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Original Research Article

Effect of Light Exposure on Secondary Metabolite Production and Bioactivities of *Syncephalastrum racemosum* Endophyte

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ABSTRACT

Endophytes have drawn the attention of researchers regarding their abilities to produce novel and bioactive compounds having medicinal values. Some studies reported that light could affect fungal secondary metabolite production. This study examined the effect of light exposure to metabolite profiles of an endophytic fungus isolated from the stem of *Coleus amboinicus* and its consequences on bioactivity. The endophytic fungi identified as *Syncephalastrum racemosum* was isolated from the stem of *C. amboinicus* and was cultured in Potato Dextrose Broth for two weeks under dark and light conditions. The metabolite profiles were examined using TLC analysis and gas chromatography. The conducted bioactivity testing included antibacterial, cytotoxic and DPPH antioxidant assays. In this study, two compounds namely methyl hexadecanoate and methyl (*Z*)-octadec-9-enoate were present in both *C. amboinicus* stem and *S. racemosum* extracts under dark condition. These substituents were diminished when the fungus was cultured and subjected to the light. Additional compounds were observed upon light exposure and this affected bioactivity. Antioxidant activity of extract exposed to light was better than that of in the dark condition (41.84 ± 0.38 vs 57.19 ± 0.88 µg/mL). A similar trend was seen in antibacterial activity, although the differences were insignificant. On the contrary, the extract from dark condition was found to be more cytotoxic against T47D cells compared to that of the one exposed to light, with IC_{50} value of 420.06 ± 12.98 and >500 µg/mL, respectively.

***Keywords*:** *Syncephalastrum racemosum, Coleus amboinicus*, Light, Bioactivity, Secondary Metabolites

Introduction

Medicinal plants have been explored for their potential of producing valuable compounds such as fragrances, colorants, flavours and bioactive substances with medicinal values i.e. anticancer, antimicrobial and antioxidant activities.[1,2] Artemisinin for example, is well known antimalarial agent produced by *Artemisia annua*.[3] Taxol, which is widely used as anticancer agent, was originally isolated from the bark of Pacific yew trees *Taxus brevifolia*.[4] Carvacrol which has antimicrobial activities, was reported to be a major component of *Plectranthus amboinicus* essential oil.[5] However, the low concentration in plant and the necessities of providing wide land for cultivation hinder crop-based manufacturing to obtain these medicinal compounds.[6] It was reported that essential oil production by *P. amboinicus* through hydrodistillation yielded only about 0.58 ± 0.03 mL/100 g dry weight of leaves.[7] Additionally, production of secondary metabolites from plant materials is also affected by geographical and environmental conditions, plant genetics and physiology as well as climate.[8] The ability of microbes in synthesising plant secondary metabolites drawn attention of many researchers as an alternative method.

The finding of endophytes capable of manufacturing secondary metabolites similar to that produced by the host plants provides strategies in overcoming obstacles faced by traditional harvesting techniques.

Many endophytes could produce secondary metabolites that were present in host plants. Endophytic fungi isolated from *A. annua* was capable of manufacturing artemisinin.[9,10] Taxol was also produced by an endophytic fungus *Paraconiothyrium* SSM001 spp., the one isolated from *Taxus x* media.[11] The production, however, was lost when the fungus was exposed by white light. This study suggested that the endophytic fungi need to grow in dark habitat to maintain its biosynthetic pathway.[11]

Previous study reported the potential of endophytic fungi isolated from *Coleus amboinicus* to produce anticancer and antimicrobial agents.[12,13] It is important to study whether the bioactivities were sustained after subculturing. It has been reported that fungal original dark habitat affected secondary metabolites production such as the case of taxol production by endophytic fungus *Paraconiothyrium* SSM001 spp.[11] This study, therefore, was aimed to examine the effect of light exposure to the secondary metabolites production by an endophytic fungi *S. racemosum* isolated from the stem of *C. amboinicus* and whether this condition influenced the bioactivities.

Materials and Methods

Materials

The reagents used in this study include Silica gel F_{254}, chloroform, ethyl acetate, methanol, were purchased from Merck (Darmstadt, Germany). Media for routine fungal culture Potato Dextrose Agar

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(PDA) and bacterial culture Nutrient Agar (NA) as well as for fermentation and antibacterial studies: Potato Dextrose Broth (PDB), Nutrient Broth (NB) and Mueller Hinton were purchased from Oxoid (Basingstoke, UK). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), quercetin dihydrate and tris-HCl were purchased from Sigma–Aldrich (Saint Louis, USA).

Fungal isolation, identification, and routine maintenance

The endophytic fungus was isolated from the stem of *C. amboinicus* collected from Faculty of Pharmacy Herbal Garden UGM. The herbarium was stored for future reference under reference number 014745. The identification of fungus was performed using fungal internal transcribed spacer regions (ITS) sequence of ribosomal DNA as previously described.[12] Phenotype and morphological characteristic of the fungi were analysed visually, and microscopic identification was conducted using an Olympus BX53 light microscope according to guidelines and monographs.[14-20] The fungi were maintained in PDA plates at 25° C and glycerol stock were made from two-week fermentation culture in PDB.

Effect of light on secondary metabolites production

Four plugs of 7 days old endophytic fungi grown on PDA agar were transferred into 500 mL Erlenmeyer containing 200 mL PDB medium. The cultures were incubated in the presence (left uncovered) and absent (covered) of natural light, agitated at 160 rpm, 25℃ for 2 weeks. The culture was filtered, and the filtrate was extracted by a liquid-liquid partition using ethyl acetate. Ethyl acetate layer was separated from fermentation broth and then evaporated until dried.

Phytochemical analysis

The ethyl acetate extract of supernatant was subject to thin layer chromatography (TLC) and GC-MS analysis. The extract was dissolved in methanol : chloroform (1:1), and spotted onto TLC plate (Silica gel 60 F_{254} (Merck)), developed in chloroform: ethyl acetate (9:1 v/v). The chromatograms were detected by visible light, UV_{254}, UV_{366}, and visualized with anisaldehyde-sulphuric acid reagent. Chemical characterization of volatile compounds from *C. amboinicus* stem, ethyl acetate extract of *S. racemosum* in dark and light conditions were analysed using GC-MS. The analysis was carried out using Gas Chromatography-Mass Spectrometry-QP2010 system (Shimadzu, Tokyo Japan) and capillary column cross bond 100% dimethylpolysiloxane (30 m x 0.25mm ID and film thickness 250 µm). Helium used as gas carrier, with a flow rate of 1 mL/min. Ion source temperature was 200°C, injector temperature was 250°C, and column temperature was set 100 – 300°C, increased 10°C/min. Column pressure was set of 100 kPa and column flow 1.33 mL/minute. A 2 mg sample was dissolved in methanol: chloroform (1:1 v/v), and 0.5 µl was injected into the GC-MS. The electron ionization (EI) was the ionization mode set at 70 eV. Identification of compounds was based on their retention time and compared to mass spectra library data WILEY7.LIB with highest similarity index.

DPPH antioxidant assay

Radical scavenging activity was conducted using DPPH assay as described by de Torre et al. (2019) with slight modification.[21] Briefly, 1 mL various concentration of ethyl acetate extract ranging from 250, 125, 62.5, 31.25, 15.63 µg/mL dissolved in methanol were mixed with 1 mL DPPH 0.04 mg/mL in methanol. The reaction was performed for 30 min at dark condition, then the sample was ready to be measured at 517 nm with quercetin as positive control.

The formula of % inhibition was used for radical scavenging activity calculation:

$$\text{Inhibition (\%)} = 1 - \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control} - \text{Abs blank}} \times 100\%$$

Where, Abs sample = absorbance of sample/positive control and DPPH solution

Abs blank = absorbance of sample (in serial concentration) and DPPH solution

Abs control = absorbance of methanol and DPPH solution.

IC_{50} value was concentration in which free radical DPPH was reduced by 50% and calculated based on sample concentration plotted against % inhibition in each concentration point.

Cytotoxicity assay

Cytotoxic study was conducted using MTT assay against various human cancer cell lines T47D, MCF7, WiDr and HeLa cells.[22] Vero cells were used as normal cell model. The RPMI (for T47D, WiDr and Hela cells) and DMEM media (for MCF7, Vero cells) were used to maintain the growth of cells at 37°C, added with 10% FBS, 1% penicillin-streptomycin and 1% fungizone, supplied with 5% CO_2. A hundred µL of medium containing $5x10^3$ cells were transferred into 96-well plate and incubated for 24 h to reach 70% – 80% confluent. The medium was discarded and substituted with new medium containing serial concentrations of extracts dissolved in DMSO (final concentration 500 – 31.25 µg/mL). Doxorubicin was used as standard control with a serial concentration (0.25 - 8 µg/mL), medium only (without cells) were included as blank control. After 24 h of incubation, the wells were gently washed with 1x warm PBS and 100 µl of medium containing MTT (0.5 mg/ml) was transferred into the well, and further incubated about 4 h at 37°C. Each well added 100µL solution of 10% SDS-0.01 N HCl, following incubation in the dark, overnight. Finally, the absorbance of each well was measured in a microplate reader (Biorad) at 595 nm. The absorbance (Abs) was converted to % cell viability as follows:

$$\text{Percentage of cell viability} = \frac{\text{Abs of treated cells} - \text{Abs of medium}}{\text{Abs of control untreated cells} - \text{Abs of medium}} \times 100$$

IC_{50} was calculated by plotting concentration vs percent cell viability which determine cell growth inhibition by 50%.

Antibacterial assay

Antibacterial activity was determined using modified microdilution method.[23,24] *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were used as model for positive and negative gram bacteria, respectively. Bacteria grown at the densities comparable to 0.5 McFarland were diluted 10 times using Mueller Hinton medium. The sample dissolved in DMSO were serially diluted using medium. The sample solution was added to the media in 96 well plate containing bacteria to obtain final concentration from 250 – 31.25 µg/mL. Streptomycin was used as standard antibacterial agent. Untreated bacterial growth was used as control, solvent and media were included in testing plate and functioned as blank. Following incubation at 37°C overnight and each well were measured at 595 nm using a microplate reader (Bio-rad). The result showed % viability of bacterial growth for each serial concentration of sample.

Statistical analysis

Data were analysed statistically using One Way ANOVA test or Kruskal Wallis test (SPSS programme version 22, depend on normality value, and independent sample T test. Value of $p < 0.05$ was considered different significantly with 95% confidence level. The experiments were conducted triplicate and expressed as mean ± SD. Dose-response curves were plotted and calculated the IC_{50} values using linear regression analysis.

Results and Discussion

Characterisation of the fungi

An endophytic fungus was isolated from the stem of *C. amboinicus*. Based on molecular identification using Internal Transcribed Spacer (ITS) ribosomal fungal DNA, the fungus showed 100% homology with *Syncephalastrum racemosum* strain CBS.441.59 (Accession number: MH857910). Macroscopically the fungus grown on PDA appeared as irregular white colony, filamentous and undulate edge with raised elevation (Figure 1). Microscopically, the colony was transparent (hyaline), hypae was septate at 3-5 µm wide. The conidiophore was with conidial head covered by merosporangium. Round conidia at 3-5 µm were arranged as chain within

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merosporangium (Figure 1). *S. racemosum* had been reportedly colonised within healthy tissue of some plants such as *Markhamia tomentosa*, *Saraca asoca*[25,26] and seaweed.[27]

Effect of light exposure to metabolite production

An attempt to examine the effect of light exposure to the production of secondary metabolites was conducted. Some studies reported that light could affect the secondary metabolites of fungi.[11,28] In this study, fungal culture of *S. racemosum* fermented for two weeks in the presence and absence of light were extracted using ethyl acetate and the extracts were subjected to TLC and GC-MS. Based on TLC analysis there were differences in metabolite profiles between *S. racemosum* extract fermented under dark and light condition as detected using UV_{254}, UV_{366} light and anisaldehyde sulphuric acid detection reagent (Figure 2). More chemical compounds were seen under dark fermentation condition. Terpenoids were detected by showing red after visualization with anisaldehyde – sulfuric acid and heated at 105°C. Based on GC-MS analysis, there were two compounds which were present both in plant stem extract and *C. racemosum* extract, namely methyl hexadecanoate and methyl (*Z*)-octadec-9-enoate (Tables 1 and 2). When the *S. racemosum* was fermented under exposure to light, these compounds were diminished (Figure 3, Table 3). Instead, additional compounds were detected at longer retention time (Figure 3). The similarity of some compounds in *S. racemosum* fermented under dark condition with those in the plant stem extract indicated that those compounds may share biosynthetic pathway which was conserved under dark condition. The fact that the compounds were disappeared under exposure to light suggested that light may regulate the production of these compounds. As reported by Kim et al. (2013), secondary metabolism and fungal development may light-dependent processes, subjected to the requirement of light for biological activity.[28]

Bioactivities

Further study was conducted to examine whether the exposure to the light also affected its biological activities. DPPH scavenging activity was performed since *C. amboinicus* was reported to have antioxidant activities.[29-31] The ethyl acetate extract of *S. racemosum* grown exposed to the light exhibited better antioxidant activity with IC_{50} of 41.84 ± 0.38 µg/mL (Table 4). The IC_{50} value was higher compared to the culture of dark condition (IC_{50} 57.19 ± 0.88 µg/mL). These data were presented as mean ± standard deviation, and each concentration of the tested extract were performed triplicates. Significant differences between means were determined and p values < 0.05 were regarded as significant. As examined from GC analysis 2-hydroxy-3-phenylpropanoic acid was the highest concentration in the ethyl acetate extract of *S. racemosum* under light condition reaching 21.3% (Table 3). However, this compound was reported inactive for DPPH radical scavenging activity,[32] instead octadecanoic acid (5.28%) was reported to function as defences against oxidative stress in cortical neurons.[33]

Considering the potency of ethyl acetate extract of *S. racemosum* as antioxidant, it is of interest to study its potency as anti-cancer agent. Preliminary in vitro cytotoxicity screening using several cancer cell lines showed that at, the highest concentration used in this study (500 µg/mL), the extracts both derived from light and dark fermentation culture did not reach 50% of growth inhibition (Table 5). Only extract at dark condition tested against T47D cells showed IC_{50} of 420.06 ± 12.98 µg/mL. IC_{50} value of this data was presented as mean ± standard deviation, and each concentration of the tested extract were performed triplicates. The level of cytotoxicity was considered moderate according to Ads et al. (2020).[34] Two major compounds as analysed by GC were present in that extract, namely methyl (*Z*)-octadec-9-enoate (21.55%) and methyl (9Z,12Z)-octadeca-9,12-dienoate (20.34%). According to previous studies, methyl (Z)-octadec-9-enoate had anticancer activity against MCF-7 and HT-29 with IC_{50} values of 252 and 266 µg/mL, respectively.[35] Meanwhile, octadecanoic acid, methyl hexadecanoate, hexadecanoic acid, and octadec-9-enoic acid were believed to have potential as anticancer.[36] Based on this study, the anticancer activities could be contributed from the detected chemical compounds in the extracts derived from dark fermentation as analyzed by GC-MS.

Searching other potency as antibacterial agent was also conducted by testing the extracts against *S. aureus* as gram-positive bacteria and *E. coli* as gram-negative bacteria. Similar with antioxidant potency, extract from fermentation under light condition showed better activity against *S. aureus* compared to dark condition, although only slight differences in viability were observed (at the concentration of 250, 125 and 31.25 µg/mL). Similar trend was seen against *E. coli*, but with less differences (only at the concentration 250 and 125 µg/mL) (Table 6).

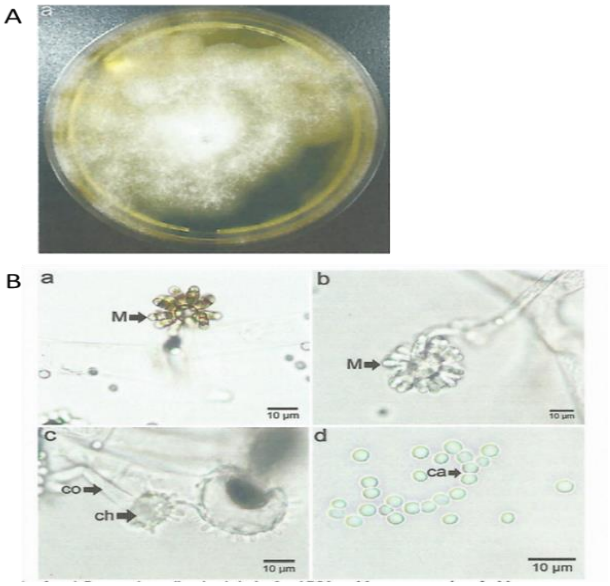

Figure 1: Endophytic fungus *Syncephalastrum racemosum*on in PDA medium (A). Microscope observation (B). M = merospongarium, co = conidiophore, ch = conidial head, ca = conidia.

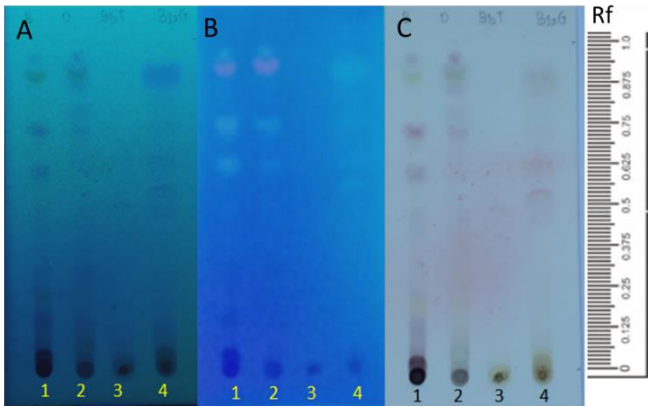

Figure 2: TLC profiles of ethyl acetate extract of fermentation broth of *S. racemosum* detected using UV_{254} light **(A)** UV_{366} light **(B)** anisaldehyde-sulphuric acid reagent **(C)** Stationary phase = Silica Gel 60 F_{254}; mobile phase = chloroform : ethyl acetate (9:1 v/v). 1. Leaves extract of *C. amboinicus*. 2. Stem extract of *C. amboinicus*. 3. Ethyl acetate extract of *S. racemosum* exposed to light. 4. Ethyl acetate extract of *S. racemosum* in dark condition.

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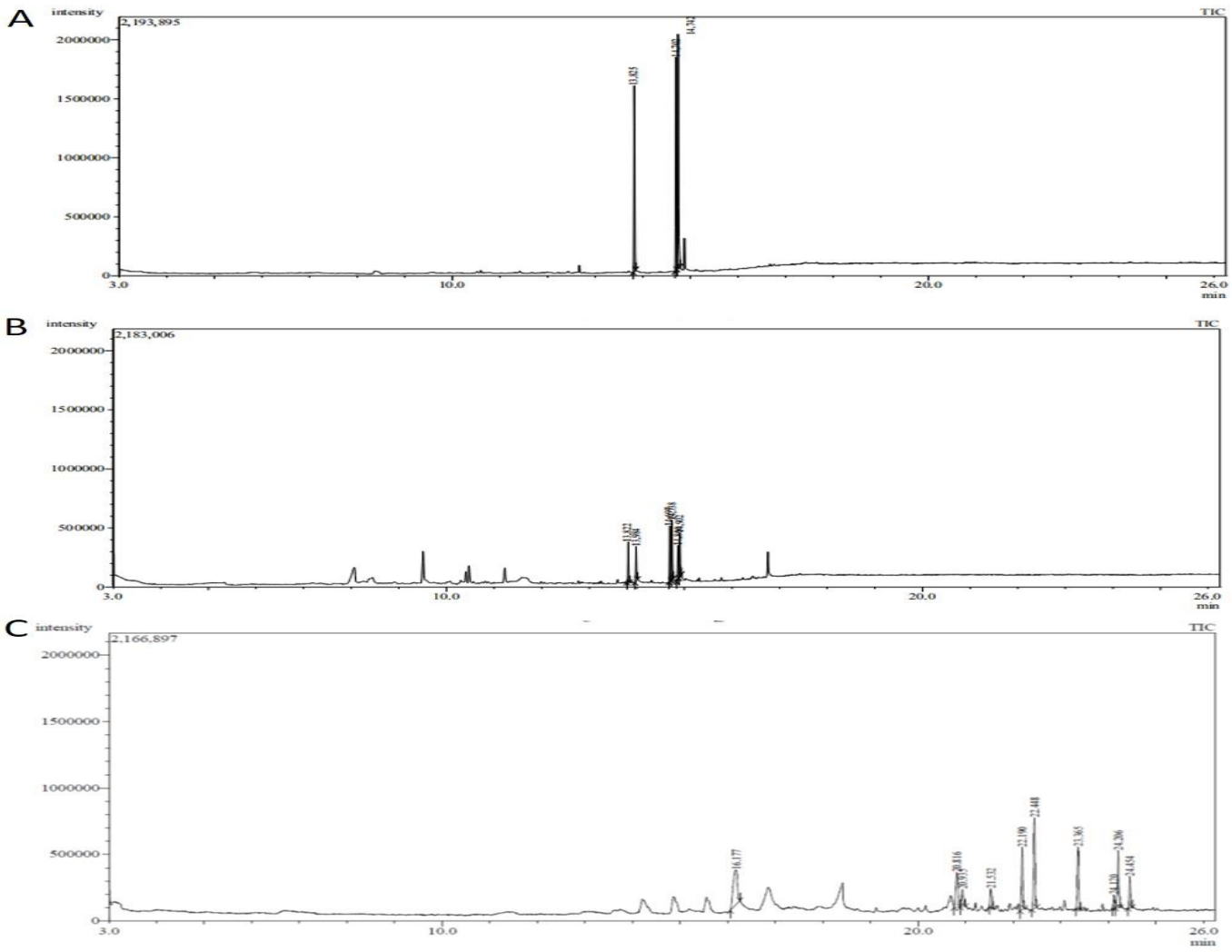

Figure 3: Gas Chromatography-MS profiles of *C. amboinicus* stem ethyl acetate extract **(A)**. *S. racemosum* fermented under dark condition **(B)**. *S. racemosum* fermented under exposure to light **(C)**.

Table 1: The Chemical Constituents of Ethyl Acetate Extract of *C. amboinicus* stem analysed by GC-MS

No	R. time (min)	Area (%)	Structure Name	Molecular Formula	Similarity Index (%)
1.	13.825	26.03	Methyl hexadecanoate	$C_{17}H_{34}O_2$	98
2.	14.702	33.24	Methyl (9*Z*,12*Z*)-octadeca-9,12-dienoate	$C_{19}H_{34}O_2$	96
3.	14.742	40.74	Methyl (*Z*)-octadec-9-enoate	$C_{19}H_{36}O_2$	98

Table 2: The Chemical Constituents of Ethyl Acetate Extract of *S. racemosum* fermented under dark condition by GC-MS

No	R. time (min)	Area%	Structure Name	Molecular Formula	Similarity Index (%)
1.	13.822	13.57	Methyl hexadecanoate	$C_{17}H_{34}O_2$	96
2.	13.984	12.89	Hexadecanoic acid	$C_{16}H_{32}O_2$	93
3.	14.697	20.34	Methyl (9*Z*,12*Z*)-octadeca-9,12-dienoate	$C_{19}H_{34}O_2$	95
4.	14.738	21.55	Methyl (*Z*)-octadec-9-enoate	$C_{19}H_{36}O_2$	97
5.	14.866	14.23	Methyl (11*E*,13*E*)-icosa-11,13-dienoate	$C_{21}H_{38}O_2$	88
6.	14.902	17.43	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	93

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Table 3: The Chemical Constituents of Ethyl Acetate Extract of *S. racemosum* fermented under exposure to light by GC-MS

No	R. time (min)	Area%	Structure Name	Molecular Formula	Similarity Index (%)
1.	16.177	21.3	2-hydroxy-3-phenylpropanoic acid	$C_9H_{10}O_3$	94
2.	20.816	10.99	2-ethyl-4-methyl-4,6-di(propan-2-yl)-1,3,2-dioxaborinine	$C_{12}H_{23}BO_2$	74
3.	20.935	4.92	Ethanone, 1-(2,6-dihydroxy-4-methoxy phenyl)	$C_{11}H_{14}O_4$	67
4.	21.532	4.27	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo [4.3.0] nonane	$C_{11}H_{18}N_2O_2$	89
5.	22.190	11.51	2,6-di*tert*-butylbenzene-1,4-diol	$C_{14}H_{22}O_2$	72
6.	22.448	16.30	Hexadecanoic acid	$C_{16}H_{32}O_2$	94
7.	23.365	11.57	2-methyl-5-(2,6,6-trimethyl cyclohexen-1-yl) pentane-2,3-diol	$C_{15}H_{28}O_2$	73
8.	24.120	2.25	Methyl (9*Z*,12*Z*)-octadeca-9,12-dienoate	$C_{19}H_{34}O_2$	92
9.	24.206	11.88	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	96
10.	24.454	5.28	Octadecanoic acid	$C_{18}H_{36}O_2$	93

Table 4: DPPH scavenging activity of *S. racemosum* ethyl acetate extract under dark and light conditions, expressed as IC_{50} values

Samples	IC_{50} + SD (µg/mL)
Ethyl acetate extract from fermentation broth under dark condition	57.19 ± 0.88*
Ethyl acetate extract from fermentation broth exposed to light	41.84 ± 0.38*
Quercetin	1.29 ± 0.01*

All values are expressed as mean ± standard deviation (n = 3). *p values < 0.05 showed significant differences between means.

Table 5: Cytotoxicity of *S. racemosum* ethyl acetate extract under dark and light conditions, expressed as IC_{50} against several cancer cell lines

	IC_{50} + SD (µg/mL)		
Cells	Ethyl acetate extract, dark condition	Ethyl acetate extract, light condition	Doxorubicin
Hela	> 500	> 500	4.30 ± 0.13
T47D	420.06 ± 12.98	> 500	3.68 ± 0.13
WiDr	> 500	> 500	2.67 ± 0.06
MCF-7	> 500	> 500	3.18 ± 0.11
Vero	> 500	> 500	7.74 ± 0.22

All values are expressed as mean ± standard deviation (n = 3)

Table 6: Antibacterial activity of *S. racemosum* ethyl acetate extract under dark and light conditions

		% Viability + SD		
Bacteria	Concentration (µg/mL)	Ethyl acetate extract, dark condition	Ethyl acetate extract, light condition	Streptomycin control at 250 µg/mL
Staphylococcus aureus	250	119.35 ± 1.90*, a	109.25 ± 3.59*, b	2.54 ± 0.49
	125	108.41 ± 3.53*, a	95.84 ± 2.36*, b	
	62.5	98.12 ± 5.17a	95.50 ± 3.18*, a	
	31.25	94.61 ± 1.90*, a	85.50 ± 2.69*, b	
	15.63	87.92 ± 1.43*, a	83.72 ± 6.80*, a	
Control bacteria	0.00	100 ± 7.21a	100 ± 2.12a	
Escherichia coli	250	83.21 ± 0.44*, a	76.16 ± 2.11*, b	0.00 ± 0.00
	125	90.53 ± 1.09*, a	85.87 ± 1.67*, b	
	62.5	93.05 ± 1.12*, a	91.96 ± 0.56*, a	
	31.25	94.30 ± 0.64*, a	94.41± 0.38*, a	
	15.63	96.75 ± 1.02*, a	96.03 ± 2.21*, a	
Control bacteria	0.00	100 ± 2.43a	100 ± 0.96a	

All values are expressed as mean ± standard deviation (n=3). *p < 0.05 showed statistically significant different in comparison to control untreated bacteria (n = 3). Different superscript alphabets in the same row indicated statistically significant different between treatment (p < 0.05).

It is observed that the antibacterial activity of extract form dark and light condition is not different significantly at all concentration. Meanwhile, it was found that the viability percentage of bacteria from extract in each concentration compared to control (untreated bacteria) was significantly different ($p < 0.05$), except at the concentration 62.5 µg/mL of extract in dark condition against *S. aureus*. The activity of 2-hydroxy-3-phenylpropanoic acid, the major compound with ethyl acetate extract of *S. racemosum* under light condition, as antibacterial agent had been reported in some studies. This compound was known as a broad-spectrum antimicrobial agent produced by lactic acid bacteria and active against bacteria and fungi.[37-39] The second highest content of the extract, hexadecenoic acid (16.30%), was also reported to possess antibacterial properties.[40] Previous studies also reported anti-bacterial activity from the 1, 4-diaza-2, 5-dioxo-3-isobutyl bicyclo [4.3.0] nonane compound.[41,42]

Conclusion

In this study, light exposure determined the secondary metabolite production of *S. racemosum*. The differences in metabolite contents influenced its bioactivity. This study showed new finding that *S. racemosum* fermentation condition affected metabolite profiles as well as its bioactivity.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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