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Investigating the antioxidant potential of *Streptomyces* sp. MUSC 11 from mangrove soil in Malaysia

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Abstract: *Streptomyces* are a distinguished group of gram-positive bacteria mostly acknowledged for their immense contribution to life-saving drugs and lines of compounds with diverse bioactivities. To date, there remains limited studies on *Streptomyces* with biological activities residing in underexplored ecosystems such as the mangrove forests. For this purpose, the present work aimed at investigating the biological activity of *Streptomyces* sp. MUSC 11 collected from soil sample in mangrove forests, situated in the State of Pahang, Peninsular of Malaysia. The cultured strain resembled phenotypic and genotypic traits of genus *Streptomyces*. Investigations of the methanolic extract from *Streptomyces* sp. MUSC 11 revealed antioxidant activities in form of scavenging free radicals ABTS, DPPH, chelating iron and reducing ferric iron. Besides the antioxidant tests, antioxidant results corresponded well to the presence of phenolic content. In summary, *Streptomyces* derived from extreme and understudied ecosystem such as the mangrove forests are potential sources of biologically active and therapeutically useful compounds.

Keywords: *Streptomyces*; antioxidative; radical scavenging; mangrove

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Introduction

The mangrove ecosystem makes up nearly 75 % of coastal margin in tropical and subtropical countries[1]. For centuries, they attracted significant attention, due to the unusual physiognomy of the trees and shrubs that reside therein. To date, much of what is known about the mangrove forests, stem from studies of macro-biodiversity-the flora and fauna; and, to a smaller extent, the micro-biodiversity[2,3]. Nevertheless, microbes are crucial to the conservation of mangrove forest, tasked with fixing nitrogen and sequestering carbon, they confer fitness to trees which are rooted in nutrient-poor, waterlogged, saline and mostly acidic soil[2].

Over the years, the rich chemical diversity generated in nature has become a minefield for natural product drug discovery researchers, who are constantly in search for better alternative drugs[4-13]. There is mounting evidence supporting the growing interest in microbes in

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underexplored habitats such as mangroves, as sources of biologically active compounds[14-16]. For instance, the review by Ancheeva and colleagues highlighted that between the years of 2014 and 2018, 163 compounds isolated mostly from mangrove-derived fungi, exhibited potent anti-cancer, antimicrobial, anti-inflammatory, cholesterol lowering and α-glucosidase activity[17]. Also, from year 2000 and onwards, microbial natural products, particular from bacteria, have accounted for 8.9 % of all the Food and Drug Administration-approved new molecular entity (NME) compared to 5.6 % that make up plant-based FDA-approved NME [18]. Given the scarcity of studies conducted on mangrove-derived bacteria such as *Streptomyces* for biologically active compounds, has prompted investigations thereof [19-23]. *Streptomyces* are gram-positive bacteria classified as a genus of the Actinobacteria phylum[24, 25]. Since the discovery of streptomycin from *Streptomyces*[26], they continue to feature among the most prominent drug-producing microbes[27-30]. Presently, *Streptomyces* are by far, the largest microbial genus studied[31,32] and hailed as prolific producers of more than 7600 bioactive compounds[33,34]. More recently, fewer new biologically active compounds have been reported from *Streptomyces*[35]. It is hoped that venturing into understudied ecological niches such as the mangrove forest, will identify underreported *Streptomyces* with rare metabolic pathways capable of producing biologically active metabolites[36-38].

Malaysia is home to the second largest mangrove forest in the South-East Asia region[39]. Much of the mangrove region in Malaysia are yet to be investigated for potential *Streptomyces* with biologically active compounds. Several published works have found that mangrove soil in Malaysia contain rare *Streptomyces* with wide spectrum of biological activities[40-47]. In this view, the present work sampled mangrove soil in Malaysia and further isolated and studied *Streptomyces* sp. MUSC 11 specifically for its antioxidant capability. Methanolic extracts from *Streptomyces* sp. MUSC 11 showed antioxidant activity against free radicals ABTS, DPPH, ferrous iron and also exerted ferric reduction power. In addition, the study established the fact that the antioxidant activities are partly caused by the presence of phenolic compounds. Overall, the study suggests *Streptomyces* sp. MUSC 11 to be a reliable producer of antioxidant metabolites and warrants further investigations.

Materials and Methods

Sampling, isolation and maintenance of *Streptomyces* sp. MUSC 11

The mangrove soil – derived strain was collected in Tanjung Lumpur, Malaysia in December, 2012 (MUSC-TLS4 3°48’21.3” N 103°20’3.3”E). The pure cultures of *Streptomyces* sp. MUSC 11 were obtained through initial heat-treatment followed by suppression of non-*Streptomyces* microbes through use of anti-fungal drugs and series of sub - cultures. They were maintained on ISP2 agar slant at 28°C and glycerol stocks (30% v/v) at - 80°C for shorter and longer storage time, respectively[48,49].

Genomic DNA extraction and phylogenetic analysis of *Streptomyces* sp. MUSC 11

The genomic DNA (gDNA) content was isolated for the purpose of amplifying the 16S rRNA gene region as detailed by the methods of Hong et al and Lee et al., respectively[43,50]. The 16S rRNA gene sequence that was eventually acquired was entered into GenBank/ EMBL/ DDBJ database to obtain several type strains that shared the closest relationship with *Streptomyces* sp. MUSC 11. Alignment of the 16S rRNA gene sequences for these *Streptomyces* type strains was carefully performed in CLUSTAL - x software[51]Julie D. Stability of generated phylogenetic tree was checked by bootstrap based on 1000 resampling method[56].

Phenotypic characterization of *Streptomyces* sp. MUSC 11

Cultural characteristics of a 7 - 14 days old *Streptomyces* sp. MUSC 11 grown at 28 °C, was assessed on different culture growth media - International *Streptomyces* Project (ISP) 2, ISP3, ISP4, ISP5, ISP6, ISP7[57], *Streptomyces* agar (SA)[58], Nutrient agar (NA)[59], Actinomycete isolation agar (AIA)[60] and starch casein agar (SCA)[61]. Its ability to produce soluble pigment as well as the colony colour on each growth media were taken note of[62]. Aside from assessing cultural characteristics, *Streptomyces* sp. MUSC 11 was also exposed to varying degrees of temperature (4 – 50 °C), salinity (0 – 10 % w/v), pH (2 - 10). This was done to determine the optimum growth condition of *Streptomyces* sp. MUSC 11. Additional biochemical tests carried out, were to investigate *Streptomyces* sp. MUSC 11 as capable of producing a number of extracellular enzymes. To determine the presence of catalase, a drop of 3 % (v/v) hydrogen peroxide was added to the culture of *Streptomyces* sp. MUSC 11. The production of bubbles suggested presence of catalase[63]. The potential of *Streptomyces* sp. MUSC 11 to induce hemolysis was further tested on a 5 - day old culture grown on blood agar media with ingredients 5 % (w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) human blood. A clear zone of inhibition around the 5 day culture, denote haemolysis and surfactant property of the culture[64]. By growing culture on ISP 2 media, the presence of chitinase, xylanase, amylase, protease, lipase and cellulose were also established[65].

Fermentation process and extract preparation

Seed culture of *Streptomyces* sp. MUSC 11 was prepared in a volume of 10 mL by growing in nutrient – rich TSB media for 10 days at 28 °C with aeration rate of 220 rpm. Afterwards, an aliquot of seed media containing *Streptomyces* sp. MUSC 11, was transferred to freshly made, sterile HFM media and incubated under same culture condition. The cell - free supernatant was later collected after centrifugation, filtration and freeze - drying were completed[43]. Methanol was selected as the organic solvent for extracting secondary metabolites of the freeze - dried supernatant. Filtrate was then evaporated to dryness by use of rotary evaporator and dried crude methanolic extract was stored at – 20 °C for future use[48] .

Antioxidant assays of methanolic extract MUSC 11

ABTS radical scavenging activity

The radical scavenging activity of the methanolic extract was examined in accordance with method of Tan et al. [66]. Briefly, the reagent, 2, 20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used to generate ABTS radical ion (ABTS•$^{+}$). This was achieved by adding together ABTS and potassium persulfate at a concentration of 7 mM and 2.45 mM, respectively. The resultant ABTS free radical solution was then allowed to react with different concentration of methanolic extract MUSC 11, in a 96 well plate, in dark, for 20 minutes. Gallic acid served as the standard for this experiment. The antioxidant activity was evaluated by taking the absorbance reading at 734 nm. The formula for calculating the percentage (%) of ABTS radical scavenging activity is as follows :

$$\%\ ABTS\ scavenging\ activity = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

DPPH radical scavenging activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was carried out in accordance to previous method stated elsewhere[66]. A solution of pre-made DPPH (0.016 % w/v) dissolved in ethanol (95 % v/v) was added into 96 well plates containing different concentrations of methanolic extract and left standing for 20 minutes, in dark at room temperature. The absorbance for each reaction well was recorded at 515 nm. Gallic acid was the standard used for this test. The formula for calculating the percentage (%) of DPPH radical scavenging activity is given below :

$$\%\ of\ DPPH\ radical\ scavenging\ activity = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Metal chelating activity

The ability of the methanolic extracts of *Streptomyces* sp. MUSC 11 to chelate iron was investigated following the method of Adjimani and Asare[67]. The ferrozine (5 mM) was allowed to react with ferrous sulfate ($FeSO_4$) 2 mM in 96 well plate for 10 minutes. The metal chelating activity takes into account the free ferrous ion in the reaction mixture by measuring the absorbance at 562nm. Ethylenediamine tetraacetic acid (EDTA) was treated as the positive control for this experiment. The following formula was used to calculate the percentage (%) of metal chelating activity :

$$\%\ of\ Metal\ chelating\ activity = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Ferric reducing activity

The ferric reducing antioxidant power (FRAP) assay was conducted following Adjimani and Asare [67] method with some modification. In brief, a series of 2 - fold concentration of methanolic extract MUSC 11 were prepared in volumes of 25 µL. In subsequent steps, 25 µL phosphate buffer (0.2 M) and 25 µL (1 %) of $K_3Fe(CN)_6$ were added into each of the 1.5 mL microcentrifuge tubes containing extracts, followed by heating at 50 °C for 20 minutes before leaving it to cool to room temperature. A 25 µL of TCA (10 %) was thereafter added which ended the reaction. From this mixture, 80 µL was removed and added into wells in 96 well plate with 20 µL of $FeCl_3$. The absorbance reading at 700 nm was recorded and presented in terms of the ascorbic acid dose equivalents.

Assessing the total phenolic content of extract

The total phenolic content (TPC) of the methanolic extract of MUSC 11 were determined following the method of Tan *et al.*[66]. A series of concentrations of the extract at 10 µL each were prepared and added into 96 well. A 50 µL of Folin - Ciocalteu's Reagent (1:10) was later transferred into individual wells and incubated in the dark for 5 minutes at room temperature. Addition of 40 µL of sodium carbonate ($NaCO_3$) at 7.5% was made to the wells containing extract to react with for another 30 minutes at room temperature. The absorbance was measured at UV wavelength of 750 nm. Results of absorbance reading were presented in terms of Gallic acid equivalents.

Gas chromatography-mass spectroscopy (GC-MS) chemical profiling of extract

The profiling of chemical constituents in the methanolic extract of *Streptomyces* sp. MUSC 11 was done as explained in detail in another study[68]. Briefly, chemical profiling was achieved by use of Agilent Technologies 6980N with a 5979 Mass Selective Detector and a HP-5MS (5 % phenyl methyl siloxane) capillary column of dimensions 30.0 m x 250 µm x 0.25 µm as a helium gas carrier (1 mL/min). Temperature was raised to 40 °C for 10 minutes; then, increased by 3 °C every minute until maximum temperature of 250 °C was reached whilst keeping peak temperature constant for another 5 minutes. MS was operational at 70 eV. Individual constituents that were detected by GC-MS had their identity verified by comparing their mass spectral data with reference compounds from NIST 05 spectral Library.

Statistical analysis

All antioxidant tests were repeated thrice and results expressed in means ± standard deviation (SD). Statistical Package for the Social Sciences software (SPSS) was used to analyse the antioxidant assays. One-way analysis of variance (ANOVA) and Tukey's *post hoc* was used to determine the statistical significance with a p-value < 0.05. The Pearson's correlation in SPSS software was employed to ascertain whether the antioxidant effect of methanolic extract of MUSC 11 were partly due to the phenolic compounds present therein.

Results

Genomic and phylogenetic analysis of *Streptomyces* sp. MUSC 11

The 1492 bp 16S rRNA gene sequence of *Streptomyces* sp. MUSC 11 (GenBank accession number MN199671) isolated from gDNA assisted in the process of accessing the 16S rRNA nucleotide sequences of closely related type strains from GenBank/EMBL/DDBJ database and subsequently aligned manually. The phylogenetic tree of *Streptomyces* sp. MUSC 11 is pictured in Figure 1. Based on the phylogenetic tree constructed, the closest relations were *Streptomyces thermocarboxydus* DSM 44293^T, *Streptomyces indiaensis* NBRC 13964^T and *Streptomyces massasporeus* NBRC 12796^T. Interestingly, both *Streptomyces thermocarboxydus* DSM 44293^T and *Streptomyces massasporeus* NBRC 12796^T displayed the closest 16S rRNA gene sequence similarity of 99.96 % proceeded by *Streptomyces indiaensis* NBRC 13964^T with 99.31 %.

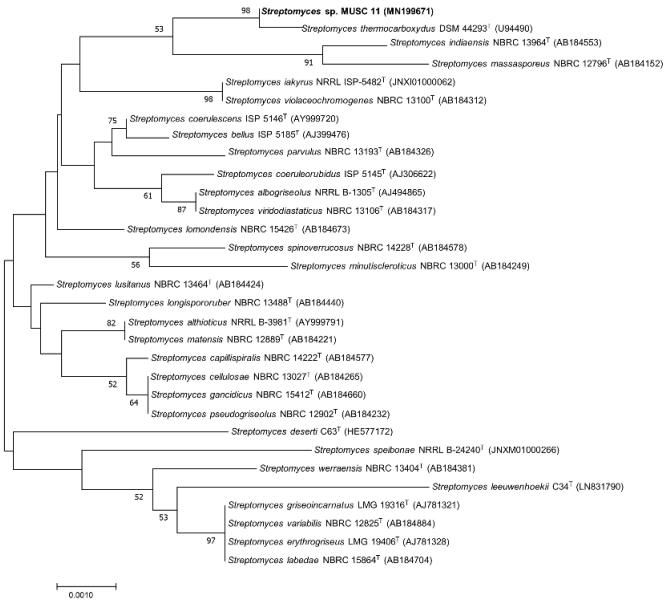

Figure 1. Neighbour-joining phylogenetic tree based on 1492 nucleotides of 16S rRNA gene sequence of Streptomyces sp. MUSC 11 and closely related type strains. Numbers and nodes indicate percentages (>50%) of 1000 bootstrap re-sampling. Bar, 0.001 substitutions per site.

Phenotypic characterization of *Streptomyces* sp. MUSC 11

The growth of *Streptomyces* sp. MUSC 11 on various media is shown in Table 1. *Streptomyces* sp. MUSC 11 showed preference to grow on ISP 2, ISP5, ISP6, ISP7 and SCA and SA after 7-14 days at 28 °C. This is in agreement by Gottlieb and Shirling who recommend ISP media for the growth of *Streptomyces*[57]. Colony colour of both the aerial and substrate mycelia were confirmed for all media grown, except ISP4. Production of soluble pigments was not observed in any of the tested media (Table 1). Optimal growth was observed at temperature of 26 – 28 °C, pH of 7 and salinity of 2 % w/v. *Streptomyces* sp. MUSC 11 was tested positive for catalase. Moreover, it was able to hydrolyse starch, casein and carboxymethylcellulose. In the case of xylan, *Streptomyces* sp. MUSC 11 was only able to hydrolyse to some extent, as shown in Table 2.

Table 1. Cultural characteristics of Streptomyces sp. MUSC 11

Media	Growth	Colony colour		Soluble pigments
		Aerial mycelia	Substrate mycelia	
ISP 2	Well	Light Yellow	Brilliant Yellow	-
ISP 3	Poor	Yellowish White	Yellowish White	-
ISP 4	No growth	-	-	-
ISP 5	Well	Dark Olive Brown	Dark Greyish Olive	-
ISP 6	Well	Greenish Yellow	Brilliant Yellow	-
ISP 7	Well	Dark Greyish Yellow	Dark Olive Brown	-
AIA	Moderate	Yellowish White	Yellowish White	-
SCA	Well	Yellowish Grey	Pale Greenish Yellow	-
SA	Well	Pale Yellow	Light Yellow	-
NA	Moderate	Pinkish Grey	Moderate Olive	-

Key: -: No growth on ISP 4 and no production of soluble pigment

Table 2. Biochemical and physiological characteristics of Streptomyces sp. MUSC 11

Catalase	+
Haemolytic	-
Enzymatic test	
Chitinase activity (2.5 % chitin)	-
Xylanase activity (0.5 % xylan)	(+)
Amylolytic activity (0.2 % starch)	+
Protease activity (2 % casein)	+
Lipase activity (1 % tributyrin)	-
Cellulase activity (0.5 % CMC)	+
Temperature	
Growth	26 - 50
Optimum	26 - 28
NaCl (%) tolerance	
Growth	0 - 6
Optimum	2
pH tolerance	
Growth	6 - 7
Optimum	7

Key: +: Activity; (+): Moderate activity; -: No activity

ABTS radical scavenging antioxidant assay

The ABTS radical scavenging assay was performed to examine whether methanolic extract MUSC 11 was able to scavenge ABTS radical cation. The ABTS radical cation was mixed with methanolic extract MUSC 11. A colour change from blue - green to colourless was observed suggesting ABTS scavenging activity. The absorbance reading of free ABTS radical was taken at 734 nm. The result of this experiment showed a concentration dependent ABTS radical scavenging activity ($p < 0.05$) with the highest measured at 4 mg/mL (Table 3).

DPPH radical scavenging antioxidant assay

The DPPH radical scavenging assay was used to determine the potential of microbial metabolites to scavenge free DPPH radical. The noteworthy colour change from purple (DPPH radical) to yellow (diphenylpicrylhydrazine) in the reaction mixture, indicated DPPH radical scavenging activity. Quantitative analysis of the antioxidant activity of methanolic extract MUSC 11 was undertaken by measuring absorbance of free DPPH radical at 515 nm. A low absorbance reading implied stronger antioxidant. The result of this experiment demonstrated the DPPH radical scavenging potential of methanolic extract MUSC 11 with an activity ($p < 0.05$) of 7.27 ± 4.73 % at its highest concentration of 4 mg/mL (Table 3).

Metal chelating assay antioxidant assay

In this experiment, the ferrozine reagent was used to determine the ability of methanolic extract MUSC 11 to chelate ferrous ion (Fe^{2+}). The metal chelating potential of the methanolic extract MUSC 11 was evaluated by measuring the absorbance of ferrous -ferrozine complex formed at 562 nm. A low absorbance reading suggested most of the complex formed were between the ferrous ion and the metabolites present in the methanolic extract MUSC 11. The result of this study indicated that methanolic extract MUSC 11 had metal chelating activity ($p < 0.05$) of 21.61 ± 1.71 % at 4 mg/mL (Table 3).

Table 3. The antioxidant activities of Streptomyces sp. MUSC 11 at different antioxidant assays.

Concentration (mg/mL)	Antioxidant activities (%)		
	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	Metal chelating activity (%)
0.125	ND¥	3.23 ± 1.16*	8.55 ± 2.39*
0.25	ND¥	5.69 ± 1.53*	6.68 ± 2.69*
0.5	0.39 ± 0.96*	5.66 ± 0.87*	8.22 ± 2.95*
1	1.22 ± 1.24*	8.46 ± 1.27*	9.73 ± 1.02*
2	7.58 ± 1.55*	16.23 ± 0.64*	14.38 ± 6.13*
4	7.27 ± 4.73*	31.42 ± 1.00*	21.61 ± 1.71*

*Statistically significant at $p < 0.05$; ¥ ND- Not detected

The ferric reducing antioxidant power (FRAP) assay

The potential of methanolic extract MUSC 11 to reduce iron in the ferric form (Fe^{3+}) to its ferrous (Fe^{2+}) form was assessed through the FRAP assay. The amount of ferric-ferrous ion complex was determined by measuring the absorbance of 700 nm. Visible colour change to Prussian blue was observed indicating the reducing power of methanolic extract. The result from the absorbance reading showed that methanolic extract MUSC 11 absorbance was 1.02 - 1.12, in the dose range of 1 - 2 mg, equivalent to 3.001 ng -3.521 ng of ascorbic acid (Figure 2).

Assessment of the Total phenolic content

The presence of phenolic compounds in the methanolic extract MUSC 11 was confirmed by the Folin -Ciocalteu's method and is positively associated with a colour change from yellow to blue. Based on this experiment, the intensity of blue colour observed was concentration dependent. To ascertain the relationship between the antioxidant activities and phenolic content in methanolic extract MUSC 11, an additional correlation analysis was undertaken. As shown in Table 4, the Pearson's correlation analysis revealed a strong relationship ($r = 0.90 - 0.99$, $p > 0.05$) between the antioxidant activities (ABTS, DPPH and metal chelation) and phenolic content of methanolic extract MUSC 11.

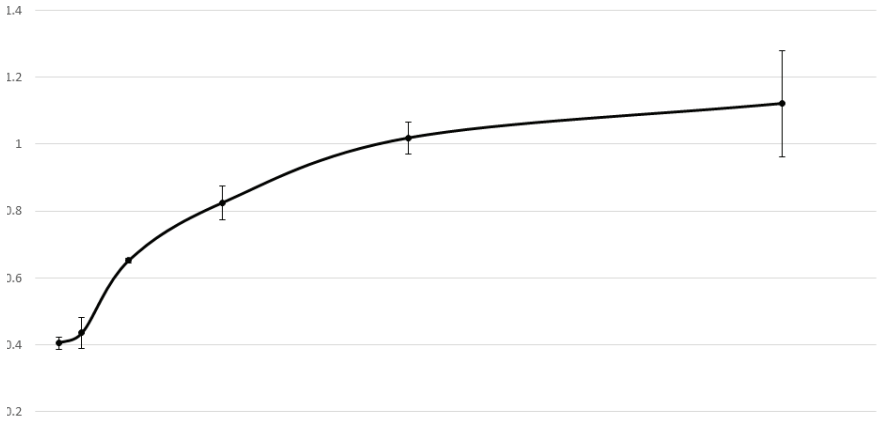

Figure 2. Ferric reducing activity of methanolic extract of Streptomyces sp. MUSC 11

Table 4. Total phenolic content of methanolic extract of Streptomyces sp. MUSC 11

Antioxidant activities	Phenolic content
ABTS radical scavenging activity	r = 0.985*
DPPH radical scavenging activity	r = 0.897*
Metal-chelating activity	r = 0.974*

*Correlation is significant at the 0.05 level.

GC-MS analysis

Chemical profiling of the various constituents contained in the methanolic extract MUSC 11 was attained through the use of GC-MS together with the mass spectral data provided by the NIST library. From this, 11 compounds belonging to alcohols, phenols, esters, fatty acids, peptidyl nucleosides, cyclic dipeptides and aminoglycoside were identified. Detailed information of individual compounds including their chemical structures are provided in Table 5 and Figure 4, respectively.

Table 5. Compounds present in the methanolic extract of and detected by GC-MS

No.	Constituents	Retention time (min)	Molecular Formula	Molecular weight	Similarity (%)
1	Benzenemethanol,2-(2-aminopropoxy)-3-methyl-	3.864	$C_{11}H_{17}NO_2$	195	98.4
2	L-Proline,5-oxo-,methyl ester	39.876	$C_6H_9NO_3$	143	81.9
3	Phenol, 2,4-bis(1,1-dimethylethyl)-	44.828	$C_{14}H_{22}O$	206	88.6
4	Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1,4-dideoxy-4-(D-2-(2-(methylamino)acetamido)hydracryl-amido)-, beta-D-	54.159	$C_{16}H_{25}N_7O_8$	443	92.3
5	Tetradecanoic acid, 12 methyl-, methyl ester	55.145	$C_{16}H_{32}O_2$	256	91.5
6	Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	55.893	$C_{11}H_{18}N_2O_2$	210	91.6
7	Oleic acid	56.359	$C_{18}H_{34}O_2$	282	92
8	2-Bromotetradecanoic acid	56.771	$C_{14}H_{27}BrO_2$	307	93.1
9	*n*-hexadecanoic acid	59.458	$C_{16}H_{32}O_2$	256	91
10	D-Streptamine, O-2-amino-2-deoxy-alpha-D-glucopyrano-syl-(1-4)-O-(O-2,6-diamino-2,6-dideoxy-beta-L-idopyrano-syl-(1-3)-beta-D-ribofuranosyl-(1-5))-2-deoxy-	72.550	$C_{23}H_{45}N_5O_{14}$	615	95.1
11	Dasycarpidan-1-methanol, acetate (ester)	77.633	$C_{20}H_{26}N_2O_2$	326	87.1

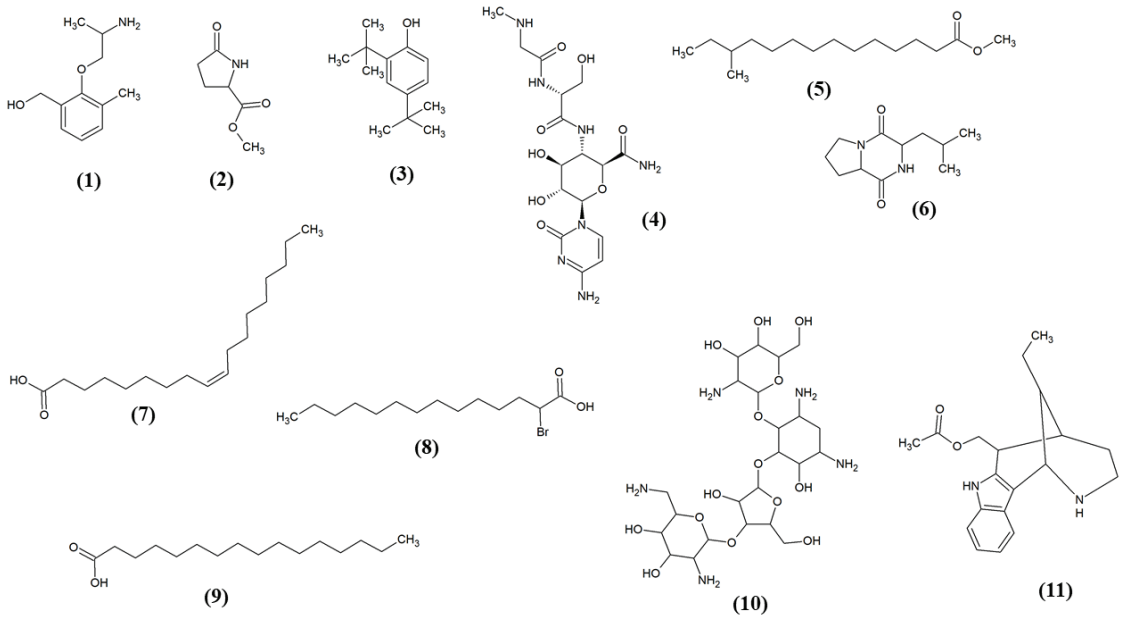

Figure 3. Chemical structures of 11 compounds that were present in the methanolic extract of *Streptomyces* sp. MUSC 11 and detected by GC-MS.

Discussion

Mangrove forest has emerged as a rich store of chemically diverse natural products[17] - any small molecules produced by living organisms[18,69]. Strategically positioned between terrestrial and marine ecosystem, mangrove forest represents a rare habitat of living organisms capable of thriving in extreme environmental conditions – saline, acidic and fluctuating tides[70]. The mangrove habitat is a treasure trove of microbes, residing mostly in the sediment[71] and in terms of their biotechnological significance, remain largely understudied.

The filamentous, aerobic, soil-dwelling, gram-positive *Streptomyces* bacteria[24] have been found residing in soil samples collected from many countries[72-76]. The traditional practice regarding the isolation of *Streptomyces* from soil samples, have over the years resulted in the rediscovery of compounds which slowly exhausted the supplies of new compounds. It has been suggested that understudied ecosystem hold *Streptomyces* species which can meet the growing demand of drug discovery and development industry[77]. Researchers who made an effort to study *Streptomyces* from less explored ecosystems such as mangrove forest were able to discover novel *Streptomyces* species and *Streptomyces* strains showing potent antioxidant activities[40,42,66,68,78,79].

In this study, *Streptomyces* sp. MUSC 11 with a 1492bp 16R rRNA gene sequence was isolated from mangrove soil in Tanjung Lumpur, Malaysia. The 16S rRNA sequence was manually aligned to an assembly of closely related member type strains accessed through the NCBI GenBank repository. Their 16S rRNA gene sequence similarities were determined by comparing them individually against the 16S rRNA gene sequence of *Streptomyces* sp. MUSC 11. Upon construction of the phylogenetic trees, it was found that *Streptomyces thermocarboxydus* DSM 44293^T, *Streptomyces indiaensis* NBRC 13964^T and *Streptomyces massasporeus* NBRC 12796^T (AB184152) shared closest relations. Highest 16S rRNA gene sequence was established between *Streptomyces thermocarboxydus* DSM 44293^T (99.96%), *Streptomyces massasporeus* NBRC 12796^T (AB184152) (99.93%) and *Streptomyces indiaensis* NBRC 13964^T (99.31%). Meanwhile, the phenotypic characteristics show that it was able to grow on all culture media with the exception of ISP 4. Furthermore, colony colour was visible on all media excluding ISP 4 (Table 1). The strain was unable to produce soluble pigment. It preferred to grow in an optimal temperature range, salinity and pH of 26 – 28 ºC, 2 and 7 respectively. Biochemical tests revealed the strain potential in producing catalase, xylanase, protease, amylase and cellulase (Table 2).

Aerobic respiration is undoubtedly one of the most fundamental life processes, in which ~90% inhaled oxygen molecule (O_2) is transported into cytoplasmic mitochondria[80] and participate in the reduction-oxidation (redox) reaction generating much of the energy-rich ATPs and water molecule. A by-product of the aforesaid reaction is the free radical reactive oxygen species (ROS). In homeostasis, ROS essentially function as intracellular and intercellular signalling molecules modulating cellular responses[81-83]. Considerable changes in ROS levels can potentially disrupt cellular functions and effects are reversed by the actions of respiratory enzymes, that assist in quenching ROS[84].Oxidative stress results from an overproduction of ROS with relatively low amounts of the antioxidant to defend the body from harmful effects of ROS[85]. It has been known that increased levels of ROS are dangerous as they tend to cause dysregulation of many cellular components involved in pathogenesis of several diseases[86-89]. Under such pathological states, synthetic or nature-based antioxidants are taken with the intent to reduce high levels of ROS. Evidence from earlier animal studies have noted synthetic antioxidants as potentially unsafe for human consumption, since higher doses and prolonged exposure can induce carcinogenesis[90,91]. Nowadays, industries prefer searching for safer and better antioxidant remedies among natural sources by utilizing variety of antioxidant assays[92-94]

Individual antioxidant assays have their own limitations and are therefore not representative of the antioxidant potential of a given extract[95]. For this reason, multiple antioxidant assays were taken into consideration to determine the antioxidant capacity of the methanolic extract MUSC 11. In fact, two of the known antioxidant methods were covered in this paper. The first method was based on the principle of scavenging preformed ROS (ABTS/ DPPH/ metal chelation)[95] whereas the second method considered the reduction power of antioxidants (ferric-reducing activity)[96]. In the experiment, ABTS and DPPH were generated as free radicals and later exposed to series of concentration from the methanolic extract MUSC 11. The measure of the antioxidant activity towards ABTS and DPPH were determined by the lending of hydrogen atom from constituents present in the methanolic extract in the process of hydrogen atom transfer (HAT)[95] thereby terminating free radical damaging effects. Both ABTS and DPPH are commonly employed to assess the antioxidant capacity of microbial extract [42, 66]. In this experiment, the methanolic extract exhibited ABTS radicals scavenging activity in the magnitude of 31.42 ± 1.00 % at 4 mg/mL. In the meantime, the result of DPPH radical scavenging was 7.27 ± 4.73 % at its highest concentration tested.

ROS such as superoxide anion radical ($O_2^{\bullet -}$) formed mainly from the reduction of O_2 molecule in mitochondria, can be further reduced to hydrogen peroxide[97] and used in Fenton reaction[98] to form hydroxyl radical. Therefore, any substrate that readily scavenges ROS can be of great therapeutic value. Transition metals such as iron found normally in cytoplasm in ferrous form (Fe^{2+}), appear to act as catalyst in the production of ROS[98]. In the presence of excess iron, Fenton reaction is enhanced resulting in the accumulation of circulating ROS. Based on previous studies, various researchers have demonstrated microbial metabolites as good antiradical agents[66,99,100] in reducing Fe^{2+} concentration and diminish ROS supplies. In this study, the metal chelating ability of the methanolic extract 11 was evaluated. The result ($p < 0.05$) showed methanolic extract was able to chelate 21.61 ± 1.17% of Fe^{2+} in the reaction mixture at the highest concentration tested (Table 3).

The reducing power of methanolic extract was tested using the ferric reducing assay. In this particular assay, the methanolic extract MUSC 11 and Fe^{3+} would simultaneously undergo oxidation (losing of electrons) and reduction (gaining of electrons), respectively. The basis of the *in vitro* ferric reducing activity was to assess the ability of the methanolic extract MUSC 11 to reduce Fe^{3+} ion to its Fe^{2+} form[67]. In the initial stage of the actual experiment, potassium ferricyanide reacted with methanolic extract which yielded a reduced ferrocyanide and a favourable oxidised methanolic extract MUSC 11[96]. Towards the end of the experiment, the ferrocyanide was reacted with ferric chloride forming a Prussian blue Fe^{3+} - Fe^{2+} complex. The ferric reducing activity ($p < 0.05$) was expressed as 1 - 2 mg of methanolic extract MUSC 11 which is equivalent to 3.521 – 3.001 ng of ascorbic acid.

Individual compounds are designated specific GC-MS retention time and molecular ion charges (m/z) which are subsequently introduced into large computer-generated databases and comparatively analysed with standards for potential match. Nowadays, GC-MS coupled with NIST mass spectral library is extensively used for determining the bioactive constituents in microbial extracts[42,68,79]. In this experiment, 11 compounds were detected in the methanolic extract MUSC 11: Benzenemethanol,2-(2-aminopropoxy)-3-methyl- **(1)**, L-Proline,5-oxo-,methyl ester **(2)**, Phenol, 2,4-bis(1,1-dimethylethyl)- **(3)**, Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1,4-dideoxy-4-(D-2-(2-(methylamino) acetamido) hydracrylamido) -, beta-D- **(4)**, Tetradecanoic acid, 12 methyl-, methyl ester **(5)**, Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- **(6)**, Oleic acid **(7)**, 2-Bromotetradecanoic acid **(8)**, *n*-hexadecanoic acid **(9)**, D-Streptamine, O-2-amino-2-deoxy-alpha-D-glucopyranosyl-(1-4)-O-(O-2,6-diamino-2,6-dideoxy-beta-L-idopyranosyl-(1-3)-beta-D-ribofuranosyl-(1-5))-2-deoxy- **(10)** and Dasycarpidan-1-methanol, acetate (ester) **(11)**.

Interestingly 11 compounds were detected by GC-MS in the microbial crude extract MUSC 11 with wide ranging biological activities. For instance, traces of compound **(1)** found in the methanolic extract of *Candida albicans*[101] and *Acinetobacter baumannii*[102] had antibacterial and antifungal activities, respectively. Methanolic extract from endophytic fungi *Xylaria* sp. containing compound **(2)** displayed strong antioxidant activity in the form of scavenging DPPH radicals[103]. Present in the methanolic extract MUSC 11 were also fatty acids- a straight chain **(9)** and a halogenated fatty acid **(8)**. The palmitic acid **(9)** was detected as one of the 3 principal compounds from S*treptomyces* sp. ECR77 extract giving rise to the anti -bacterial effect on several fish pathogens[104] A halogenated form of the 14-carbon fatty acid **(8)** was produced by fungus *Aspergillus niger*[105] and *Saccharomyces cerievisiae*[106] with antimicrobial activities. Compound **(11)** was previously reported from *Pseudomonas aerigunosa* as having activities against *Aspergillus fumigatus*[107].

Phenolic compounds constitute hydroxyl functional groups attached to aromatic compounds[108]. The lower bond dissociation energy (BDE) between hydroxyl functional group, renders hydrogen a good leaving group, and, the parent compound -phenolic compounds – overall, as good scavengers of ROS[109,110]. Given that phenolic compounds are good antioxidants, the total phenolic content of methanolic extract MUSC 11 was investigated. The results of this study indeed showed a strong correlation ($p < 0.05$) between the methanolic extract and phenolic content, suggesting the antioxidant activities was possibly due to presence of such constituents (Table 4). Further information on the nature of phenolic compound **(3)** was provided by the GC - MS analysis (Table 5 and Figure 3). Studies of *Streptomyces* involving antioxidant on numerous occasions have mentioned the detection phenolic compounds including compound **(3)**[41,42,48,66,68,79,111]. Hence, compound **(3)** was perceived to have caused the antioxidant activity to some extent, provided the contribution may have also come from other constituent in methanolic extract MUSC 11.

Bioactive pyrrolopyrazines have been previously recovered from microbial fermentation. For example, Azman

and her colleagues investigated biological activities of *Microbacterium mangrovi* MUSC 115^{T}, *Sinomonas humi* MUSC 117^{T} and *Monashia flava* MUSC 78 ^{T} isolated from the mangrove forest of Malaysia. From their study, it was shown that methanolic extracts from 3 bacterial species contained a number of pyrrolopyrazines and either one of these extracts were effective against batteries of pathogenic bacteria, cancer cell lines and neuroprotection models[108]. Given that past studies have highlighted the presence of compound **(6)** in microbial extract with antioxidant potentials[40,48,100], further points to its likely role in inducing antioxidant effect.

Several bioactive compounds in the methanolic extract MUSC 11 accounted for in this study were elaborated elsewhere and includes compound **(4)** also referred to as aspiculamycin, a peptidyl nucleoside antibiotic isolated from *Streptomyces toyocaensis* var. *aspiculamyceticus*[112]. The antileishmaniasis aminoglycoside (**10**) under the common name of paromomycin, was first reported from a *Streptomyces* species[113] and to date, appears on the WHO essential 20th list of medicines[23]. Previous work by Ser *et al*. detected compound **(5)** in *Streptomyces* extract of with potent antioxidant and anticancer activity[41].

Conclusion

The mangrove-derived *Streptomyces* sp. MUSC 11 has exhibited antioxidant activities against a number of comply used antioxidant assays. The study was also able to justify that its antioxidant property was in part due to the presence of a phenolic compound. Investigation of the chemical constituents in methanolic extract revealed 11 chemical compounds with interesting bioactivities. In general, the study identified *Streptomyces* sp. MUSC 11 emerging from mangrove forest as an untapped resource of antioxidant metabolites warranting future investigation.

Author Contributions

K-HM, LT-HT, L-HL, and B-HG performed the experiments, data analysis and writing of the manuscript. Technical supports and proofreading were provided by LT-HT, H-LS, JW-FL. L-HL, B-HG, K-GC contributed to the funding of the project. L-HL and B-HG founded the research project.

Conflict of Interest

The authors declare no conflict of interest pertaining to the publication of this article.

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