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METABOLITE PROFILING AND BIOLOGICAL ACTIVITIES OF EXTRACTS FROM ENDOPHYTIC FUNGI ISOLATED FROM MANGROVE PLANT *Avicennia lanata*

(Profil Metabolit dan Aktiviti Biologi Ekstrak daripada Kulat Endofitik Dipencilkan daripada Tumbuhan Bakau *Avicennia lanata*)

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Abstract

Endophytic fungi are important in drug discovery for their unique secondary metabolites, besides the extract obtained are sustainable, reproducible and culturable under laboratory conditions as compared to their host plants. However, the use of single culture leads to the production of known compounds with similar bioactivities. Therefore, single culture and co-culture between *Fusarium* sp. and *Curvularia* sp. which have been isolated from mangrove plant *Avicennia lanata* were cultured on malt agar and rice media on 7, 15 and 30 days to trigger the production of metabolites that may absent in their single cultures. In this study, dereplication study was applied to speed up the identification of secondary metabolites in the extracts. Then, the data was processed utilising MZmine 2.40.1 and SIMCA P+ 15.0 software coupled with macro analysis and Dictionary of Natural Product (DNP) database for dereplication studies. The antioxidant activity of the extracts were evaluated by using DPPH scavenging assay. Amongst the extract obtained from malt agar medium, the extract of co-culture on day 7 (MAFC7) showed the most potent activity with IC_{50} value of 0.6 mg/mL. For the rice medium, the extract from single culture *Curvularia* sp. on day 15 showed the most significant activity with IC_{50} value of 1.1 mg/mL. While, the agar disk-diffusion method was used to evaluate the antibacterial activity of the extracts and the results indicated that the extracts exhibited activity against Gram-positive bacteria, specifically *Micrococcus* sp., *Staphylococcus aureus*, and *Bacillus cereus*. The antibacterial activity of the co-culture extracts were the most active with minimum inhibition concentration (MIC) values of 0.256 mg/mL – 5.0 mg/mL. In conclusion, the extracts obtained from co-culture showed potent antioxidant and antibacterial activities that could be further scaled-up and investigated for future pharmacological properties.

Keywords: *Fusarium* sp., *Curvularia* sp., co-culture, antioxidant, antibacterial

Abstrak

Kulat endofit adalah penting dalam penemuan ubat untuk metabolit sekunder yang unik, selain itu ekstrak yang diperolehi adalah mampan, boleh dihasilkan semula dan dikultur di bawah keadaan makmal berbanding dengan tumbuhan perumah. Walau

bagaimanapun, penggunaan kultur tunggal kebiasaannya membawa kepada penghasilan sebatian yang diketahui dengan bioaktiviti yang sama. Oleh itu, kultur tunggal dan kultur bersama antara *Fusarium* sp. dan *Curvularia* sp. yang telah dipencilkan daripada tumbuhan bakau *Avicennia lanata* telah dikulturkan pada agar malt dan media beras pada hari ke-7, 15 dan 30 hari untuk mencetuskan penghasilan metabolit yang berbeza yang mungkin tidak hadir dalam kultur tunggal mereka. Dalam kajian ini, kajian dereplikasi telah digunakan untuk mempercepatkan pengenalpastian metabolit sekunder dalam ekstrak. Kemudian, data telah dianalisis menggunakan perisian MZmine 2.40.1 dan SIMCA P+ 15.0 beserta analisis makro dan pengkalan data Kamus Bahan Semulajadi (DNP) bagi kajian dereplikasi. Aktiviti antioksidan bagi ekstrak telah dinilai menggunakan asai skaveng DPPH. Antara ekstrak yang diperolehi daripada medium agar malt, ekstrak kultur bersama pada hari ke-7 (MAFC7) menunjukkan aktiviti yang berpotensi dengan nilai IC_{50} sebanyak 0.6 mg/mL. Bagi medium beras, ekstrak daripada kultur tunggal *Curvularia* sp. pada hari ke-15 menunjukkan aktiviti yang paling ketara dengan nilai IC_{50} sebanyak 1.1 mg/mL. Sementara itu, kaedah penyerapan cakera agar telah digunakan untuk menilai aktiviti antibakteria ekstrak dan keputusan menunjukkan bahawa ekstrak menunjukkan aktiviti melawan bakteria Gram-positif termasuk *Micrococcus* sp., *Staphylococcus aureus* dan *Bacillus cereus*. Aktiviti antibakteria bagi ekstrak kultur bersama adalah yang paling aktif dengan nilai kepekatan rencat minima (MIC) pada 0.256 mg/mL– 5.0 mg/mL. Kesimpulannya, ekstrak yang diperolehi daripada kultur bersama menunjukkan aktiviti antioksidan dan antibakteria yang bagus yang boleh dilanjutkan untuk skala besar dan disiasat untuk sifat farmakologi masa hadapan.

Kata kunci: *Fusarium* sp., *Curvularia* sp., kultur bersama, antioksidan, antibakteria

Introduction

Natural products are molecules derived from plants, microorganisms or animals that naturally produce primary and secondary metabolites which continuing provide varities bioactive metabolites. Overall, more than 70% of antibacterial and anticancer compounds are natural products origin [1]. Endophytic fungi are the organism live commonly within most plant species' tissues. The endophyte occupies host tissues without causing any diseases and found mostly in terrestrial plants [2]. Endophytic fungi play an important role in plant habitat adaptation, enhancing plant production and defending plant against biotic and abiotic stress [3]. In the past few years, the metabolites isolated from the endophytic fungi including steroids, alkaloids, anthraquinones, terpenes, flavonoids and cyclic peptides that are known for their biological activities, such as antibacterial, antitumour, antiviral, antifungal and anti-inflammatory [4]. In this study, endophytic fungi *Fusarium* sp. and *Curvularia* sp. have been isolated from the mangrove plant *Avicennia lanata*. *Fusarium* genus is recognised as one of the major sources of secondary metabolites with a variety of biological activities, including antioxidant activity [5]. *Fusarium* sp. has produced a variety of secondary metabolites, including peptides, alkaloids, terpenoids, amides, pyranones, and quinones with pharmacological potential [6]. Meanwhile, *Curvularia* sp. is known as pathogen associated with humans and plants. Some of *Curvularia* species are economically important pathogens worldwide, while other species are found in combination with different hosts as pathogens or epiphytes [7]. Many biologically active secondary metabolites were previously reported from *Curvularia*, for example, 11-α-methoxycurvularin, curvularin, cytochalasin B, dehydrocurvularin, radicinol and radicinin [8]. Endophytic fungi *Curvularia* sp. also produce a variety of bioactive secondary metabolites including polyketides and steroids [9]. However, the single fungi reproduce the same compounds and similar biological activities. Therefore, co-culture has been a useful research method which effectively triggers the new metabolites that are silent in single culture.

A co-cultivation is a method to challenge two or more microorganisms by imitating the actual natural microbial community competition that is carried out in a laboratory scale, becomes a successful strategy to produce novel bioactive natural products [10]. The co-culture between endophytic fungi may trigger the production of new secondary metabolites that are silent under normal condition. Production of bioactive secondary metabolites during co-culture may be related to various mechanisms, including competition for space and nutrients in which the parameter is the most important for microbial growth and survival in a competitive environment [11, 12]. Marine-derived microorganisms such as bacteria and fungi proved to be prolific sources of novel bioactive compounds which are not only considerable interest as a new drug for the

treatment of cancer, but also for other malignancies such as chronic pain [13]. Conventional method only focused on major compounds due to diversity of metabolites, thus it is challenging especially in data interpretation. Therefore, modern technologies and techniques have been developed in natural product discovery to produce a great outcome in research.

Metabolomics study is considered as a modern technique that is developed for systematic procedures to explore various mechanism as well as detecting metabolites changes in specific organism [14]. The presence of metabolites in microbes can easily be measured and compared via metabolomics. Metabolomics is used to fast track the identification of metabolites in a mixture of extract prior to isolation work to avoid repetitive works. The metabolomic study uses multivariate analysis to classify mass data collection and groups by monitoring their distribution accordingly with subjected parameter. Therefore, a clear overview of metabolites in the extract can be detected by liquid chromatography-mass spectrometry (LC-MS) coupled with multivariate analysis such as principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) [15]. In this study, the extracts from a co-culture between *Fusarium* sp. and *Curvularia* sp. was carried out to provide a better understanding on the metabolites as compared to the metabolites in their independent cultures.

Materials and Methods

Preparation of fungal strain

Stock cultures of endophytic fungi *Fusarium* sp. and *Curvularia* sp. isolated from the root of *Avicennia lanata* were obtained from Fungus Laboratory, Centre of Research and Field Service, Universiti Malaysia Terengganu (UMT). The single colony of both endophytic fungi were inoculated on malt agar plate independently and incubated in an incubator at 28 ± 1 ºC for 7 – 15 days for further work.

Inoculation of fungal on malt agar and rice media

Malt agar medium consists of 10.0 g of agar (Oxoid, Malaysia) and 7.5 g of malt extract (Oxoid, Malaysia) and were transferred into 500 mL of Schott Duran bottle. 500 mL of milli-Q water was added into the bottle and autoclaved. A 25 mL of warm agar medium was poured into labelled agar plates, left to cool and stored inverted at 4 °C. The fresh malt agar containing actively growing culture of *Fusarium* sp. and *Curvularia* sp. were cut into 5 x 5 mm cubes, placed on malt agar plates for single and co-culture and incubated at 28 ± 1 °C for 7, 15 and 30 days. The growth of mycelia of *Fusarium* sp. and *Curvularia* sp. in single and co-culture agar plates were monitored daily based on the shape and colour. Once the incubation period was completed, the agar plates containing the actively growing mycelia of single and co-culture *Fusarium* sp. and *Curvularia* sp. were cut and transferred into conical flasks, then 50 mL of ethyl acetate (Fisher Scientific, Malaysia) was added, sonicated for 1 h and filtered into round bottom flasks by using cotton wool (50 mL/extraction, three times). The organic phase was concentrated under vacuum by using a rotary evaporator (Buchi, Switzerland) to give an extract. The extracts were kept at 4 ºC prior to further analysis.

Meanwhile, cultivation of single and co-culture between the fungi in sterile rice medium was also carried out for up-scaling. 15 g of rice and 15 mL of milli-Q water were added into 250 mL of conical flasks. The conical flasks were covered with cotton wool and aluminium foil and kept overnight before autoclaved. The flasks were left to cool. A fresh malt agar plate containing actively growing culture of *Fusarium* sp. and *Curvularia* sp. were cut and placed on the rice medium for single and co-culture and incubated at room temperature for 7, 15 and 30 days. Once the incubation period was completed, 100 mL of ethyl acetate (Fisher Scientific, Malaysia) was added into each flask, sonicated for 1 h and filtered into round bottom flasks by using cotton wool (100 mL/extraction, three times). The organic phase was concentrated under vacuum by using a rotary evaporator (Buchi, Switzerland). The extracts were kept at 4 ºC prior to further analysis [16].

Antioxidant activity

The antioxidant activity of extracts from single and co-cultures were evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Malaysia) *in vitro* assay. The free radical scavenging activity of each sample was determined by measuring the changes of

absorbance colour from violet of DPPH into yellow by using microplate spectrophotometer (Thermo, United States) at 520 nm [18]. 50% of inhibition concentration (IC_{50}) value was defined as the concentration of the sample that scavenged 50% of DPPH radical.

5 mg/mL of extracts obtained from malt agar culture and 10 mg/mL of extract from rice culture were dissolved in dimethyl sulfoxide (DMSO) with 2-fold serial dilution method to give eight concentration values of 0 mg/mL to 5 mg/mL and 0 mg/mL to 10 mg/mL, respectively. Quercetin was used as a positive control and DMSO was used as the negative control. 4.73 mg of DPPH powder was dissolved in 200 mL of methanol (Merck, Germany), transferred into 500 mL of Schott Duran bottle and covered with aluminium foil. 20 µL of each concentration was pipetted into respective wells of the 96-well plate. 200 µL of DPPH solution was then added to each well containing extract and incubated for 30 min in dark at room temperature. The absorbance was measured by using a microplate spectrophotometer (Thermo, United States) at 520 nm. All determinations were performed in triplicate to obtain a mean value and the radical scavenging activity was calculated by using the following formula:

$$\text{DPPH scavenging effect (\%)} = ((A_0 - A_1) / A_0) \times 100 \quad (1)$$

Where as A_0 = the absorbance of the control, and A_1 = the absorbance of the extract mixed by DPPH

The half percentage of inhibition samples concentration (IC_{50}) was determined from the linear regression curve.

Antibacterial activity

The antibacterial activity of extracts from single and co-cultures used the disk-diffusion method [19]. Three Gram-positive strains – *Micrococcus* sp., *Staphylococcus aureus*, and *Bacillus cereus*, and three Gram-negative bacteria – *Salmonella* sp., *Escherichia coli* and *Vibrio parahaemolyticus* were obtained from Institute of Marine Biotechnology, UMT. 28 g of nutrient agar (Oxoid, Malaysia) /1 L, 55.1 g of marine agar (Difco, Malaysia) /1 L and 35 g of Mueller-Hinton agar (Merck, Germany) /1 L of milli-Q, stirred until homogenised and autoclaved. Each extract was prepared with 2-fold dilution to give eight concentration values of 0 mg/mL to 10 mg/mL in DMSO. Then, 20 µL of extract was loaded onto Whatman paper AA disks (Whatman, China).

Each bacterium strain was streaked on nutrient and marine agar plates, incubated at 37 °C for 24 h. After 24 h, 2 mL of sterile milli-Q water was transferred into McFarland tube and bacteria strains were added. Optical density (OD) (BioMērieux, France) was adjusted at 0.5 MFU (McFarland Unit) and 100 µL of bacterial inoculum was uniformLy spread by using sterile cotton swab onto Mueller-Hinton agar plates. The Whatman paper AA disks (Whatman, China) containing samples were placed inverted onto agar plates (3 replicates) and incubated at 37 °C for 24 h. 30 µg oxytetracycline, 10 µg ampicillin, 10 unit penicillin and 10 µg gentamycin antibiotic disks (Oxoid, United State) were used as positive control, and DMSO as negative control. For initial screening, 10 mg/mL of extract was tested on the Gram-positive and Gram-negative bacteria strains in order to observe the presence of inhibition growth zone between the extracts and bacteria.

After 24 h of incubation, inhibition zone diameter (IZD) around the Whatman paper AA disks (Whatman, China) containing extract was observed. The IZD was measured and expressed in millimetre (mm), and minimum inhibitory concentration (MIC) was taken as the lowest concentration of the extracts that inhibited the visible growth of bacteria after overnight incubation.

HRESI-LCMS analysis and data processing

1 mg of each extract was dissolved in 1 mL of methanol prior to analysis by using Accela high performance liquid chromatography (HPLC) (Thermo Scientific, UK) coupled with a 280 nm and 360 nm UV detector and an Exactive-Orbitrap high-resolution mass spectrometer (Thermo Scientific, UK) including a methanol blank. The column size used was HiCrom, ACE (Berkshire, UK) C_{18}, of 75 mm x 3.0 mm, 5 µm.

The mobile phase used was micropore water (A) and acetonitrile (B) with 0.1% formic acid at a flow rate of 300 µL/min was applied linearly. The gradient program started at 10% B and linearly increased to 100% B within 30 min and remained isocratic for 5 min before linearly decreasing back to 10% B in 1 min. The column was then reequilibrate with 10% B for 9 min before the next injection. The total analysis time was 45 min for each sample. The injection volume was 10 µL with tray temperature maintained at 12 ºC. High-resolution mass spectroscopy was carried in both positive and negative ESI ionisation switch modes with a spray voltage of 4.5 kV. The capillary temperature was set at 320 ºC with mass range from *m/z* 150 – *m/z* 1500 for ESI-MS range. HRESI-LCMS was used to conduct the dereplication study on the extracts, then data processed with MZmine 2.40.1 software, an in-house macro coupled with the Dictionary of Natural Product (DNP) 2015 and SIMCA P+ 15.0 (Umetrics AB, Umeå, Sweden). The mass spectral data was processed by using the procedure by MacIntyre et al., which was established in the Natural Products Metabolomics Group Laboratory at Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS) as described here [16, 17]. The LC-MS chromatograms and spectra were viewed by using MZmine 2.40.1. Raw data were initially sliced into negative and positive data sets utilising the MassConvert software package from ProteoWizard (pwiz).

Results and Discussions

Extract yields of *Fusarium* sp. and *Curvularia* sp.

Daily monitoring of *Curvularia* sp. resulted in black colour of mycelia and grew all over the plate by day 9. Meanwhile, *Fusarium* sp. had yellow and slightly pink colour and occupied the whole plate by day 8. In a co-culture, *Fusarium* sp. ramified faster than *Curvularia* sp. as shown in Figure 1. Previous study reported that the fungi contained a variety of bioactive compounds with unique structures including benzopyranones, alkaloids, flavonoids, chinones, quinones, phenolic acids, terpenoids, steroids, xanthones, tetralones, and others. The bioactive metabolites are commonly used as antibiotics, agrochemicals, antiparasitics, immunosuppressants, anticancer and antioxidants agents [3]. The extract from single culture of *Fusarium* sp. 30 days (F30) from rice medium gave the highest extract yield with 204.76±12.6 mg (Table 1). Meanwhile, the extract yield from co-culture also increased from day 7 (FC7 = 151.23±17.4 mg) to 189 mg for day 15 and day 30 (FC15 & FC30 extracts). The use of sterile rice as an alternative substrate promoted a better amount of fungus mycelial that led to the production of higher yield of extract that could be used in pharmacological purposes and other applications as compared to inoculation on agar plates.

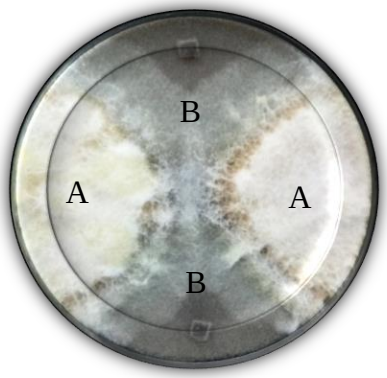

Figure 1. The co-culture between *Fusarium* sp. (A) and *Curvularia* sp. (B) on malt agar plate after 21 incubation days

Antioxidant activity

The scavenging activity of the extracts that were obtained from both media showed a significant activiy (Table 1). Extracts from malt agar plates showed a potent activity with IC_{50} values of 0.6 mg/mL – 2.4 mg/mL. While, the activity of extracts from single culture day 7 of *Fusarium* sp. and *Curvularia* sp. was also observed; MAF7 (IC_{50} = 1.7 mg/mL) and MAC7 (IC_{50} = 0.85 mg/mL), respectively. However, the extracts from agar culture that were incubated between day 15 and day 30 decreased in activity. Interestingly, the activity of extracts from co-culture grown on agar plates increased as compared to their single culture's extract. The co-culture between two or more fungi in a confined vessel promoted the competition of nutrients and space, thus triggering the production of metabolites that may responsible in increased activity. Therefore, a co-culture between *Fusarium* sp. and *Curvularia* sp. in a larger volume was introduced in this study. The use of rice as an alternative substrate gave essential nutrient for

the growth of fungi, besides it is more economic to produce better amount of extract that may have the same metabolites with similar biological activities to the extract that obtained from malt agar culture. Amongst the co-culture extracts, culture of FC15 grown on day 15 possessed in enhanced scavenging activity with IC_{50} value of 2.8 mg/mL as compared to FC7 (IC_{50} = 4.1 mg/mL) and FC30 (IC_{50} = 4.8 mg/mL). The endophytic fungi were reported possessing bioactive compounds including phenols, in which the hydroxyl groups have a potent antioxidant activity. The phenolic group is very active agent in DPPH solution because the bond dissociation energy in O-H is lower, thus it is easier to lose H atom [34]. A study on *Curvularia* sp. reported that various functional groups such as aromatic, carboxylic acid [35], phenol, ester and ketone were identified [36].

Table 1. Yield and antioxidant activity of extract at 50% of inhibition concentration value (mg/mL)

Substrate	Extracts	Extract yield (mg)	IC_{50} (mg/mL)
Malt agar medium	***Fusarium* sp.**		
	MAF7	4.08±0.4	1.7
	MAF15	11.19±1.1	2.4
	MAF30	13.53±1.2	2.4
	***Curvularia* sp.**		
	MAC7	6.63±0.2	0.85
	MAC15	7.06±0.5	0.9
	MAC30	8.4±0.3	1.0
	Co-culture		
	MAFC7	6.4±0.2	0.6
	MAFC15	4.03±0.2	0.8
	MAFC30	7.00±0.2	0.9
Rice medium	***Fusarium* sp.**	**Extract yield (mg)**	**IC_{50} (mg/mL)**
	F7	143.2±4.7	9.2
	F15	201.8±43.8	4.6
	F30	204.76±12.6	8.2
	***Curvularia* sp.**		
	C7	98.03±12.9	3.0
	C15	134.50±9.1	3.0
	C30	170.13±9.6	1.1
	Co-culture		
	FC7	151.23±17.4	4.1
	FC15	189.16±9.1	2.8
	FC30	189.45±14.5	4.8
Quercetin			0.3

*Legend: MA = malt agar; F7 = extract from single culture *Fusarium* sp. day 7; F15 = extract from single culture *Fusarium* sp. 15 days; F30 = extract from single culture *Fusarium* sp. 30 days; C7 = extract from single culture *Curvularia* sp. day 7; C15 = extract from single culture *Curvularia* sp. 15 days; C30 = extract from single culture *Curvularia* sp. 30 days; FC7 = extract from co-culture between *Fusarium* sp. and *Curvularia* sp. day 7; FC15 = extract from co-culture between *Fusarium* sp. and *Curvularia* sp. 15 days (FC15); FC30 = extract from co-culture between *Fusarium* sp. and *Curvularia* sp. 30 days. The data were expressed as mean ±SD (n=3).

Antibacterial activity

The antibacterial activity of the extracts showed more selectivity towards the Gram-positive bacteria with MIC values of 0.156 mg/mL – 10 mg/mL (Table 2), however, no activity was observed against the Gram-negative bacteria, except on *Salmonella* sp. Amongst the extracts, extracts from agar plate showed a potent activity as compared to the extracts from rice medium, except extracts from single culture of *Curvularia* sp. day 7 (C7) and day 15 (C15) also inhibited the growth of *Salmonella* sp. with MIC values of 5.0 mg/mL. The antibacterial results showed there was a significant loss of biaoactivity in the extracts grown in rice medium as compared to extracts from rice culture. Althought, the ability of the extracts to inhibit more specific activity on the bacteria used in this study, cultivation of the fungi on agar plate also can be optimised in rice medium for their mass production purposes by monitoring the diversity of chemical profiles in the extracts.

Dereplication study on extracts

Amongst the the extracts, extracts from a co-culture between *Fusarium* sp. and *Curvularia* sp. day 15 (FC15) in rice as a substrate was the optimum condition for mass production of their bioactive metabolites in these two fungi. The other parameters that have to be considered were shorter period and yield of the extract in order to have an adequate starting material for further isolation work. Dereplication studies on HRESI-LCMS data coupled with multivariate analysis on the extracts were evaluated that correlated with their bioactivities. Further investigation on FC15 extract was carried out by observing the occurrence of the metabolites in total ion chromatogram (Figure 2A). Distribution of the known and unknown compounds (highlighted rows) in the FC15 extract (Table 3) was dereplicated and putatively identified by using Dictionary of Natural Product (DNP) database. The FC15 extract possessed microlides, cyclic peptides and polyketides compounds that were putatively identified as 24-demethyl bafilomycin C1, cyclosporine C and monocerin, respectively, earlier were isolated from *Streptomyces* sp. and fungi [20].

A PCA model (Figure 2B) was also used to examine the variation and the diversity of the various extracts. However, the PCA model gave a low predictability score with Q^2 = 0.276. No significant variation can differentiate the extracts. Thus, the differences between the extracts were visualised by a supervised model. The supervised OPLS-DA (Figure 2C) scores scatter plot showed the difference between the most antioxidant active extract (colored violet) of *Fusarium* sp. (FC15), and other extracts (colored blue) that have moderate activity (FC7, FC30, C7, C15, and C30). Meanwhile, from the S-loadings (Figure 2D) scatter plot, metabolites can be predicted to be responsible for the most active group and be targeted for mass production prior to isolation work. From FC15 extract, the metabolite at *m/z* 276.159 $[M+H]^+$ was putatively identified as annotine that previously has been isolated from *Lycopodium annotinum* [24]. While, the ion peak at *m/z* 367.124 $[M+H]^+$ matched with 3-O-α-L-rhamnopyranosyl-D-galactose; 2-ac earlier reported from bacterial cell wall polysaccharides, and the ion peak at *m/z* 515.3331 $[M+H]^+$ was putatively identified as serratamolide A, that previously were isolated from bacteria *Serratia marcescens* [25]. The ion peak at *m/z* $[M+H]^+$ 309.132 was putatively identified as monocerin, that has been isolated from *Helminthosporium monoceras*, *Fusarium larvarum* and *Exserohilum rostratum* [26]. Other metabolites were also observed at *m/z* 213.112 $[M+H]^+$ and $[M-H]^-$ 257.045 that were putatively identified as canadensolide; 1',3β-Dihydro and simonyellin; 5-Deoxy, respectively, earlier were isolated from *Penicillium* sp. [27]. The ion peaks at *m/z* 243.050 $[M+H]^+$ and 301.075 $[M-H]^-$ were putatively identified as 3,4,5-trihydroxy-1,2-benzenedicarboxylic acid; 3,5-di-me ether and 1-deoxyxylitol; D-form, 2,4-methylene, 5-tosyl, respectively. While, the *m/z* $[M+H]^+$ 226.071, 241.143, 276.195 and $[M-H]^-$ 265.111, 595.359 were putatively identified as serine; (R)-form, N-(2-hydroxybenzoyl), armillariol C, 6-[2-(1-Hydroxybutyl)-4-methylphenyl]-5-hexenamide; (E)-form, vasconine and thiomarinol H, respectively. These metabolties have been isolated from *Actinomadura madurae* IFM 0745, *Armillaria* sp. strain 488, *Streptomyces* X537 [21], *Narcissus vasconicus* [28] and *Pseudoalteromonas* sp. SANK73390 [29], respectively. Other ion peaks in the FC15 extract have been dereplicated as presented in Table 3. Five discriminationg metabolites were also observed at *m/z* $[M+H]^+$ 369.131, 309.110 and *m/z* [M-

H]$^-$ 307.113, 593.343, 181.094, which did not find any match from the database.

Table 2. Antibacterial activity of extracts at minimum inhibition concentration (MIC) values (mg/mL)

		Gram positive bacteria			**Gram negative bacteria**		
Substrate	**Extracts**	***Micrococcus* sp.**	***S. aureus***	***B. cereus***	***Salmonella* sp.**	***E. coli***	***V. parahaemolyticus***
Malt agar medium	***Fusarium* sp.**	**Minimum Inhibitory Concentration (MIC) (mg/mL)**					
	MAF7	n.d	n.d	n.d	n.d	n.d	n.d
	MAF15	0.156	n.d	5.0	n.d	n.d	n.d
	MAF30	n.d	n.d	5.0	n.d	n.d	n.d
	***Curvularia* sp.**						
	MAC7	2.5	5.0	5.0	n.d	n.d	n.d
	MAC15	5.0	5.0	5.0	n.d	n.d	n.d
	MAC30	5.0	2.5	10.0	5.0	n.d	n.d
	Co-culture						
	MAFC7	5.0	2.5	5.0	5.0	n.d	n.d
	MAFC15	5.0	5.0	n.d	5.0	n.d	n.d
	MAFC30	5.0	2.5	5.0	5.0	n.d	n.d
Rice medium	***Fusarium* sp.**	**Minimum Inhibitory Concentration (MIC) (mg/mL)**					
	F7	n.d	n.d	n.d	n.d	n.d	n.d
	F15	n.d	n.d	n.d	n.d	n.d	n.d
	F30	n.d	n.d	n.d	n.d	n.d	n.d
	***Curvularia* sp.**						
	C7	n.d	n.d	5.0	n.d	n.d	n.d
	C15	n.d	n.d	5.0	n.d	n.d	n.d
	C30	n.d	n.d	n.d	n.d	n.d	n.d
	Co-culture						
	FC7	n.d	n.d	n.d	n.d	n.d	n.d
	FC15	n.d	n.d	n.d	n.d	n.d	n.d
	FC30	n.d	n.d	n.d	n.d	n.d	n.d

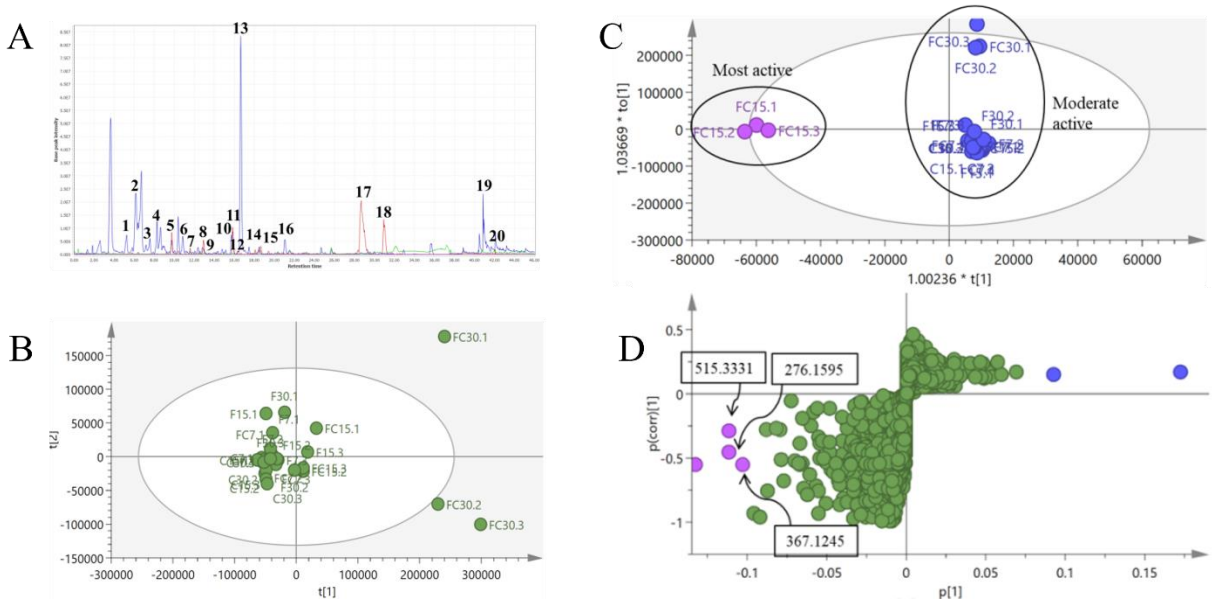

Figure 2. (A) Total ion chromatogram of co-culture extract between *Fusarium* sp. and *Curvularia* sp. FC15 (blue and red represent positive and negative ionisation modes, respectively; green and pink represent positive and negative ionisation modes of blank methanol, respectively). Dereplication of numbered peaks is shown on Table 3. (B) PCA scores scatter plot of single and co-culture extracts *($R^2(Y) = 0.72$; $Q^2 = 0.276$)* (C) OPLS-DA scores scatter plot *($R^2 = 0.951$; $Q^2 = 0.620$; Q^2(Y intercept))* from permutation test = −0.623) and (D) S-loadings plot . Legend: set F7.1-F7.3 = single culture *Fusarium* sp. incubation day 7; set F15.1-F15.3 = single culture *Fusarium* sp. incubation 15 days; set F30.1-F30.3 = single culture *Fusarium* sp. incubation 30 days; set C7.1-C7.3 = single culture *Curvularia* sp. incubation day 7; set C15.1-C15.3 = single culture *Curvularia* sp. incubation 15 days; set C30.1-C30.3 = single culture *Curvularia* sp. incubation 30 days; set FC7.1-FC7.3 = co-culture between *Fusarium* sp. and *Curvularia* sp. incubation day 7; set FC15.1-FC15.3 = co-culture between *Fusarium* sp. and *Curvularia* sp. incubation 15 days; set FC30.1-FC30.3 = co-culture between *Fusarium* sp. and *Curvularia* sp. incubation 30 days, n=3.

Table 3. List of compounds indicated on the total ion chromatogram for the extract of co-culture between *Fusarium* sp. and *Curvularia* sp. (FC15) that were putatively identified using the Dictionary of Natural Product database. Highlighted rows represent the unknown compounds as indicated with their MZmine IDs

Peak ID	ESI Modes/ MZmine ID	Rt (min)	MS (*m/z*)	Molecular Weight (g/mol)	Chemical Formula	Chemical Name	Tolerance (ppm)	Sources	Peak Area
1	P_7286	5.67	243.0500	242.0427	$C_{10}H_{10}O_7$	3,4,5-trihydroxy-1,2-benzenedicarboxylic acid; 3,5-di-me ether	0.3258		1.19E+03
2	P_8297	7.52	226.0711	225.0638	$C_{10}H_{11}NO_5$	serine; (R)-form, N-(2-hydroxybenzoyl)	0.3462	*Actinomadura madurae* IFM 0745	5.89E+04
3	P_7603	7.60	276.1595	275.1522	$C_{16}H_{21}NO_3$	annotine	0.1932	*Lycopodium annotinum*	1.25E+08
4	P_16421	8.94	213.1122	212.1048	$C_{11}H_{16}O_4$	canadensolide; 1',3β-dihydro	0.1758	*Penicillium canadense* *Aspergillus indicus*	2.07E+06
5	N_4	9.76	301.0758	302.0831	$C_{13}H_{18}O_6S$	1-deoxyxylitol; D-form, 2,4-methylene, 5-tosyl	2.3556		8.11E+02
6	P_15803	11.06	241.1435	240.1363	$C_{13}H_{20}O_4$	armillariol C	0.3857	*Armillaria* sp. strain 488	2.00E+04
7	N_808	11.10	307.1131	308.1204	$C_7H_{26}N_4OP_4$	No hits			2.86E+04
8	N_7080	12.51	265.1116	266.1189	$C_{17}H_{16}NO_2$	vasconine	2.8423	*Narcissus vasconicus*	2.67E+06
9	P_559	13.33	707.3983	706.3911	$C_{38}H_{58}O_{12}$	bafilomycin C1; 24-demethyl	-2.4354	*Streptomyces* sp. CS	5.19E+04
10	P_20981	15.03	369.1319	368.1246	$C_{18}H_{12}N_{10}$	No hits			1.57E+05
11	N_14832	15.74	367.1245	368.1318	$C_{14}H_{24}O_{11}$	3-O-α-L-rhamnopyranosyl-D-galactose; 2-ac	-0.1576	Bacterial cell-wall polysaccharides	9.21E+07
12	P_558	16.34	515.3331	514.3258	$C_{26}H_{46}N_2O_8$	Serratamolide A	0.7494	*Serratia marcescens*	2.25E+04
13	P_21898	14.94	309.1102	308.1029	$C_8H_{12}N_{12}S$	No hits			1.08E+03
14	N_8	18.79	257.0459	258.0532	$C_{14}H_{10}O_5$	simonyellin; 5-deoxy	1.5973	*Penicillium allii*	6.84E+04
15	P_60	18.75	1218.8435	1217.8362	$C_{62}H_{111}N_{11}O_{13}$	cyclosporin C	-0.0526	*Acremonium luzulae* *Fusarium solani*	4.02E+04
16	P_42112	22.90	276.1958	275.1886	$C_{17}H_{25}NO_2$	6-[2-(1-hydroxybutyl)-4-methylphenyl]-5-hexenamide; (E)-form	0.164	*Streptomyces* X537	7.05E+04
17	N_14830	28.67	593.3433	594.3506	$C_{34}H_{57}PS_3$	No hits			5.10E+08
18	N_14831	30.94	595.3591	596.3663	$C_{31}H_{52}N_2O_9$	thiomarinol H	-1.5705	*Pseudoalteromonas* sp. SANK73390	1.93E+08
19	P_7605	42.15	309.1329	308.1256	$C_{16}H_{20}O_6$	monocerin	-1.1412	*Helminthosporium monoceras* *Fusarium larvarum* *Exserohilum rostratum*	2.06E+08
20	N_14889	40.86	181.0946	182.1019	$C_5H_8N_8$	No hits			8.10E+05

Previous studies reported that serratamolide A possessing antimicrobial activity on the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA) [30], while, bafilomycin C1; 24-Demethyl inhibited *Penicillium avellaneum* UC-4376 with minimal inhibitory amount at 10 µg/disc and possessed cytotoxic activity against tumour (P388) and lung cancer (A-549) cells at concentration 10^{-4} M with cell line inhibitory rate at 97.9% and 96.9%, respectively [21]. Meanwhile, compound cyclosporine C possessed a strong antifungal activity on fungi *Alternaria* sp. (MIC = 0.1 µg/mL – 0.5 µg/mL) and *Penicillium* sp. (MIC = 2.0 µg/mL - 5.0 µg/mL) [23]. Monocerin from *Fusarium* sp. possessed antioxidant activity at more than 324 µM [31] and antibacterial activity on *S. aureus* (MIC = 62.5 µg/mL) and *E. coli* (MIC = 15.62 µg/mL), while thiomarinol H also inhibited *S. aureus* [32, 33].

Conclusion

The extracts from co-culture on malt agar plate possessed a stronger antioxidant activity as compared to their independent extracts. Meanwhile, the antibacterial activity of the extracts demonstrated more selective inhibition on the Gram positive bacteria. Optimisation of the co-culture between *Fusarium* sp. and *Curvularia* sp. in sterile rice medium for up-scaling, increased the antioxidant activity in the co-culture FC15 extract as compared to other extracts. The supervised OPLS-DA scores scatter plot showed the difference in diversity of various metabolites between the most active antioxidant FC15 extract as compared to others. Therefore, the co-culture between two fungi triggers the silent gene to produce difference metabolites that absent in their independent extracts that can be further explored for pharmaceutical purposes.

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