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In-vitro Anti-food Poisoning Bacteria and Cytotoxic Activities of Bioactive Compounds Produced by *Streptomyces* sp. A10 Isolated from Fermented Food

Running Title: Anti Bacteria and Cytotoxic Activities of Bioactive Compounds

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Abstract: *Streptomyces* sp. A10 was isolated from fermented food, PLA-SOM, collected from Nakhon Si Thammarat province, Thailand and identified by means of MALDI Biotyper, designated as *Streptomyces violaceoruber*. It could produce bioactive compounds (BCs) that showed spectrum of antibacterial activity against representative of Gram-positive bacteria (*Staphylococcus aureus* TISTR 517 and *Bacillus cereus* ATCC 11778) and Gram-negative bacteria (*Pseudomonas aeruginosa* TISTR 1467 and *Salmonella typhimurium* TISTR 292) by using cross streak method. Moreover, it could excrete the BCs into medium broth (half-formula of Luria Bertani) within 4 days of incubation period at 30 ℃ in shaking incubator 200 rounds per minute (RPM). The excreted BCs from cell-free supernatant (CFS) was determined the protein concentration by means of Bradford assay and investigated the antibacterial activity and cytotoxicity against brine shrimp nauplii by using broth microdilution method and brine shrimp lethality bioassay, respectively. Moreover, cell cytotoxicity activity of CFS was also investigated using MTT assay in HepG2 cells. The results demonstrated that the BCs which had protein concentration equal to 254 μg protein/ml showed strong activity against representative of Gram-positive bacteria, moderate activity against representative of Gram-negative bacteria, but *Ps. aeruginosa* TISTR 1467 is resist to these BCs. The MIC, MBC and LC_{50} of BCs were in the range of 0.49 - 63.50, 0.49 - 127.00, and 131.58 μg protein/ml, respectively. Moreover, CFS (10-80%) had no cytotoxic effect on HepG2 cells. It implied that BCs may contain the important polypeptide substances that can be used as model for development of novel polypeptide antibiotic or food preservative agents.

Key words: Antibacterial activity, Bioactive compounds, Cytotoxic activity, *Streptomyces violaceoruber*.

1. Introduction

Bioactive compounds (BCs) from natural resources have been studied worldwide as models in the development of novel or important medical agents [1] and biopreservative agents. One important source of BCs is microorganisms, especially terrestrial bacteria in the order actinomycetes [2]. Actinomycetes are Gram-positive bacteria that are widespread in nature, including food and feed. Among the order actinomycetes, the members of the genus *Streptomyces* (in the family Streptomycetaceae) are considered important BCs producers. About 80% of total BCs that are used to develop antibiotics, antifungal-, antitumoral-, antihelminthic-, antiviral-, immunomodulators-agents, herbicides and insecticides are produced by genus *Streptomyces* [3]. They are chemoorganotrophic, non-fastidious, filamentous and aerobic bacteria, spore forming and non-motile with high G + C content (69-78 mol %) in their DNA. The most *Streptomyces* usually produce BCs or secondary metabolites to kill

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or inhibit the growth of the related microorganisms [4]. Therefore, most BCs are generally secreted outside the cell, which often occurs at the late growth phase. The temporal nature of BCs is controlled to synthesize through genetic biosynthesis, which is greatly connected to primary metabolism and influenced by environmental manipulation. Most BCs have a low molecular weight (MW < 3000), and are chemically and taxonomically extremely diverse compounds which are not essential for normal growth, development or reproduction. From several medical problems, especially a surge in the development and spread of antibiotic resistance has become a major cause for concern. No major new types of antibiotics have been produced and almost all known antibiotics are increasingly losing their activity against pathogenic microorganisms. The levels of multi-drug resistant bacteria have also continuously increased [5]. As a result, BCs have been intensively studied in the development of new antibiotics [6]. Many BCs, however, should not be used in the development of new antibiotics because of cytotoxic activity etc. Therefore, this study aimed to investigate both the antimicrobial and cytotoxic activities of BCs excreted to medium broth, produced by *Streptomyces* sp. A10, to explore the possibility of new antibiotics, medicinal agents or the development of biopreservatives for food safety agents in the future.

2. Materials and Methods

2.1 Samples Selection and Isolation of Streptomyces Species

Twenty samples of fermented food, PLA-SOM, were randomly collected from the retail markets in the Nakhon Si Thammarat province of southern Thailand. The samples were transferred at room temperature for investigation. Briefly, 25 mL of each sample was enriched in a 225 mL of Luria-Bertani (LB) broth (HiMedia, India) and incubated for 24 hours at 30 ℃. The enriched broth samples were serially diluted in 1× phosphate-buffered saline (PBS; HiMedia) and plated on a half-formula of Luria Bertani (LB/2) agar medium (5 g/l tryptone, 2.5 g/l yeast extract, 5 g/l NaCl and 15 g/l agar powder). These plates were incubated at 30 ℃ for 48-72 hours. The fungal-like embedded colonies were selected randomly and subcultured onto LB/2 agar plates. After incubation, each isolated colony was selected and stored at -80 ℃ with 15-20% glycerol as cryoprotectant for further analysis.

2.2 Bacterial Strains and Culture Conditions

A total of 4 indicator bacteria caused food poisoning, including representatives of Gram-positive bacteria viz. *Staphylococcus aureus* TISTR 517 and *Bacillus cereus* ATCC 11778, Gram-negative bacteria viz. *Salmonella typhimurium* TISTR 292 and *Pseudomonas aeruginosa* TISTR 1467. Both representatives of Gram-positive and Gram-negative bacteria were cultured in Luria-Bertani (LB) agar medium (Himedia, India) at 37 ℃ for 24 hours in a static incubator. After incubation, a single colony of each isolate was inoculated into LB broth and incubated at 37 ℃, 200 PRM in a shaking incubator for 24 hours. The cells of indicator bacteria which grew to the late logarithmic phase were stored in 15% glycerol at -80 ℃ until use.

2.3 Streptomyces Identification

Gram staining, microscopic examination, were evaluated and confirmed by matrix-assisted laser desorption ionization (MALDI)-Biotyper (Bruker Daltonics, Germany) as per the manufacturer's instructions. In brief, 3–5 colony isolates were transferred into 300 µL sterile water and suspended in 900 µL of ethanol. After centrifugation, the pellets were suspended with formic acid and incubated for 30 min. Then, 100% acetonitrile was added, and the supernatant was collected. Alpha-cyano-4-hydroxycinamic acid (HCCA) matrix solution (10 mg HCCA in 1 mL of OS [500 µL of 5% triflouroacetic acid and 500 µL of 100% acetonitrile]) was added to the supernatant, and 2 µL of mixture was spotted on the MALDI plate. The

MALDI plate was generated by double-measurement using adequate quality control criteria. MALDI-time-of-flight mass spectrometry (TOF MS) was performed using BRUKER ULTRAFLEX III TOF/TOF in a linear positive mode with a mass range of 2–20 kDa. All spectra were analyzed using BioTyper 2.0 software.

2.4 Preliminary Antibacterial Activity Screening

The efficiency of BCs produced by isolated *Streptomyces* sp. was preliminarily tested against the indicator bacteria using the cross-streak method [7]. Isolated *Streptomyces* sp. was streaked across diameter on a LB/2 agar plate, and the unstreaked LB/2 agar plate was assigned as the control plate. Both streaked and unstreaked plates were incubated at 30 ℃ for 7 days in a static incubator. After incubation, a loopful of each freshly prepared indicator bacteria was adjusted for turbidity with sterile normal saline solution equal to McFarland No. 0.5 and streaked perpendicular to the central line of *Streptomyces* sp. A10. All plates were incubated at 37 ℃ for 24 hours in a static incubator and the growth inhibition was measured and compared with the control plate.

2.5 Cell-free Culture Broth Preparation

1 cm^2 of initial streaked, 5-day old culture of isolated *Streptomyces* sp. was inoculated into 10 ml LB/2 medium broth in a 25 x 150 mm screw cap test tube. Inoculum was incubated at 30 ℃, 200 RPM in a shaking incubator for 7 days. After incubation, culture broth was centrifuged to separate the cell sediment at 4 ℃, 12,000 RPM, for 20 minutes. The supernatant was filtered through a membrane with a pore size of 0.45 m (Millipore). Later, the cell-free culture broth which contained BCs was harvested using the aseptic technique. The cell-free culture broth was stored at -20 ℃ until use.

2.6 Anti-S. aureus by Agar Well Diffusion

S. aureus TISTR 517 was cultivated on LB (HiMedia) agar broth at 37 ℃ for 24 h. The broth cultures of test bacteria were swabbed on sterile LB agar plates followed by punching wells of 6 mm diameter using sterile cork borer. 100 µl of cell free culture was transferred into labeled wells and the plates were incubated at 37 ℃ for 24 h. The plates were observed for zone of inhibition formed.

2.7 Partial Characterization of the Anti-S. aureus Products

The sensitivity to heat was examined by heating the cell free culture of selected strain to 60 ℃ for 30 min, 100 ℃ for 30 min and 121 ℃ for 15 min then the treated cell free culture was tested against *S. aureus* TISTR517 to determine its anti-*Staphylococcus* activity by the agar well diffusion method. After incubation for 24 h at 37 ℃, the inhibition zone was measured.

2.8 Protein Concentration Measurement

The protein concentration of BCs was determined using the Bradford assay [8]. Bovine serum albumin (BSA) was prepared at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml concentrations. 20 µl of each concentration of BSA and the cell-free culture broth were mixed with 1 ml working solution of the Bradford reagent. Each mixture was incubated at room temperature for 5 minutes and the absorbent value was measured at 595 wavelengths (OD_{595}). After the absorbent value measurement, the relation between absorbent values and the concentration of BSA was plotted as a standard curve. Absorbent values of the cell-free culture broth were used to calculate the concentration of BCs from a linear equation.

2.9 Biological Activities

2.9.1 Minimum inhibitory concentration (MIC) assay

The MIC was determined using the broth microdilution method. The cell-free culture broth was diluted as 2-fold serial by 4x Muller Hinton (MH)

broth media for bacteria in 96 well plates. The wells, which contained 100 µl of the diluted cell-free culture broth, were added to the 100 µl of 1.5 x 10^6 CFU/ml of bacteria suspension and assigned as the experimental group. In one well, which had 100 µl of media alone with 100 µl of 1.5 x 10^6 CFU/ml of cell suspension, was assigned as the control group. Both the experimental and the control group were incubated at 37 ℃ for 24 hours for bacteria. After incubation, the well with the least concentration of BCs showing no growth was taken as the MIC value for the respective bacterium.

2.9.2 Minimum bactericidal concentration (MBC) assay

The MBC was determined using the sub-culture technique. The 100 µl well of each indicator microorganism cell suspension that showed an MIC value, and the 3 wells that showed a value greater than the MIC value, were all spread onto fresh LB and SD agar medium without antibiotics and incubated at 37 ℃ for 24 hours for bacteria in a static incubator. After incubation, if the growth of the indicator bacteria was lower than 5 colonies, it indicated that the concentration of the BCs in these wells had an MBC value.

2.9.3 Cytotoxicity bioassay

The cytotoxic activity of bioactive secondary metabolites was determined using the brine shrimp lethality bioassay [9]. In this method, the eggs of the brine shrimp, *Artemia salina* Leach, were obtained from the faculty of Aquaculture Technology (Walailak University, Thailand) and hatched in artificial sea water (3.8% NaCl solution) and grown for 48 hours into mature shrimp called nauplii (Larvae). Meanwhile, BCs were dissolved into LB/2 broth medium to attain 500 µl of series concentrations of 225.00, 200.00, 175.00, 150.00, 127.00, 63.50, 31.75, 15.87, 7.94, 3.97, 1.98, 0.99, 0.49 and 0.25 µg protein/ml. 500 µl of LB/2 broth medium without BCs was assigned as the negative control. 500 µl of 7.6% NaCl solution was added to both experimental and control vials to attain final NaCl concentration of 3.8%. Then 10 brine shrimp nauplii were applied to each of all the experimental vials and the control vial. The number of nauplii that died after 24 hours was counted. The findings were presented graphically by plotting the concentration of BCs compared to the percentage of nauplii that died, from which LC_{50} was determined by extrapolation.

2.10 Cell Culture

The human hepatoma (HepG2) cell line was purchased from the American Type Cell Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's medium DMEM (DMEM, Gibco, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin–streptomycin at 37 ℃, 5% CO_2.

2.11 Cell Viability Assay

HepG2 cells were seeded at a density of 1 × 10^4 cells/well on a 96-well microplate in 100 µL of DMEM for 24 h at 37 ℃, 5% CO_2. After washing with phosphate buffered saline (PBS), HepG2 cells were treated with 100 µL of cell-free supernatant (A10) at several concentrations (0, 10, 20, 40, 60, 80, 100%), or deionized water (control) for 24 hours at 37 ℃. To assess cell viability, the cells were washed with PBS and then incubated with 200 µL/well of thiazolyl blue tetrazolium bromide (MTT 0.5 mg/ml) for 4 hours at 37 ℃. The mixture was then discarded from the wells and 200 µl dimethyl sulfoxide (DMSO) was added into the wells. The microplate was gently shaken for 5 minutes, and then read at 560 nm using a microplate reader (Thermo Scientific Multiskan FC microplate photometer, USA), and the background at 670 nm was subtracted.

3. Results and Discussion

3.1 Selection and Isolation of Interesting Bacterial Isolate, A10

After LB medium plate which was used to collect

microorganisms from fermented food, PLA-SOM. It was incubated at 30 ℃ for 7 days. The embed bacterial colony which showed no growth of another microorganisms around certain colony in impure culture plate and fungal-like formation was isolated as pure culture by using aseptic technique for Gram staining. The certain bacterial isolate colony showed Gram positive branching formation, known as actinomycetes and named as A10. Actinomycetes are the largest number of environmental microorganisms such as air, soil, water, mangrove sediment etc [10], including fermented food [11, 12] or feed containing agricultural materials or natural products. From extensive studies in the past three decades, Actinomycetes have been proven themselves as reliable sources of novel BCs and richest source of BCs [13-15].

3.2 Antibacterial Activity Screening

Isolate A10 was preliminarily screened to produce the BCs and its antibacterial activity against indicator bacteria viz. *B. cereus* ATCC 11778, *S. aureus* TISTR 517, *Ps. aeruginosa* TISTR 1467 and *S. typhimurium* TISTR 292 by means of cross streak method. The cross-streaking is an easy and relatively rapid method for screening cultures in search for new antibiotics substances or BCs. This method, isolate A10 will produce BCs and excreted into agar around colony of isolate A10. As a result, inhibitory activity of isolate A10 could preliminarily screen against many indicator bacteria in one-time [7]. The preliminary screening found that isolate A10 showed broad spectrum of antibacterial activity against both representative of Gram-positive bacteria (*B. cereus* ATCC 11778 and *S. aureus* TISTR 517) and Gram-negative bacteria (*Ps. aeruginosa* TISTR 1467 and *S. typhimurium* TISTR 292) (Fig. 1). These finding indicated that isolate A10 could produce interesting BCs which might be develop as medically important agent in the future.

3.3 Protein Concentration of Cell-free Culture Broths

The protein concentration had found in cell-free culture broth had equal to 254 μg protein/ml and LB/2 broth medium alone which was defined as negative control had protein concentration equal to 0 μg protein/ml. This experiment was performed to design the scale unit of BCs for interpreting result as quantitative concentration. In preliminary of this experiment found that cell-free culture broth showed positive result after it was tested by ninhydrin reaction which was assay for detecting of protein or peptide. These findings indicated that the cell free culture broth had a composition of protein or peptide or imines such as pipecolic acid and proline, the guanidino group of arginine, the amide groups of asparagine, the indole ring of tryptophan, the sulfhydryl group of cysteine,

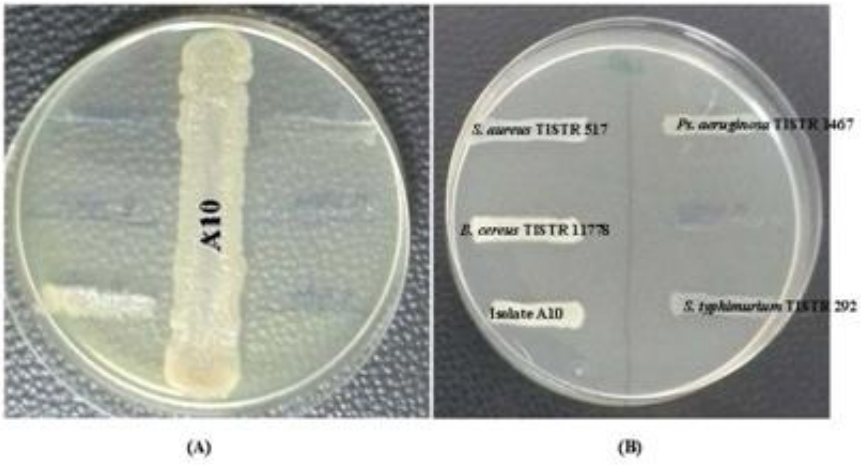

Fig. 1 Preliminary antibacterial activity screening of isolate A10 was evaluated by comparing clearance inhibition of each indicator bacteria compare with experimental plate (A) and control plate (B).

amino groups of cytosine and guanine, and cyanide ions also react with ninhydrin to form various chromophores of analytical interest, it results in the formation of the same soluble chromophore by all primary amines which react. be they amines, amino acids, peptides, proteins, and even ammonia. It is not lost when the insoluble substrate is removed by centrifugation or filtration after the reaction is completed.

The review articles related about *Streptomyces* and antibiotics produced by *Streptomyces* have been reported [16, 17]. For example, natamycin, also known as pimaricin, is a polyene macrolide antibiotic, produced in submerged cultures of certain *Streptomyces* strains such as *Streptomyces natalensis*, *Streptomyces chattanoogensis*, and *Streptomyces lydicus* [17]. Expected isolate A10 exhibited good antimicrobial activity against *S. aureus* TISTR 517. Such, the fermented broth from isolate A10 was used for testing the stability of anti-Staphylococcal compounds and biological activities. Commonly, BCs which produced by genus *Streptomyces* had two major groups include (i) Polyketide- and (ii) polypeptide substance [5]. The most were reported as polyketide substance [14, 18]. Whereas in this study, it is possible that BCs might be as polypeptide substance similar to polypeptide antibiotic that produced by *Streptomyces parvulus* [19].

The anti-*S. aureus* activity was decreased after placing at temperature more than 100 ℃. However, at temperature 25 ℃ was still had anti-*S. aureus* activity of BCs. It implied that the effective molecule from BCs may be the protein because it cannot resist to high temperature (100 ℃) for 30 min. Further studies should be needed to confirm results by means of NATIVE or sodium dodecyl sulphate polyacylamide gel electrophoresis analyzing for anti-*S. aureus* activity [20]. Because culture is growing at a constant growth rate and no obvious stress is being imposed on the mycelium, particularly at pH 7.0, the research data provide strong support that protein secretion is not occurring through some cultural perturbation and may be linked to antibiotic production as an important element of secondary metabolism in isolate A10.

3.4 Biological Activities of BCs

BCs was diluted as 2-fold serial dilution (0.25–127.00 μg protein/ml) for using in broth microdilution method and prepared at different concentrations level from 0.25 to 225.00 μg protein/ml for using in brine shrimp lethality bioassay. In this experiment found that both MIC and MBC value of BCs against represent of Gram-positive bacteria were in the range 0.49–0.99 μg protein/ml. For represent of Gram-negative bacteria, MIC and MBC value of BCs were in the range 1.98 – 63.50 and 3.97–127 μg protein/ml, respectively (Table 1). From these finding indicated that BCs showed strongly inhibitory efficiency against Gram-positive bacteria, moderately inhibitory efficiency against Gram-negative bacteria, especially *Ps. aeruginosa* TISTR 1467.

In brine shrimp lethality bioassay, the BCs on mortality rate of nauplii showed a concentration dependent activity (Table 2). Protein concentration of BCs versus percentage of mortality rate of nauplii was plotted as linear correlation graph. From this graph

Table 1 MIC and MBC values of BCs on each indicator bacteria.

Indicator bacteria	MIC (g protein/ml)	MBC (g protein/ml)
Gram-positive		
B. cereus TISTR 11778	0.49	0.49
S. aureus TISTR 517	0.99	0.99
Gram-negative		
Ps. aeruginosa TISTR 1467	63.50	127.00
S. typhimurium TISTR 292	1.98	3.97

found that 50% of mortality rate of nauplii cause from BCs at protein concentration showed LC_{50} value of 131.58 g protein/ml (Fig. 2). In generally, if MBC is less than 4-times of the MIC or equals to MIC then the agents is known to be bactericidal [21]. It is noticed that the MBC of BCs was equal to MIC against indicator bacteria, indicating its bacteriocidal properties (Table 1). Interestingly, the LC_{50} value found that it is higher than both MIC and MBC value of BCs against all indicator bacteria (Table 2). Moreover, MTT assay showed that CFS (10-80%) had no cytotoxic effect on HepG2 cells (Fig. 3). Therefore, this experiment demonstrated that BCs in cell-free culture broth had a weak cytotoxic activity against brine shrimp nauplii and also might be including to normal cell. For determining the cytotoxic activity of BC, several methods are employed. A widely used method in this field is brine shrimp lethality bioassay because it is a rapid method utilizing only 24 hours, inexpensive, and needs no special equipment. Moreover, the interest of this method was the use of the zoological organism to investigate the cytotoxic activity [22]. In this thesis, brine shrimp lethality bioassay was used to preliminarily assess the cytotoxicity of BCs because the animal cytotoxicity test show variations depending on the chemical nature of the compound and the route of administration to the experimental animal [23]. Interestingly, the LC_{50} value was higher than both the MIC and the MBC value of BCs against all indicator bacteria (Table 2).

Table 2 Percentage of mortality rate of nauplii after exposed with BCs at different concentration level for 24 h. and LC_{50} value of BCs on nauplii.

Protein concentration of BCs (μg protein/ml)	Mortality rate of nauplii after exposed with BCs for 24 h. (%)	LC_{50} (μg protein/ml)
225.00	87.5	131.58
200.00	77.5	
175.00	70.0	
150.00	55.0	
127.00	40.0	
63.50	22.5	
31.75	20.0	
15.87	5.0	
7.94	2.5	
3.97	0.0	
1.98	0.0	
0.99	0.0	
0.49	0.0	
0.25	0.0	
Negative control	0.0	

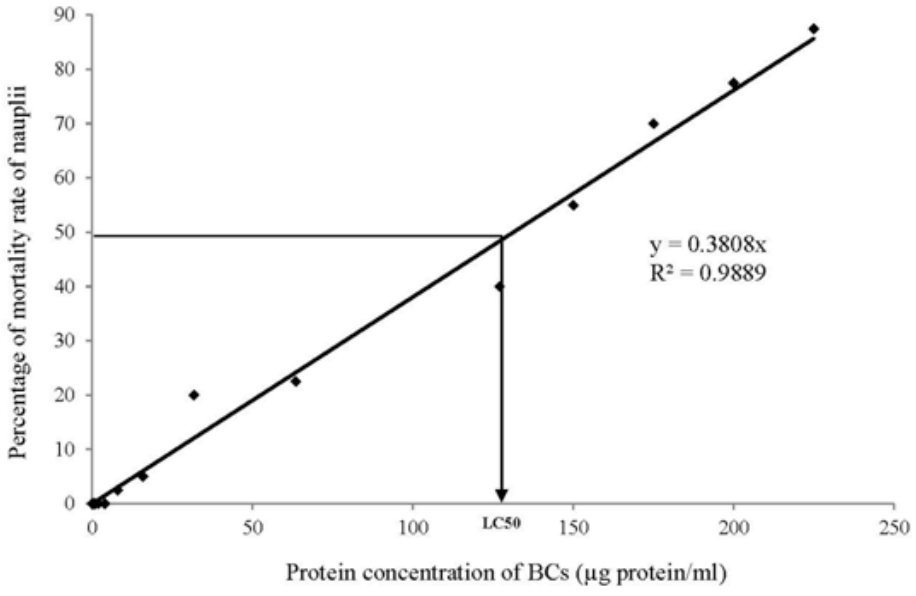

Fig. 2 Linear correlations between protein concentrations of bioactive secondary metabolites versus percentage of mortality rate of nauplii..

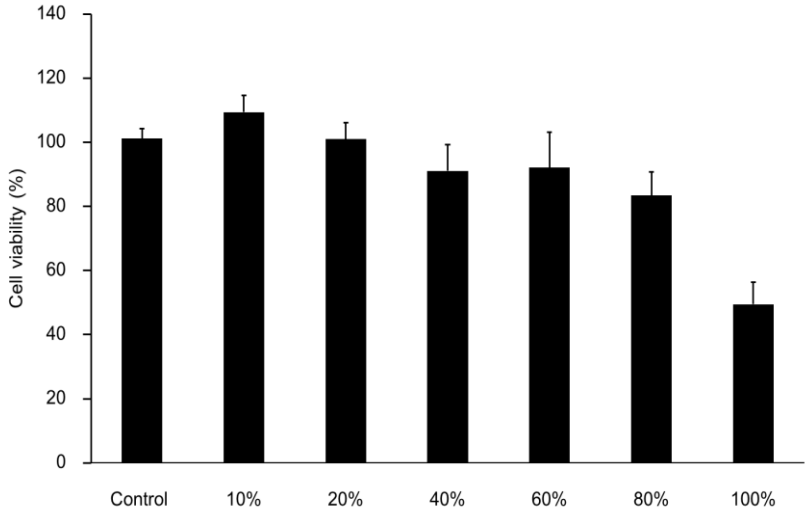

Fig. 3 Cell cytotoxicity of cell-free supernatants (CFS) on HepG2 cells. Cell viability was determined using the MTT assay after treatment with CFS. The data is presented as mean ± SD (n = 3).

Therefore, this experiment demonstrated that BCs in a cell-free culture broth had weak cytotoxic activity against brine shrimp nauplii. The weak cytotoxic activity may also extend to normal cells when tested by MTT assay as shown in Fig. 3.

4. Conclusions

The isolate A10 described in this study was identified as *Streptomyces violaceoruber*. BCs in cell-free culture broth which had protein concentration at 254 µg protein/ml showed MIC and MBC on each indicator bacteria at different concentration level in the range 0.49 to 63.50 and 0.49 to 127.00 µg protein/ml, respectively. Whereas, the LC_{50} value of BCs on nauplii which equaled to 131.58 µg protein/ml which is higher than both MIC and MBC value. CFS (10-80%) had no cytotoxic effect on HepG2 cells. This result demonstrated that these BCs have been interested and also might be as important polypeptide compounds that can be used as model for development of novel polypeptide antibiotic or biopreservative in the future. Therefore, the future works will increase the yield of BCs and extract, purify and analyze the chemical structure of these pure active compound including confirm the cytotoxicity in *in vivo* experiments.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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