:2:2) and sprayed with dragendorff reagent. The presence of alkaloid showed orange colour bands/spots on the TLC plate. For detecting terpenoids, we applied the same extract to a TLC plate and developed it with a mobile phase of *n*-hexane:ethyl acetate (4:1). The plate was sprayed with an anisaldehyde reagent and then heated at 105ºC. The presence of terpenoid was detected as reddish purple spots on TLC plate. For flavonoid test, the TLC plate loaded with sample extracts was developed with buthanol:acetic acid glacial:water (4:1:3) and the flavonoid was detected under ammonia vapour as yellowish colour. For the polyphenolic test, we

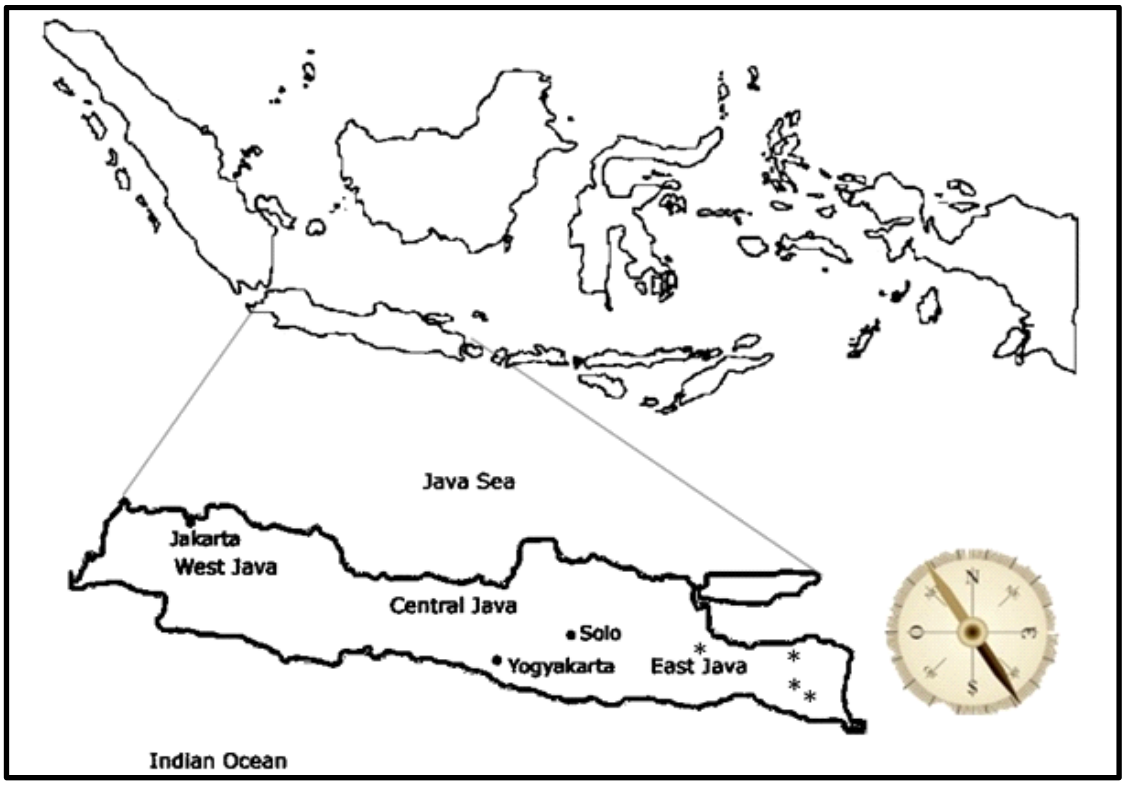

Figure 1. Map of Indonesia and the study sites in Java Island. *Lichen collection site in Java Island (Meru Betiri National Park, Jember, Bondowoso and Pasuruhan Districts)

Table 1. Crude extracts of lichens and their % yield

Sample	Weight of fresh lichen (mg)	Crude extract (mg)	Yield (%)
Candelaria fibrosa (Fr.) Müll. Arg.	1,165.00	6.99	0.60
Cladonia scabriuscula (Duby) Leight	6,433.64	761.10	11.83
Parmelia aurulenta Tuck.	4,999.95	971.49	19.43
Parmelia caroliniana Nyl.	2,119.73	471.64	22.25
Parmelia cetrata Ach.	42,903.02	6,680.00	15.57
Parmelia dilatata Vain.	55,750.15	9,550.00	17.13
Parmelia tinctorum Nyl.	4,649.00	649.40	13.97
Physcia cf. *millegrana* Degel.	2,919.81	495.20	16.96
Teloschistes flavicans (Sw.) Ach.	1,536.73	506.66	32.97

developed the pre-loaded TLC plate with chloroform:ethyl acetate (1:9) and dipped in $FeCl_3$ reagent. The TLC spots that contained polyphenolics turned into black colour.

Antibacterial microdilution assay

For assessing the antibacterial activity of the crude extracts of lichens against the gram negative bacteria (*Pseudomonas aeuginosa* ATCC 27853), we used microdilution method as described in CLSI [12]. Briefly, the bacterial stock were grown in Mueller-Hinton media (37°C, 24 h). Standardized inoculum was made and the final microbial concentration was adjusted to 0.5 Mc Farland turbidity. The adjusted inoculum was then diluted to obtain 5 x 10^5 CFU/mL bacteria in each well. Ninety six well plate was inoculated with *P. aeruginosa* bacteria and the lichen extracts in a

series concentration of 1024, 512, 256, 128, 64 and 32 µg/mL followed by incubation at 37°C for 20 h. IC_{50} value was calculated from correlation graph between extract concentration and absorbance.

Cytotoxicity/anticancer assay

For assessing the cytotoxicity or anticancer activity of the lichen extracts, we used a well-established colorimetric MTT assay [13]. Vero (ATCC CCL-81), MCF7 (ATCC HTB-22), HeLa (ATCC CRM-CCL-2) and WiDr (ATCC CCL-218) cells were seeded in a 96-well plate at a density of 1x104 cells/well and allowed to adhere for 24 hours at 37°C in a CO_2 incubator. Eagle's Minimum Essential Medium supplemented with 10 % fetal bovine serum was used as the culture media for all cell lines. After 24 hours of incubation, culture media was replaced with a fresh medium as and when necessary. Cells were then treated with various concentrations of the desired lichen extracts (1024, 512, 256, 128, 64, 32 µg/mL) for 24 h at 37°C in a CO_2 incubator. Subsequently, 10 µL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 4 h at 37°C in a CO_2 incubator. The medium was then aspirated, and the formazan crystals were solubilized by adding 50 µL of DMSO per well for 30 min at 37°C in a CO_2 incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 540 nm.

Results and discussion

Collection, identification and the explorative status of lichens

The earliest report on Indonesian lichen was reported in 1913 [10] and no explorative studies were conducted after that year. This study was the first biochemical and bioactivity screening conducted on Indonesian lichens. The study resulted in the collection and identification of leaf-like lichens from four different districts in Java Island - Indonesia, namely Meru Betiri National Park, Jember, Bondowoso and Pasuruhan (Fig. 1). A total of nine lichens (Fig. 2) were identified and most of them belonged to Parmelia genus. Through SciFinder global database searches, we found that most of these lichens were under-studied and hence there was no report regarding their secondary metabolites/constituents and their pharmacological activities.

Phytochemical screening for major classes of chemotypes

Freshly collected lichen samples (see table 1 for mass details) were frozen with liquid nitrogen, ground them into coarse powder and then soaked in methanol for 24 h. The solution was filtered, concentrated and the crude methanol extract (see Table 1 for the yield of crude extracts) of each lichen was tested for the presence of major classes of phytochemicals using TLC plate and chemical reactive-based visualisation.

The phytochemical test confirmed that all nine lichens were devoid of alkaloid and flavonoid but were rich in terpenoid, steroid and polyphenol (see Table 2). Amongst the nine lichens tested, *Physcia* cf. *millegrana* gave positive test for two major classes of phytochemicals including terpenoid and polyphenol. While *Candelaria fibrosa, Parmelia aurulenta* and *Teloschistes flavicans* contained major chemotypes (terpenoid), *Cladonia scabriuscula* and *Parmelia tinctorum* tested positive for only one chemotype (polyphenol). Related species of *Cladonia* genus including *C. fimbriata, C. furcata, C. subulata, C. foliacea* and *C. Rangiferina* were previously reported to contain hypoprotocetraric acid, fumarprotocetraric acid, usnic acid and atranorin [14].

Antibacterial activity of nine lichens

Ever since the anti-bacterial activity of the lichen was first reported in 1944 [15], the search for antibiotic from lichen was pursued and subsequently revealed several activities against both gram negative and positive bacteria [9]. The fact that gram negative bacteria is less sensitive to antibiotics led us to focus our study on *Pseudomonas aeruginosa* (ATCC 27853) using the microdilution method as described in CLSI [12]. Methanol extracts of all nine lichens were tested against this bacterial strain. Of nine lichen extracts tested at a concentration of 1024 µg/mL, only *Parmela cetrata* and *Parmela dilatata* extracts showed moderate anti-bacterial activity with per-

Figure 2. Nine lichen samples collected from Meru Betiri National Park, Jember, Bondowoso and Pasuruhan Districts. *Candelaria fibrosa* (Fr.) Müll., *Parmelia aurulenta* Tuck., *Physcia cf. millegrana* Degel., *Cladonia scabriuscula* (Duby) Leight, *Parmelia caroliniana* Nyl., *Parmelia tinctorum* Nyl., *Parmelia cetrata* Ach., *Teloschistes flavicans and Parmelia dilatata*

Table 2. Major classes of phytochemicals detected in the crude methanol extracts of nine Lichens collected from Java Island

Species	Alkaloid	Terpenoid	Flavonoid	Polyphenol
Candelaria fibrosa (Fr.) Müll. Arg.	-	+	-	-
Cladonia scabriuscula (Duby) Leight	-	-	-	+
Parmelia aurulenta Tuck.	-	+	-	-
Parmelia caroliniana Nyl.	-	-	-	-
Parmelia cetrata Ach.	-	-	-	-
Parmelia dilatata Vain.	-	-	-	-
Parmelia tinctorum Nyl.	-	-	-	+
Physcia cf. *millegrana* Degel.	-	+	-	+
Teloschistes flavicans (Sw.) Ach.	-	+	-	-

-: not detected; +: positive

centage inhibition of 50 % and 37 %, respectively. Parmelia genus belong to the family of Parmeliaceae. This genus along with Usnea, Parmotrema, Cetraria, Pseudevernia and Hypo-gymnia are known to possess potent antibacterial activities [16]. Common antibiotic found in Parmelia genus are salazinic acid, usnic acid and protocetraic acid, which presented a broad-spectrum antimicrobial activity against, not only gram positive and negative bacteria, but also fungi [9].

Usnic is acid isolated from *Evernia divaricata* was reported significantly inhibit the growth of *Pseudomonas aureginosa* [9]. This same compound was isolated from *Parmelia caperata* and was found to possess antibacterial activity against *Bacillus mycoides*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* with minimum inhibition concen-

tration (MIC) value of 0.0008, 0.0008, 0.25, 0.0625, 0.125 mg/mL, respectively [17]. Protocetraric acid (isolated from *P. caperata*) exhibited antibacterial activities against *Bacillus mycoides*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* with MIC value of 0.015, 0.015, 1.0, 0.5, 0.015 mg/mL, respectively [17]. Salazinic acid (isolated from *Parmelia saxatilis*) was also reported to exhibit high antibacterial activity against several gram-positive and gram-negative bacteria including *Bacillus mycoides*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* with MIC value of 0.015, 0.0312, 1, 0.5, 0.125 mg/mL, respectively [17].

Fumarproto-cetraric acid of *Cladonia rangiferina* exhibited antibacterial activity against *Bacillus mycoides, Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Klebsiella pneumoniae*, with MIC value 0.062, 0.62, 0.125, 0.125, 0.03 mg/mL, respectively [18].

Cytotoxicity activities and anticancer potential of lichens

The crude methanol extract obtained from nine lichen species were tested against breast cancer cell MCF7, colon cancer cell Widr and cervical cancer cell Hela and normal Vero cell (Table 3). Amongst nine lichens, the methanol extract of *Cladonia scabriuscula* showed the best cytotoxicity against breast cancer cell-MCF7 and colon cancer cell -Widr with same IC_{50} value of 324 µg/mL. However, this lichen extract also showed cytotoxicity against normal Vero cell with IC_{50} value of 283 µg/mL. Its closely related species including *C. rangiformis*, *C. convolute*, *C. fimbriata, C. furcata, C. subulata, C. foliacea* and *C. rangiferina* were previously reported to significantly (with IC_{50} value range of 54 - 63 µg/mL) inhibit MCF-7 human breast cancer cells proliferation and Hela cell growth [19]. Another study involving *C. rangiferina* showed that this lichen also has relatively strong anticancer activity against FemX (human melanoma) and LS174 (human colon carcinoma) cell lines with IC_{50} value of 19.97 and 10.97 µg/mL respectively [18]. Methanol extract of *Physcia* cf. *millegrana* possessed most significant cytotoxicity againts cervical cancer cell- Hela with IC_{50} value of 137 µg/mL. This agrees to the general hypothesis that plants containing diverse classes of chemicals are of superior biological activities [11]. Thus, it may be also deduced here that such strong anticancer activity could be the collective result of the highest number of chemotypes/chemical diversity (two chemotypes including terpenoid, and polyphenol) present in *Physcia* cf. *millegrana*. The fact that it showed very mild cytotoxicity against normal Vero cell line gives (<600 µg/mL) an edge on selective therapeutic index and makes this lichen attractive for further chemical and bioprospecting studies in near future.

Table 3. Cytotoxicity and the anticancer activities of nine lichens against breast cancer cell - MCF7, colon cancer cell - Widr, cervical cancer cell - Hela and the normal Vero cell

Species	IC_{50} (µg/mL)			
	MCF7	Widr	Hela	Vero
Candelaria fibrosa (Fr.) Müll. Arg.	997	392	733	581
Cladonia scabriuscula (Duby) Leight	324	324	476	283
Parmelia aurulenta Tuck.	628	563	552	538
Parmelia caroliniana Nyl.	765	595	328	1415
Parmelia cetrata Ach.	430	642	751	749
Parmelia dilatata Vain.	>1000	769	981	1000
Parmelia tinctorum Nyl.	420	574	629	578
Physcia cf. *millegrana* Degel.	640	332	137	597
Teloschistes flavicans (Sw.) Ach.	806	799	959	649

Conclusion

This study identified nine macro lichen of Java island and showed that they are devoid of alkaloids and flavonoids. However, they were rich in terpenoid, steroid and polyphenol chemotypes. While *Parmelia cetrata* and *Parmelia dilatata* showed moderate antibacterial activity against gram negative, *Pseudomonsa aeruginosa*; *Cladonia scabriuscula* and *Physcia* cf. *millegrana* exhibited significant anticancer properties, especially against breast cancer cell- MCF7, colon cancer cell -Widr and cervical cancer cell- Hela. Since four of these lichens, especially *Physcia* cf. *millegrana,* possess interesting phytochemicals and biological activities, future work to isolate their bioactive compounds would be worthwhile**.**

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