

Antibacterial, Anticancer and Neuroprotective Activities of Rare Actinobacteria from Mangrove Forest Soils

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Abstract Mangrove is a complex ecosystem that contains diverse microbial communities, including rare actinobacteria with great potential to produce bioactive compounds. To date, bioactive compounds extracted from mangrove rare actinobacteria have demonstrated diverse biological activities. The discovery of three novel rare actinobacteria by polyphasic approach, namely *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T from mangrove soils at Tanjung Lumpur, Peninsular Malaysia have led to the screening on antibacterial, anticancer and neuroprotective activities. A total of ten different panels of bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, ATCC 70069, *Pseudomonas aeruginosa* NRBC 112582 and others

were selected for antibacterial screening. Three different neuroprotective models (hypoxia, oxidative stress, dementia) were done using SHSY5Y neuronal cells while two human cancer cells lines, namely human colon cancer cell lines (HT-29) and human cervical carcinoma cell lines (Ca Ski) were utilized for anticancer activity. The result revealed that all extracts exhibited bacteriostatic effects on the bacteria tested. On the other hand, the neuroprotective studies demonstrated *M. mangrovi* MUSC 115^T extract exhibited significant neuroprotective properties in oxidative stress and dementia model while the extract of strain *M. flava* MUSC 78^T was able to protect the SHSY5Y neuronal cells in hypoxia model. Furthermore, the extracts of *M. mangrovi* MUSC 115^T and *M. flava* MUSC 78^T exhibited anticancer effect against Ca Ski cell line. The chemical analysis of the extracts through GC–MS revealed that the majority of the compounds present in all extracts are heterocyclic organic compound that could explain for the observed bioactivities. Therefore, the results obtained in this study suggested that rare actinobacteria discovered from mangrove environment could be potential sources of antibacterial, anticancer and neuroprotective agents.

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Keywords *Microbacterium mangrovi* MUSC 115^T · *Sinomonas humi* MUSC 117^T · *Monashia flava* MUSC 78^T · Antibacterial · Anticancer · Neuroprotective

Introduction

Actinobacteria is common soil inhabitant and have a high proportion of total microbial biomass in soil [1]. They are considered as the most economically significant as well as biotechnologically valuable microbe, producing bioactive compounds including antibiotics, antimicrobial, anticancer,

antitumor, enzyme, enzyme inhibitors and immunosuppressive agents [2]. Actinobacteria such as *Streptomyces* are excellent producer of bioactive compounds especially secondary metabolites [3–8]. Over 10, 000 bioactive compounds were derived from actinobacteria species, 7600 (76%) compounds were derived from *Streptomyces* while 2400 (24%) compounds isolated from rare actinobacteria [9]. Unfortunately, repeated isolation of known compounds and a reduced hit-rate of novel compounds have limited the development of new and effective drugs to treat ever increasing human diseases. [10–12]. At the same time, the arising multi drug resistance (MDR) pathogen and other deadly diseases caused the dramatic increase in demand to look for new compounds [13] from other sources such as rare actinobacteria.

Previously, the numbers of rare actinobacteria being discovered were low, as compared to *Streptomyces*. This is due to the facts that they are difficult to isolate, cultivate and maintain under conventional conditions [14]. However, the number of novel rare actinobacteria is increasing, from only 11 genera in 1970 to 220 genera by 2010 [15]. At the time of writing (March 2016), there are approximately 340 genera of rare actinobacteria (www.bacterio.net) discovered from various environment thus demonstrating that rare actinobacteria are widely distributed in the biosphere. According to Goodfellow [16], there are a low number of rare actinobacteria isolated from marine environments such as the mangrove. Thus, mangrove environment has gained attention from the researcher due to its location where it situated at the inter-phase between the terrestrial and marine environment, and have a special condition such as high moisture, high salinity and hypoxia tolerant [17]. This condition breeds many novel microorganism including rare actinobacteria that contained special and unique metabolic pathways to adapt with those conditions and lead to the production of valuable metabolites [17].

The rare actinobacteria strains discovered in previous work were *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T. These strains were isolated from mangrove soils located at Tanjung Lumpur, Peninsular Malaysia. The taxonomic status of these strains was described in previous publications using polyphasic approach [18–20]. Currently, the study of bioactive compounds from mangrove rare actinobacteria become popular as they possess great potential to pharmaceutical industry [3, 10, 14]. An example, Mangamuri et al. [21] reported that the bioactive metabolites from *Pseudonocardia endophytica* VUK-10 was able to inhibit the growth of Gram-positive and Gram-negative bacteria, yeast, fungi and also exhibited potent cytotoxic activity against human breast adenocarcinoma

cell line (MDA-MB-231, MCF-7), human cervical cell line (HeLa), human ovarian cyst adenocarcinoma cell line (OAW-42). Janardhan et al. [22] showed the extracts of strain *Nocardioopsis alba* isolated from mangrove soil of Nellore regions, Andhra Pradesh, India, exhibited potent total antioxidant property. Novel anticancer and anti-infection compounds are being isolated from mangrove rare actinobacteria, as represented by the discovery of the salinosporomide A, an anti-cancer compound produced by *Salinispora tropica* [23].

In light of this, the present study was initiated to investigate the extracts of rare actinobacteria collected from Tanjung Lumpur, Peninsular Malaysia for its biological activity such as antibacterial, anticancer or neuroprotective activity.

Materials and Methods

Preparation of *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T Extracts

All novel strains were grown on ISP2 medium for 5 days prior to fermentation process. The fermentation medium used was FM3 [10, 24] with slight modification and autoclaved at 121 °C for 15 min. The strains were cultured at 200 rpm, for 7–10 days at 28 °C. The resulting fermentation media were separated from the mycelium by centrifugation at 4500 rpm at 4 °C for 30 min. The supernatant was collected and subjected to freeze dry process. Upon freeze-drying, the sample was extracted with methanol for 72 h (ratio 3:1; methanol:sample) and the residue was re-extracted under the same condition twice at 24 h interval with ratio of 2:1 and 1:1, respectively. All the methanol-containing extract was filtered and evaporated using a rotary vacuum evaporator and the extract were kept in –20 °C until further analysis [25].

Bacterial Strains

Ten different pathogens were used for the antibacterial screening; namely *Acinetobacter calcoaceticus* NBRC 13006, *Salmonella typhi* ATCC 19430, *Escherichia coli* ATCC 25922, *Vibrio parahaemolyticus* VP103 (Jeffrey Cheah School of Medicine and Health Science laboratory), *Pseudomonas aeruginosa* NRBC 11258, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, ATCC 70069, ATCC 33591, ATCC BAA-44, *Bacillus subtilis* ATCC 31098. The test organisms were maintained on Mueller–Hinton agar (MHA).

Minimal Inhibitory Concentration (MIC) Determination

Minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that able to inhibit the growth of particular bacterium after overnight incubation. The work was performed by the broth microdilution method in 96 well plate as described by Wiegand et al. [26], with slight modification. Chloramphenicol (0.1 mg/mL) was used as positive control and untreated bacterial culture was used as negative control. One hundred microliters aliquot of the bacteria was added into the wells with an approximate inoculum of 1×10^6 CFU/mL, previously prepared as a 0.5 McFarland's standard. Serial dilutions of the extracts were done to achieve the final concentration of 5, 2.5, 1.25, 0.625 and 0.313 mg/mL. Aliquot (100 μ L) of the extract with different concentration was added into each of the wells and incubated at 37 °C for 24 h. The MIC was determined by assessment of turbidity by optical density readings at 600 nm.

Minimal Bactericidal Concentration (MBC) Determination

Minimal bactericidal concentration (MBC) is the lowest concentration of an antimicrobial that prevent the growth of particular microorganism. The MBC was determined by sub-culturing 100 μ L from well that exhibited no growth onto MHA and incubated at 37 °C for 24 h.

Cell lines Maintenance and Growth Condition

The human cancer cell lines (HT-29 and Ca Ski) and the neuronal cell lines (SH-SY5Y) involved in this study was maintained in Roswell Park Memorial Institute (RPMI) and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% fetal bovine serum and $1 \times$ antibiotic–antimycotic at 37 °C humidified incubator containing 5% CO₂ [6].

Neuroprotective Assay

Cell viability of neuronal cells were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, SHSY-5Y cells were seeded into a microtitre plate at a density of 3×10^4 cells/well and allowed to adhere overnight. 20 μ L of each extracts were added into the cells with the final concentration ranging from 6.25 to 200 μ g/mL. Catechin (100 μ M) (oxidative stress and hypoxia model) or gallic acid (1 μ g/mL) (dementia model) were used as a positive control in the experiments of the study. The pre-treated cells were incubated for 2 h followed by either 250 μ M hydrogen

peroxide (H₂O₂), 400 μ M streptozotocin (STZ) or 5 mM cobalt (II) chloride (CoCl₂) treatment for 24 h.

Anticancer Activity of the Extract on Human Cancerous Cells

The effect of extracts on cell viability of human cancer cells lines was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into a microtitre plate at a density of 5×10^3 cells/well and allowed to adhere overnight. 20 μ L of each extracts were added into the wells with the final concentration ranging from 6.25 to 200 μ g/mL and incubated at 72 h. Curcumin (3 μ g/mL) was included as positive control.

MTT Assays

The MTT assay for neuroprotective and anticancer activities were performed by adding 20 μ L of MTT (5 mg/mL) into each well and the plates were incubated at 37 °C containing 5% CO₂ for 4 h [25]. After the incubation period, the medium was then aspirated carefully and 100 μ L of DMSO was added. The absorbance of the product was determined spectrophotometrically at 570 nm, with 650 nm as reference using a microplate reader. The percentage of cell viability was calculated as follows:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells} \times 100\%}$$

Statistical Analysis

All values expressed as mean \pm standard deviations (SD) by Microsoft Excel. Data were analyzed for statistical significance using one-way ANOVA, followed by Dunnett's test as a post hoc test with GraphPad Prism 6.0 software for Windows (Inc., San Diego, USA).

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was performed in accordance with our previous developed method with slight modification [27, 28]. The instrument used was Agilent Technologies 6980N (GC) equipped with 5979 Mass Selective Detector (MS), HP-5MS (5% phenyl methyl siloxane) capillary column of dimensions 30.0 m \times 250 μ m and helium as carrier gas at 1 mL/min. The column temperature was programmed initially at 40 °C for 10 min, followed by an increase of 3 °C/

min to 250 °C and was kept isothermally for 5 min. The MS was operating at 70 eV. The constituents were identified by comparison of their mass spectral data with those from NIST 05 Spectral Library.

Results and Discussions

Antibacterial Assays

Results of antibacterial activity are represented in Table 1. Showed that all extracts demonstrated good inhibitory activity against different bacteria tested, with MICs values of 2.5 mg/mL, except for *P. aeruginosa* NRBC 112582, *V. parahaemolyticus* VP103 and *E. coli* ATCC 25922, where the inhibitory concentration was 1.25 mg/mL. These findings indicate that the extracts inhibited the growth of the bacteria tested at low concentrations.

The MBC result in Table 1 presented *M. mangrovi* MUSC 115^T and *S. humi* MUSC 117^T extracts were completely inhibiting the growth of *P. aeruginosa* NRBC 112582, *S. typhi* ATCC 19430 and *E. coli* ATCC 25922 at a concentration of 5 mg/mL. However, a higher concentration might be needed for complete inhibition of the growth of the other bacteria tested.

In addition, some of the extracts displayed bactericidal effects on few numbers of bacteria. According to Ocampo et al. [29], bacteriostatic can be defined as the agent inhibit

the growth of bacteria without killing effects, while bactericidal means agents that kill bacteria. An extract is considered as bactericidal when the ratio of MBC/MIC is ≤ 4 and bacteriostatic when this ratio is >4 [30]. This effect was observed with the *M. mangrovi* MUSC 115^T extract against *P. aeruginosa* NRBC 112582, *S. typhi* ATCC 19430, and *E. coli* ATCC 25922 with the ratios of MBC/MIC equal to 4, 2, and 2 respectively. The *S. humi* MUSC 117^T extract was also bactericidal against *E. coli* ATCC 25922 with the ratio of MBC/MIC equal to 2. Overall, the extracts of each strains possessed activity to inhibit the growth of bacteria tested.

Neuroprotective Assays

In this study, the neuroprotective assays were performed by using three different experimental models focusing on hypoxia, oxidative stress and dementia. Results of each experimental model were shown in Figs. 1, 2 and 3, respectively.

Neuroprotective Property of Extracts on Hypoxia Induced Cytotoxicity

Hypoxia can be defined as the reduction or lack of oxygen in organs, tissues or cells. A common experimental model of hypoxia was created using a transition metal, cobalt (II) chloride (CoCl₂) [31, 32]. CoCl₂ is a chemical agent that

Table 1 Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and MBC/MIC ratios of the extracts of strains *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T

Bacterium tested	MUSC 115 ^T MIC	MUSC 117 ^T MBC	MUSC 78 ^T MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
* <i>Staphylococcus aureus</i> ATCC 43300	2.5	>5	–	2.5	>5	–	2.5	>5	–
* <i>Staphylococcus aureus</i> ATCC 70069	2.5	>5	–	2.5	>5	–	2.5	>5	–
* <i>Staphylococcus aureus</i> ATCC 33591	2.5	>5	–	2.5	>5	–	2.5	>5	–
* <i>Staphylococcus aureus</i> ATCC BAA-44	2.5	>5	–	2.5	>5	–	2.5	>5	–
<i>Acinetobacter calcoaceticus</i> NBRC 13006	2.5	>5	–	2.5	>5	–	2.5	>5	–
<i>Bacillus subtilis</i> ATCC 31098	2.5	>5	–	2.5	>5	–	2.5	>5	–
<i>Pseudomonas aeruginosa</i> NRBC 112582	1.25	5	4	1.25	>5	–	1.25	>5	–
<i>Salmonella typhi</i> ATCC 19430	2.5	5	2	2.5	>5	–	2.5	>5	–
<i>Vibrio parahaemolyticus</i> VP103	1.25	>5	–	1.25	>5	–	1.25	>5	–
<i>Escherichia coli</i> ATCC 25922	2.5	5	2	1.25	5	2	1.25	>5	–

* Methicillin-resistant *Staphylococcus aureus* (MRSA)

* (–): not calculated for MBC/MIC as the MBC value was >5 mg/mL

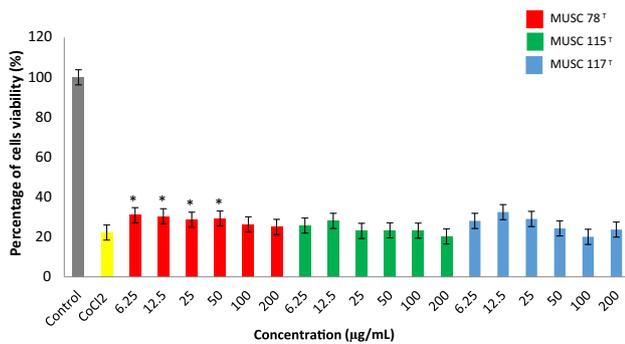


Fig. 1 The neuroprotective activity of methanolic extracts on the cell viability of SH-SY5Y cells treated with CoCl₂. Cells viability was measured using MTT assay. **p* < 0.05 indicates statistically significant differences compared to CoCl₂ induced cells

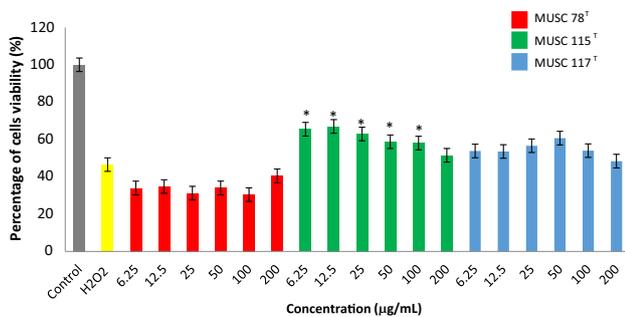


Fig. 2 The neuroprotective activity of methanolic extracts on the cell viability of SH-SY5Y cells treated with H₂O₂. Cells viability was measured using MTT assay. **p* < 0.05 indicates statistically significant differences compared to H₂O₂ induced cells

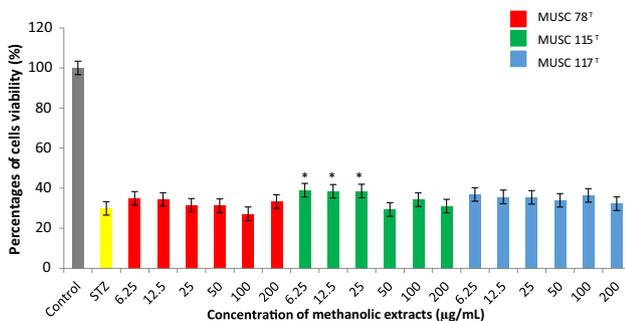


Fig. 3 The neuroprotective activity of methanolic extracts on the cell viability of SH-SY5Y cells treated with STZ. Cells viability was measured using MTT assay. **p* < 0.05 indicates statistically significant differences compared to STZ induced cells

reportedly induces a biochemical and molecular response similar to that observed under low-oxygen conditions in mammalian systems [33]. Beside it is widely used to establish the model of hypoxia in both in vitro and in vivo study. Theoretically, the Co²⁺ will replace the Fe²⁺ in heme on the cell surface, thus weaken the oxygen signaling and transport, leading to the generation of reactive oxygen species (ROS) and cell death [34]. In fact, a study done by

Lee et al. [35] and Vengellur and LaPres [36] have shown that both hypoxia and cobalt affecting a similar group of genes on a global gene expression level. This implies that the robustness and suitability use of this model of experiment for experimental purposes.

Figure 1 showed that the neuronal cells subjected to CoCl₂ exposure showed a significant reduction in viability of cells up to 77.8%. Based on the analysis, the extract of *M. flava* MUSC 78^T was able to protect the neuronal cells from the CoCl₂ insult at lower concentration; 6.25–50 μg/mL. The neuroprotective activity reduced when the concentration of the extracts reached at 50 μg/mL. The statistical analysis of *S. humi* MUSC 117^T and *M. mangrovi* MUSC 115^T extracts showed the percentages of cell viability for each concentration tested were not significant when compared to CoCl₂ induced cells and concluded that these two extracts were not able to protect neuronal cells from the hypoxia induced neuronal damage.

Neuroprotective Property of Extracts on Oxidative Stress Induced Cytotoxicity

One of the most common methods applied for studying the in vitro neuroprotective activity of antioxidants is H₂O₂ induced cytotoxicity [37, 38], hence this method was employed to study the extracts of each strain. The insults of H₂O₂ have been linked to the formation of oxidative stress which is known to cause neurodegenerative diseases such as Alzheimer’s [39] and Parkinson diseases [40]. H₂O₂ has a short half-life, and its dissociation into hydroxyl and superoxide ions may affect the membrane integrity and leading to cellular damage [39, 41]. In fact, H₂O₂ has been observed to exert toxic effect on different cell types while neuron was found to be most susceptible to H₂O₂-induced toxicity [42].

Based on Fig. 2, it was observed that only *M. mangrovi* MUSC 115^T extract was able to protect the neuronal cells against H₂O₂ challenge at low concentration, 6.25 μg/mL. Furthermore, there is a significant decreased in cell viability in *M. mangrovi* MUSC 115^T extract treated cells from 50 μg/mL to 200 μg/mL as compared to H₂O₂ control. This indicates the effect of the treatment reached its maximum efficacy at around 12.5 μg/mL. Further increase of treatment will eventually found to be toxic towards the neuronal cells. On the other hand, the *S. humi* MUSC 117^T and *M. flava* MUSC 78^T extracts were found to exhibit no protective activity on SH-SY5Y neuronal cells when challenged by H₂O₂.

Neuroprotective Property of Extracts on Dementia Induced Cytotoxicity

Dementia is known as a multisystem-related neurodegenerative disorder. A set of symptoms are associated to this

disease which include impairment in short- and long-term memory, impairment in thinking, judgment, other disturbance of higher cortical function, or personality change [43]. In order to understand the pathological aspect of dementia in human, researcher have made use of STZ as an inducer in rats to create the experimental model of dementia [44]. It was also commonly utilized in preparing the in vitro dementia model of experiment particularly on SH-SY5Y neuronal cells [45]. The induction of STZ was found to generate excessive free radicals which leading to formation of oxidative stress [46], inflammation [47], abnormal protein [48] and leads to mitochondrial dysfunction and apoptosis in cell [49].

Figure 3 demonstrated the result of neuroprotective activity of extracts on dementia model of experiment. From the data, the percentage of cell viability of SH-SY5Y neuronal cells treated with STZ only was found to be significantly ($p < 0.05$) reduced up to about 70.0%. The pre-treatment of *M. flava* MUSC 78^T and *S. humi* MUSC 117^T extracts on the STZ treated cells showed that both of these extracts were unable to protect SH-SY5Y cells from the STZ induced neuronal damage. However, only *M. mangrovi* MUSC 115^T extracts treatment was found to demonstrating neuroprotective activity at different concentration ranging from 6.25 to 25 $\mu\text{g/mL}$.

Anticancer Activity of the Extracts on Human Cancerous Cells

Rare actinobacteria, represent a promising reservoir of different kinds of therapeutics drugs. In this study, the anticancer effect of the extracts were tested on two different types of human cancer cell lines; human colon cancer cell lines (HT-29) and human cervical carcinoma cell lines (Ca Ski). The effects of the extract on the tested cancerous cells are shown Figs. 4 and 5.

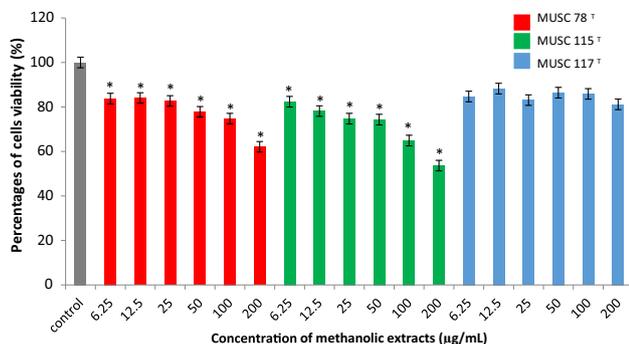


Fig. 4 The anticancer activity of methanolic extracts on the cell viability of Ca Ski cells. Cells viability was measured using MTT assay. * $p < 0.05$ indicates statistically significant differences compared to untreated cells

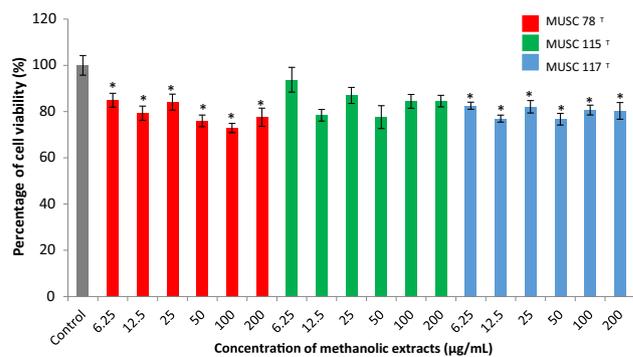


Fig. 5 The anticancer activity of methanolic extracts on the cell viability of HT-29 cells. Cells viability was measured using MTT assay. * $p < 0.05$ indicates statistically significant differences compared to untreated cells

All the extracts displayed varying levels of anticancer against the Ca Ski cells (Fig. 4). Interestingly, a dose-dependent response was observed for the *M. flava* MUSC 78^T and *M. mangrovi* MUSC 115^T extracts treatment as there was a significant reduction of the viability of cells when compared to the untreated cells. The Ca Ski cells was found to be the most vulnerable to the treatment of *M. mangrovi* MUSC 115^T extracts with the strongest growth inhibition at high concentration tested (200 $\mu\text{g/mL}$), seconded by the treatment activity displayed by *M. flava* MUSC 78^T. In the case of *S. humi* MUSC 117^T extract, there was no significant reduction of the viability of Ca Ski cells as compared to control. Overall, *M. flava* MUSC 78^T and *M. mangrovi* MUSC 115^T extracts are effective in inhibiting the growth of Ca Ski cells.

Similarly, the anticancer effect of *M. flava* MUSC 78^T, *M. mangrovi* MUSC 115^T and *S. humi* MUSC 117^T extracts on HT-29 cells were examined as well. Results in Fig. 5 displayed that there is a mild growth inhibition activity of HT-29 cells as the viability of HT-29 cells was significantly reduced especially at the highest concentration of treatment at 200 $\mu\text{g/mL}$.

Taken altogether, the extracts of *M. mangrovi* MUSC 115^T, *S. humi* MUSC 117^T and *M. flava* MUSC 78^T were shown to be effective in causing cytotoxic effect on this two different cancer cell lines namely human colon cancer cell lines (HT-29) and human cervical carcinoma cell lines (Ca Ski). The results of the studies also demonstrated that two cancer cell lines showed different reaction towards the concentration of extracts tested. *M. mangrovi* MUSC 115^T and *M. flava* MUSC 78^T extracts exhibiting a cytotoxic activity on Ca Ski cells except for *S. humi* MUSC 117^T, meanwhile all the extracts exhibiting a low anticancer activity against HT-29 cells. In general, varying strength at the effect possessed by extracts are most likely affected by the differences in the chemical composition that present in.

Chemical Profiling Analysis

Following the assessment of bioactivities possessed by *M. mangrovi* MUSC 115^T, *S. humi* MUSC 117^T and *M. flava* MUSC 78^T extracts, GC–MS analysis were performed in order to analyze the chemical constituents that present in the extracts. GC–MS is an effective combination of technologies which meant for the analysis of chemical compounds. Basically, the compounds will be separated by GC while MS generates the characteristic mass profile for each of the compounds detected [50, 51]. As shown in Table 2, a total of six chemical compounds were identified in *M. mangrovi* MUSC 115^T extract, ten compounds were detected in *S. humi* MUSC 117^T extract while *M. flava* MUSC 78^T extract analysis yielded a total of twenty compounds. Through GC–MS analysis, the obtained results indicating the majority of the compounds that present in extracts are consisted of organic heterocyclic compounds. These heterocyclic compounds include phenolics, pyrazines, and pyrrolopyrazine.

Phenolic are a class of organic compound consisting of a hydroxyl group bonded to an aromatic hydrocarbon group. Among other heterocyclic organic compound, phenolic compounds have attracted the attention of researchers as they are well known for their antioxidant and free radical-scavenging abilities. These potent bioactivities are associated with potential beneficial effects on human health [52]. In fact, phenolic compounds have been reported to possess potent antioxidative, anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities [53–56]. Through GC–MS analysis, the phenolic compound known as, 2,4-di-tert-butyl phenol (2,4 DTBP) (**2**, **9**, **27**) was detected in all of the extracts tested in current study. Literature has shown that 2,4 DTBP can be produced by microorganisms such as fungus [57] and bacteria [58]. For example, this compound has been detected in *Pseudomonas monteilii* PsF84 and was found to be effective against *Fusarium oxysporum* [59]. Besides, the existence of this compound in *Lactococcus* sp was associated to its antifungal and antioxidant properties as well as its cytotoxic activity [58]. It was also reported that, the antibacterial activity of *Monochaetia kansensis* could be due to the presence of 2,4 DTBP as well [60].

Another group of compound that was found in this study was pyrazines. Pyrazines are known to exist in form of complex structure with the present of nitrogen atoms in their aromatic ring. These compounds are greatly known for their strong odor properties and have been detected in several bacteria. The value of this group of compound reside with their bioactivities, as pyrazines are commonly known to exhibit antimicrobial, anticancer, antioxidant as well as neuroprotection properties [6, 61–64]. In current study, the pyrazines compounds; **19**, **21**, **22**, **23** and **24**

were found in *M. flava* MUSC 78^T extract. Previous study has shown that compound **19** and **21** were detected in myxobacteria *Stigmatella* WXNJ-B which known to exhibit a significant high level of antitumor activities [65]. Meanwhile, compound **22**, **23** and **24** were detected previously in *Streptomyces antioxidans* and were found to have a strong antioxidant activities [64].

The complex structure form with incorporation of one or more pyrrole compound into a pyrazine is normally known as pyrrolopyrazine. The GC–MS characterization analysis have also demonstrated the existence of pyrrolopyrazine compounds in all of the extracts. For example, compounds such as pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (**4**, **11**, **32**) and (3R,8aS)-3-methyl-1,2,3,4,6,7,8,8a-octahydro-pyrrolo[1,2-a]pyrazine-1,4-dione (**3**, **10**, **31**) were found as the constituents of the mixture. Literature has shown that these compounds were detected in different *Streptomyces* species [6, 64, 66] which include *Nocardia* sp. [67], and *Bacillus* sp. [68] and was associated to antioxidant activity. Another pyrrolopyrazine compound identified in MUSC 115^T and MUSC 78^T strains was 3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane (BDDB) (**6**, **36**). Gohar et al. [69] have reported the present of BDDB in *Burkholderia cepacia* may responsible for the antibacterial activity against *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio ordalii*. Besides, the detection of BDDB in *Streptomyces cacaoi* GY525 [70] was believed to contribute for the mortality of second-stage juvenile and hatch inhibition of *Meloidogyne incognita*. Therefore, the detection of pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (**16**) in *S. humi* MUSC 117^T and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (**35**) in *M. flava* MUSC 78^T might contribute for the observed bioactivities. In fact, these compounds were seen to occur in quite a number of different *Streptomyces* species which have demonstrated to exhibit a wide range of bioactivities [6, 64, 66, 71, 72]. For example, the present of these compounds in microorganism has been associated to the strong antibacterial activity against *E. coli*, *P. aeruginosa* and *E. faecalis* [73]. Besides, Hong et al. (2008) [74] have also showed **16** was able to inhibit expression of serine/threonine kinase Akt which may be useful for inhibition of cell proliferation and activation of apoptosis activity in cancer cells. Perhaps, the detection of 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane (**6**, **17**) in *M. mangrovi* MUSC 115^T and *S. humi* MUSC 117^T may explain for the anticancer activity exhibited by these microorganisms. As the present of those compounds in *Streptomyces* strains of previous studies were suggested to be responsible for the observed cytotoxic effect on human cancer cell line [5, 75]. Overall, majority of the pyrrolopyrazine compounds detected are known to exhibit antioxidant activity. Since antioxidants were suggested to play important role in

Table 2 Compounds identified from *Microbacterium mangrovi* MUSC 115^T, *Sinomonas humi* MUSC 117^T and *Monashia flava* MUSC 78^T by using GS-MS

Extract	No	Retention time (min)	Compound	Formula	Molecular weight (MW)	Quality (%)
MUSC 115 ^T	1	9.684	Methylaurate	C ₈ H ₁₀	106	80
	2	44.422	2,4-di-tert-butyl phenol	C ₁₄ H ₂₂ O	206	97
	3	51.592	(3R,8aS)-3-methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	C ₈ H ₁₂ N ₂ O ₂	168	90
	4	53.188	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C ₇ H ₁₀ N ₂ O ₂	154	96
	5	59.008	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	C ₁₁ H ₁₈ N ₂ O ₂	210	83
	6	70.761	3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	C ₁₄ H ₁₆ N ₂ O ₂	244	76
MUSC 117 ^T	7	9.936	Butanoic acid, 3-methyl-	C ₅ H ₁₀ O ₂	102	72
	8	10.880	Butanoic acid, 2-methyl-	C ₅ H ₁₀ O ₂	102	53
	9	44.428	2,4-di-tert-butyl phenol	C ₁₄ H ₂₂ O	206	95
	10	51.563	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	C ₈ H ₁₂ N ₂ O ₂	168	90
	11	53.137	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C ₇ H ₁₀ N ₂ O ₂	154	96
	12	54.865	Methyl n-pentadecanoate	C ₁₆ H ₃₂ O ₂	256	93
	13	59.025	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	C ₁₁ H ₁₈ N ₂ O ₂	210	64
	14	59.174	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine	C ₁₄ H ₂₂ N ₂ O ₂	250	53
	15	61.462	Methyl 14-methylhexadecanoate	C ₁₈ H ₃₆ O ₂	284	93
	16	70.749	pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C ₁₄ H ₁₆ N ₂ O ₂	244	92
MUSC 78 ^T	17	7.538	2-Methylpyrazine	C ₅ H ₆ N ₂	94	80
	18	9.181	Pyrrole, 2-methyl-	C ₅ H ₇ N	81	80
	19	13.438	Pyrazine, 2,5-dimethyl-	C ₆ H ₈ N ₂	108	80
	20	17.094	2,3,4-Trithiapentane	C ₂ H ₆ S ₃	126	72
	21	19.383	Pyrazine, 2-ethyl-6-methyl-	C ₇ H ₁₀ N ₂	122	60
	22	19.480	Pyrazine, 2-ethyl-5-methyl-	C ₇ H ₁₀ N ₂	122	95
	23	19.555	Pyrazine, trimethyl-	C ₇ H ₁₀ N ₂	122	87
	24	24.184	Pyrazine, 3-ethyl-2,5-dimethyl-	C ₈ H ₁₂ N ₂	136	90
	25	25.900	4H-Pyran-4-one, 3-hydroxy-2-methyl-	C ₆ H ₆ O ₃	126	70
	26	34.935	1H-Indole	C ₈ H ₇ N	117	95
27	44.439	2,4-di-tert-butyl phenol	C ₁₄ H ₂₂ O	206	96	
28	45.567	1H-Pyrrole, 2-phenyl-	C ₁₀ H ₉ N	143	87	
29	49.475	1-Naphthalenamine, N-ethyl-	C ₁₂ H ₁₃ N	171	90	
30	50.213	3,4-Dimethyl-2-phenyl-1H-pyrrole	C ₁₂ H ₁₃ N	171	72	
31	51.649	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	C ₈ H ₁₂ N ₂ O ₂	168	90	
32	53.349	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C ₇ H ₁₀ N ₂ O ₂	154	97	
33	54.596	Methyl 13-methyltetradecanoate	C ₁₆ H ₃₂ O ₂	256	98	
34	57.995	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270	93	
35	59.122	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C ₁₁ H ₁₈ N ₂ O ₂	210	95	
36	70.743	3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	C ₁₄ H ₁₆ N ₂ O ₂	244	92	

cellular mechanisms [76], the detection of pyrrolopyrazine compounds in these strains of bacteria could be contributing to the observed cytotoxic effects on cancer cells and neuroprotective effect on SH-SY5Y cells against the insults of H₂O₂.

Taken altogether, the existence of heterocyclic compounds such as phenolics, pyrazines and pyrrolopyrazines as part of the constituents of *M. mangrovi* MUSC 115^T, *S. humi* MUSC 117^T and *M. flava* MUSC 78^T extracts may account for the observed antibacterial, anticancer activities

as well as the neuroprotective properties. Based on current findings, rare actinobacteria may serve as important sources for the potential new drugs development.

Conclusions

The results have demonstrated *M. mangrovi* MUSC 115^T, *S. humi* MUSC 117^T and *M. flava* MUSC 78^T possessed antibacterial, anticancer and neuroprotective activities. The chemical analysis study afforded a further in depth understanding on the mixture of chemical constituents that present in these strains of bacteria. Based on the literature evidences, the occurrence of these chemical compounds might accounted for the observed bioactivities. In short, the current study has showed these novel rare actinobacteria were able to produce a wide range of bioactive compounds which could serve as potential sources for future drug development. Further in depth studies focusing on isolation and characterization of bioactive principle(s) through bioassay-guided isolation is currently undertaking. As we deeply believe the procedure will eventually enabling us to identify the bioactive principle(s) that present in these mixtures and the findings might potentially generate useful knowledge for the future development of new drug(s).

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