

Anti-Microbial Properties of Secondary Metabolites of Marine *Gordonia tearrae* Extract

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Abstract

The objective of this study is to screen four species of marine actinomycetes: *Gordonia* (SPTG111), *Rhodococcus* sp (BP33), *Gordonia* sp (BP5), *Brevibacterium antaracterium* (SPTG45). These bacteria were grown in seven different media, namely Braine Heart Infusion broth (BHIB), Nutrient broth, Marine broth, Trypticasein Soy Broth, Tryptic Soy Broth, Luria Bertani broth, and Lauryl Sulfate Broth. Brine heart infusion broth (BHIB) was used to growth bacteria for the antibacterial activity test. Crude extract of *Gordonia tearrae* was tested for antimicrobial activity against *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Candida albicans* using agar diffusion method. The minimal inhibitory concentration (MIC) of *G. tearrae* crude extract was determined by the microbroth dilution method. Ethyl acetate extract of this isolate showed significant antibacterial activity against Gram negative bacteria *E. coli* (20 mm), *Pseudomonas aeruginosa* (9 mm), *Salmonella typhi* (12 mm) and Gram positive bacteria *E. faecalis* (7 mm), *Bacillus cereus* (21 mm), and MRSA (30 mm), and yeast *C. albicans* (21 mm). The MIC value of antimicrobial activity ranged from 25 to 6.3 µg/ml.

Keywords: *Gordonia tearrae*, BHIB, yield of extraction, antibacterial, antifungal, MIC

1. Introduction

Infectious disease mortality rates are increasing in developed countries (Pinner et al., 1996). Jones et al. (2008) reported the emergence of 335 infectious diseases between 1940 and 2004 in the global human population. These negative health trends call for a renewed interest in infectious disease as well as effective strategies for treatment and prevention. Natural products are one of the most important sources of antibiotics (Bull & Stach, 2007). With respect to the development of new antimicrobials, the marine environment holds great promise for the discovery of novel bioactive compounds. Marine sponges are among the most ancient multicellular animals. These sessile, filter feeding animals are a rich source of novel biologically active metabolites and offer great potential for drug discovery and, in the long term, for treatment of cancer and infectious diseases (Blunt et al., 2007). Members of the phylum Actinobacteria and specifically the order Actinomycetales have been identified as abundant members of sponge-associated microbial communities (Hentschel et al., 2002; Zhang et al., 2006). Their existence in the marine environment has been further shown in marine sediments as well as in the deepest ocean trenches (Bredholdt et al., 2007; Maldonado et al., 2005). Actinomycetes are of considerable interest owing to their ability to produce new chemical entities with diverse pharmacological activities. Marine actinomycetes in particular have yielded numerous novel secondary metabolites (Lam, 2006). Several antibiotics have already been derived from marine actinomycetes (Baltz, 2008) and at present, two thirds of natural antibiotics are obtained from actinomycetes. They also serve as alternative sources of biologically active substances (Behal, 2003) especially in combating infectious diseases which today are leading health problems with high morbidity and mortality in the developing countries (Black et al., 1982). Several reports are available on antibacterial and antifungal activity of marine actinomycetes (Suthindhiran & Kannabiran, 2009; Bredholt et al., 2008) but relatively few studies are available for *Gordonia* species. *Gordonia tearrae* is one of 27 *Gordonia* species of marine actinomycetes (Arenskotter et al., 2004). Previously known as "*Gordonia tearrae*" and "*Rhodococcus tearrae*", it is a nocardioform actinomycetes isolated from soil and water (Tsukamura, 1971; Stackebrandt, 1997). In this study,

we report the antimicrobial activities of methanol crude extract of *Gordonia tearrae* (SPTG 11-1) which isolated from pulau tinggi sponge.

2. Materials and Methods

2.1 Actinomycetes Bacteria Collection

Four actinomycetes includes *Gordonia* (SPTG 11-1), *Brevibacterium antarctarium*. (SPTG 45), were isolated from (Pulau Tinggi sponge), and *Gordonia* sp (BP5), *Rhodococcus* sp (BP 33), were isolated from (Langkwi Kedai Sediment) in Malaysia. They were collected from National University of Malaysia microbe culture unit, and cultured in starch casein agar, marine agar, Actinomycetes isolation agar. These Incubated at 30°C, 1-2 weeks.

2.2 Microbial Pathogens

Bacterial and fungal pathogens tested include. *Escherichia coli* (ATCC 10536), *Salmonella typhi* (ATCC51312), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC14506), *Bacillus cereus* (ATCC11774), *MRSA* (ATCC11632) and *Candida albicans* (ATCC10231). This strain was maintained on nutrient agar slants (NA; Difco, USA) at 4°C. Test cultures were obtained from the National University of Malaysia microbe culture collection unit.

2.3 Physiological and Morphological Characterization of the *Gordonia*

Physiological and morphological characterization of *Gordonia* SPTG111 isolate was determined as described by the Bergey's Manual of determinative bacteriology. Following the observation of the colony morphology of *Gordonia* on BHIA, Gram staining of overnight pure cultures was subsequently done. The presence of respiratory enzymes, Oxidase and Catalase were investigated using standard methods as described as Kovacs (1956) and Vera and Power (1980). Motility test was conducted using SIM medium. Different salt concentrations of different media were used for salt tolerance assay.

2.4 Molecular Taxonomy, Sequencing and Phylogenetic Analysis

The genomic DNA of *Gordonia* SPTG111 was isolated by CTAB/NaCl method. The isolate was grown in 50 ml of BHI broth using the universal primers (published forward primer (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer (5' GGT TAC CTT GTT ACG ACT 3') for the amplification of DNA. These sequences were acquired from FirstBase Laboratory, Sdn. Bhd., Malaysia. A final PCR reaction volume of 50 µl containing 1 µl supernatant containing DNA, 1 x GoTaq Flexi Buffer (Promega, USA), 2 mM MgCl₂ (Fermentas, USA), 1 µM of each forward and reverse primer, 200 µM each dNTP and 2.5 U Taq polymerase. Amplification was carried out in a Mini-cycler (MJ Research, USA). The PCR protocol used was 95°C for 2 min, 51°C for 30 s, 72°C for 45 s, 22 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 45 s. Amplification was followed by a final extension at 72°C for 2 min. After the PCR reaction was completed, 10 µl of obtained PCR product was electrophoresed on a 1% 1× Tris-acetate-EDTA agarose gel containing 1:10,000 gel red. A 1kb DNA ladder (Promega, USA) was also included in the run. PCR products were purified using QIAquick Purification Kit (QIAGEN, Germany) according to manufacturer's instructions. Sequencing was carried out by automatic DNA sequencing machine (ABI Prism 377) at FirstBase Laboratory, Sdn Bhd, Malaysia. Phylogenetic tree was generated by means of neighbour relationships using MEGA software version 4.0.2.

2.5 Scanning Electron Microscopy (SEM)

BHI agar containing isolates that have been incubated for 5 days were cut into small (1 cm³) pieces using a sterile spatula. For the purpose of fixing, these pieces were immersed in glutaraldehyde 2.5% (w/v) and kept at 4°C for 24 hours. The samples were subsequently washed three times with phosphate pH 7 buffer solution for 10 minutes. Then the samples were dehydrated by an ethanol series beginning with 30% and 50% for 15 minutes and 70% for 24 hours at 4°C. Extended hydration process was achieved using ethanol 80, 85, 90 and 95% for 15 minutes. Specimens in 100% ethanol were critical-point dried in a CO₂ atmosphere, gold-coated for 5 minutes and observed under scanning electron microscope (SEM Philips XL30 model).

2.6 Evaluation of Different Media Broth for Bacterial Growth

A modified method of (Augustine et al., 2005) was used. The bacterial isolates were inoculated into seven different media, Brain Heart Infusion Broth (BHIB), Nutrient Broth (NB), Marine Broth (MB), Trypticasein Soy Broth (TrySB), Tryptic Soy Broth (TSB), Luria Bertani Broth (LBB), and Lauryl Sulfate Broth (LSB). These were then incubated for 5-7 days at 30°C and a shaking speed of 200rpm. To extract bioactive metabolites compounds, the bacterial cultures obtained were thereafter centrifuged for 15 min at 10,000 rpm. Cell-free supernatants obtained were extracted and mixed with an equal volume of ethyl acetate and kept in rotary shaker

overnight (200 rpm). The solvent layers were collected and then evaporated in a rotary evaporator to obtain crude extracts filtered using 0.45 µm membrane filter, for use in screening for antimicrobial activity.

2.7 Antimicrobial Activity Assay of *Gordonia Tearrae* Strain

The crude extract of *Gordonia* SPTG111 obtained was dissolved in methanol used for testing against bacterial and fungal pathogens by disc diffusion method as described by Berghe and Vlietinck (1991). This crude was pipetted on the sterile paper disk (AA Whatman, Healthcare, UK, Limited) and left to air dry room temperature around 6 hours. Using sterile swab to streak the pathogens bacteria were following a 0.5 McFarland turbidity standard followed by placing the disc containing 30 µg crude on the agar. The plates were incubated at 37°C for 24 h during which antimicrobial activity was evidenced by the presence of a clear zone of inhibition surrounding the disk. Each test was repeated three times and the antimicrobial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolite when compared to chloromphenicol 30 µg as a control.

2.8 Minimum Inhibition Concentration (MIC)

The MIC of the crude extract of *Gordonia tearrae* was determined using microbroth dilution method (Carbounelle et al., 1987) in Mueller Hinton broth. The MIC was considered as the lowest concentration of the crude extract which inhibited visible growth of the microbe in Mueller Hinton or Sabouraud broth supplemented with 10% glucose and 0.5% phenol red. For susceptibility testing, in a first step Mueller Hinton broth (50 µl) was distributed from the first to the twelfth test tubes. Dry extracts was dissolved in DMSO (100 µl) and subsequently in Mueller Hinton broth, to reach a final concentration of 1000 µg/ml. These solution (50 µl) was added to the first well of each microtiter line. The dilutions was done by transferring the solution (50 µl) from the first to the eleventh tube. A volume of 50 µl was finally discarded from the eleventh tube. The twelfth tube served as growth control where no sample (extract, reference antibiotics) was added. A microbial suspension (50µl, 10⁵ colony forming units), obtained from an overnight growth at 37°C was added to each well. The final concentration of the extracts adopted to evaluate the antimicrobial activity ranged from 200 to 0.8 µg/ml. Tests were incubated aerobically at 37°C for 24 h before being read. The MIC was considered as the lowest concentration of the sample that prevented visible growth or changed in color from red to yellow due to the formation of acidic metabolites corresponding to microbial growth.

2.9 Statistical Analysis

The data were presented as mean ±S.D. Statistical computations were calculated using ANOVA one way for windows software. Differences were considered significant when $p < 0.05$.

3. Results

3.1 Physiological and Morphological Characterization of the *Gordonia Tearrae*:

This strain of *Gordonia tearrae* was observed to be deeply orange pigmented colony, and irregular and rough, aerobic, gram-positive, catalase- urease- oxidase positive, non motile, nocardioform (mixture of rod-shaped or coccoid elements) confirmed by the SEM image (Figure 1) that do not generate spores.

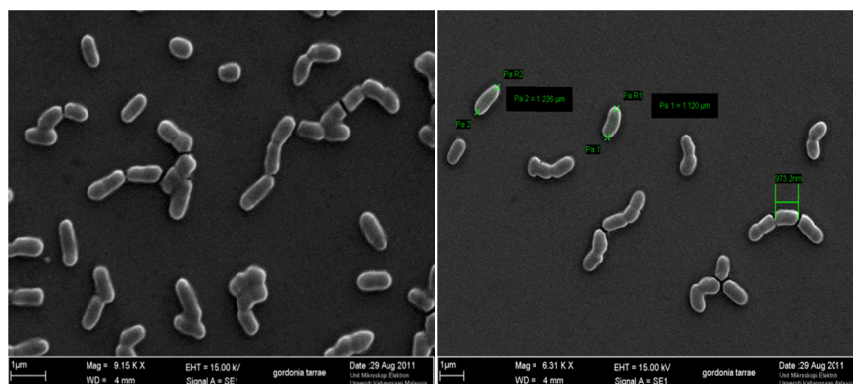


Figure 1. Scanning electron microscopy of *Gordonia tearrae*. Bar indicates 1 µm

3.2 Molecular Taxonomy, Sequencing and Phylogenetic Analysis

The almost complete 16S rRNA gene sequencing and NCBI blast search retrieved from GenBank showed a similarity of 100% alignment with the genus *Gordonia* and *Gordonia tearrae* being the closest fit (Figure 2). The size of PCR products obtained was ~1.5 kb, detected using 1% agarose gel electrophoresis.

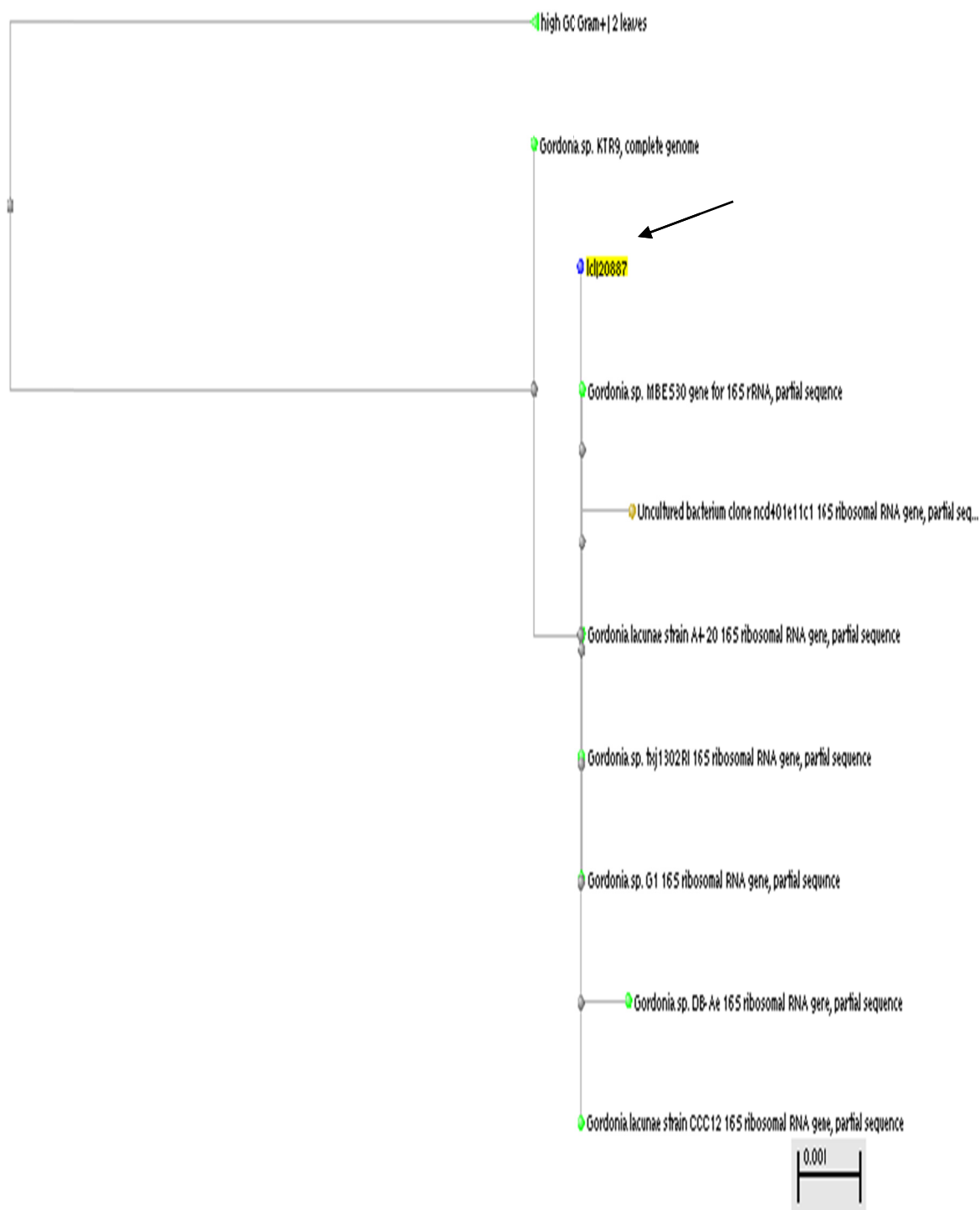


Figure 2. Phylogenetic relationship of *Gordonia* to related bacteria based on neighbour-joining tree analysis of 16S rRNA gene sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

3.3 Evaluation of Different Media Broth for Bacterial Growth

Gordonia tearrae showed maximum yield of extraction in BHIB compared with Trypticasein SB, LBB, TSB, MB, NB and LSB respectively. The significantly high level of secondary metabolites compounds ($p < 0.005$, $n=3$) (Figure 3).

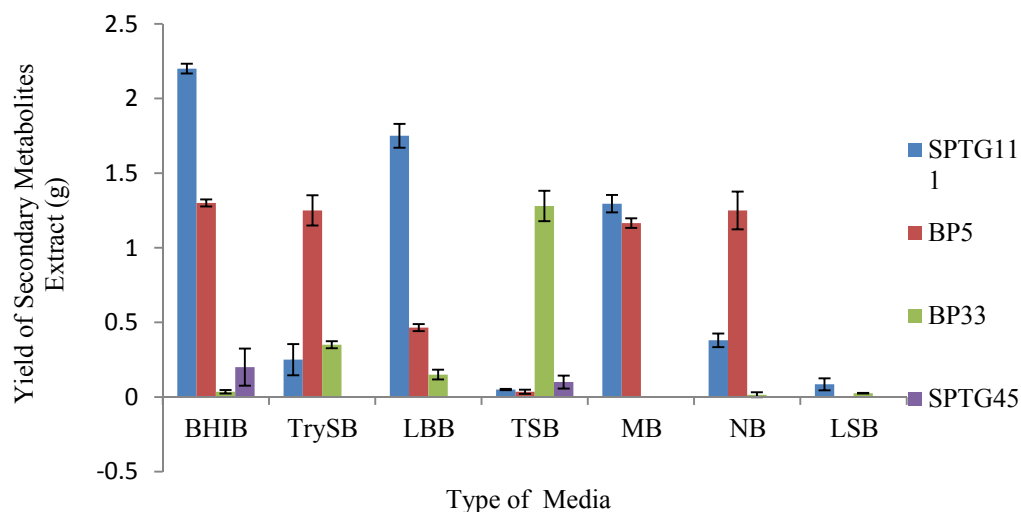


Figure 3. The yield of secondary metabolites of *Gordonia tearrae* SPTG111

3.4 Antimicrobial Activity Assay

The 30 µg/disc concentration of ethyl acetate crude extract of *Gordonia tearrae* principally extracted by BHIB, showed higher antimicrobial activities (Figure 4) with inhibition zones ranging from 7-30 mm as compared with BP3, BP5 and SPTG45 which were extracted with same media (range 1-25 mm) (Table 1). Of all the tested bacteria used in this study, antimicrobial effect was highest among *S. aureus* for *Gordonia tearrae* isolate in addition to BP33 and BP5. Notable also was the fact that extract from isolate SPTG45 was not active against most of the tested isolates on all four growth media. However, there were exemptions for *S.typhi* and *P. aeruginosa* where only small zones of inhibition were noticeable using BHI broth (Table 1). Only extract from *G. tearrae* was effective against MRSA in levels comparable to the control drug, Chloramphenicol.

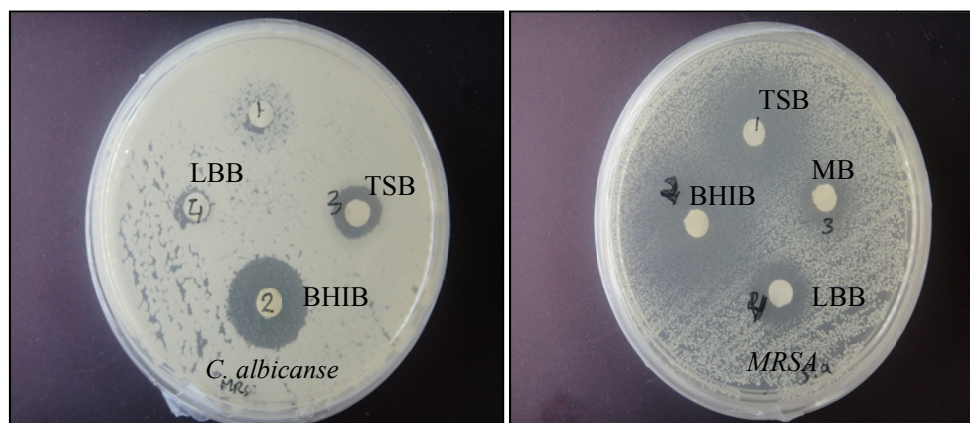


Figure 4. No 2 is antimicrobial activity of *Gordonia tearrae* crude extracted by BHI broth against *C.albicans* and *MRS*

Table 1. Antimicrobial activity of crude extracts of four marine actinomycetes (mm) extracted with different four selected media

Media	BHIB				LBB				MB				TSB				CH
Tested isolate	SPTG	BP	BP	SPTG	SPTG	BP	BP	SPTG	SPTG	BP	BP	SPTG	SPTG	BP	BP	SPTG	
	111	33	5	45	111	33	5	45	111	33	5	45	111	33	5	45	
<i>Escherichia. coli</i>	20	5	15	/	15	/	12	/	17	4	/	/	16	3	7	/	15
<i>Enterobacter.faecalis</i>	7	/	10	/	11	/	14	/	0	/	/	/	5	/	8	/	12
<i>Bacillus. cereus</i>	21	/	12	/	20	/	12	/	15	/	/	/	19	2	2	/	30
<i>MRSA</i>	30	/	/	/	1	/	/	/	10	/	/	/	4	/	/	/	30
<i>Salmonella. typhi</i>	12	2	5	3	6	/	6	/	6	2	1	/	1	/	1	/	17
<i>Pseudomonas .aeruginosa</i>	9	1	7	7	3.5	/	2	/	4	1	/	/	1.5	2	/	/	12
<i>Candida. albicans</i>	25	5	10	/	5	/	10	/	0	3	/	/	19	5	8	/	20

- (/) the sample was not active.

- (CH) Chloramphenicol (30 µg /disc).

- Brain Heart Infusion Broth-BHIB, Luria Bertani Broth- LBB, Marine Broth- MB, Trypticase soya broth-TSB.

3.5 Minimum Inhibition Concentration (MIC)

The MIC values of the *Gordonia tearrae* extract obtained by microbroth dilution method are presented in Table 2. These values ranged from 3.12 -12.5 µg/ml, most of them were less than those obtained from the reference drug Chloramphenicol. This indicated *G.tearrae* crude extract have strong activity on pathogen bacteria.

Table 2. Minimum inhibitory concentration (MIC) of *Gordonia tearrae* crude extract

Tested microbe	Extracts MIC	CH*
<i>MRSA</i>	6.3	25
<i>Bacillus cereus</i>	3.12	25
<i>Enterococcus faecalis</i>	25	25
<i>Escherichia coli</i>	6.3	25
<i>Salmonella typhi</i>	12.5	50
<i>Pseudomonas earoginosa</i>	25	50
<i>Candida albicans.</i>	6.3	25

(CH)*Chloramphenicol 30µg.

4. Discussion

Marine actinomycetes are of a great importance due to their astounding ability to produce numerous biologically active compounds including antibiotics, anticancer and antiprastic agents. Marine actinomycetes thus present an arsenal of current and potential drugs particularly in the global battle against the rising menace of antibiotic resistance in both clinical and environmental settings (Williams, 2009). The *Gordonia tearrae* (SPTG 111) strain used in this study was obtained from the UKM culture collection unit, where it was deposited and labelled as previously isolated from marine sponge at Pulau Tinggi. The sequencing of PCR amplicons of the 16S rRNA gene showed 100% sequence identity to *Gordonia* sp. Isolate Bg17 (HF548413.1), a bacteria isolate from Rubinskoe water reservoir, Russia; *Gordonia* sp. G1 16S ribosomal RNA gene, partial sequence FJ939311.1 isolated in a study of degradation of crude oil and utilization of hydrocarbon compounds by bacterial isolates from used engine oil-contaminated soil; *Gordonia tearrae* strain MRbS27 (FJ959396.1), an isolate from a study on the diversity of bio-compound producing heterotrophic, rhizosphere bacteria from *Stachytarpheta crassifolia*,

a Brazilian semi-arid plant and *Gordonia tearrae* strain K22-39 (EU333873.1) isolated from a study on culturable bacteria from a cold desert of the Indian Himalayas. The selected strain when cultured in Brain Heart Infusion Broth (BHIB), showed higher activity against tested microbial pathogens. Our observation of BHIB supporting the highest level of accumulation of crude secondary metabolites is in concert with the findings of Marinho et al. (2009) who reported that BHI supported growth of a bacteriocin producer strain of *P. putida*. In this study, we used the zones of inhibition of the lawn of bacterial cells around extracts filled disc as an indication of antibacterial activity. This zone of inhibition ranged from 5.5 mm for extracts at 30 µg/disc concentration to 6.3 for 25 µg/ml of crude extract. The crude extract of ethyl acetate of *Gordonia tearrae* showed an activity that was significantly different from that of chloramphenicol, which in this case was used as reference drug. This observation was recorded when crude extract was tested against *Staphylococcus aureus*, *Candida albicans*, *Bacillus cereus*, and *E. Coli*. Notably, the observed MIC values for the crude extract was higher than that of chloramphenicol (Table 2).

5. Conclusion

In conclusion, secondary metabolites produced by marine actinomycetes usually have distinct chemical structures, which may form the basis for the synthesis of novel drugs. Much undoubtedly, these targets may pave the way for more highly effective antibiotics. This study investigated the antibacterial effect of ethyl acetate extract of *Gordonia tearrae* (marine actinomycetes) against pathogenic bacteria. Brain heart infusion broth media was able to stimulate the highest level of production of metabolic compounds as compared to other growth media. The marine actinomycetes described here show promising activity in the area of antimicrobial activity. The crude extract of *Gordonia tearrae* was active (MIC ≤ 3.12 µg/ml) against at least one of the tested microorganisms *Bacillus cereus*. Further work is currently going on to elucidate possible toxicity studies associated with the extracts.

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