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Extraction of antimicrobial compounds from soil *Actinomycetes* strain smc256 against nosocomial pathogen

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Abstract

Antimicrobial compounds are chemical substances inhibiting growth of microorganisms, such as bacteria, fungi and virus. Most antimicrobials are synthesized by soil bacteria, particularly actinomycetes. *Streptomyces*, a majority of the actinomycetes is abundant in natural resources, has ability to produce several types of antimicrobials including antibacterials, antifungals and antivirals. The antibiotic productions are species specific, and are important for competition with other microorganisms that come in contact. Rapidly emerging infectious diseases and overuse of antibiotics by self-medication are important factors that contribute to drug-resistance and shortening antibiotic lifetime. As a result, the need for searching new antibiotics should be focused on. In this report, antimicrobial compound against *Acinetobacter baumannii* could be extracted from soil actinomycetes strain SMC256. After 14 days of culture incubation at 28℃, the supernatant was extracted with ethyl acetate twice. Antibacterial activity of the crude extract was shown against *A.baumannii* by disc diffusion method. The crude extract was subjected to chromatographic separation on silica gel column, and the antibacterial compound was mainly eluted by 20% dichloromethane in hexane as monitored at wavelength of 254 nm and disc diffusion method.

Introduction

Antibiotics, also called antibacterials are chemical substances inhibiting growth of microorganisms, such as bacteria. Antibiotics have ability to either kill or inhibit the growth of bacteria, and are classified by their interactions to the targets in cellular functions, such as DNA replication, RNA synthesis, cell wall synthesis and protein synthesis. After discovery of the first antibiotic (penicillin) in 1940, the history of antibiotics originated from *Streptomyces* began with the discovery of streptothricin and streptomycin two years later. Scientists were interested to discover antibiotics within the genus *Streptomyces*, a majority of actinomycetes. Nowadays, 80% of the antibiotics around the world are come from them.[1]

Streptomyces spp. are Gram-positive filamentous bacteria whose genomes have high GC-contents (70%) in contrast to the others, e.g. *Escherichia coli* (50%). They grows up in various environments especially in soils, sediments and marine waters. The *Streptomyces* morphology involves the formation of hyphae that can differentiate into a chain of spores. The ability of spores to survive in the hostile environments has been increased due to the presence of pigment and aroma in the spores, which stimulates cell development and secondary metabolite production. The *production* of *most antibiotics* is *species specific* and these secondary metabolites are important for their species in order to compete with other microorganisms that come in contact.

Despite hundreds of antibiotics have been discovered and synthesized for treating human infectious diseases, the diseases have remained the leading cause of death worldwide. Self-medication and overuse of antibiotics are other important factors that contributes to resistance and reducing the lifetime and potency of antibiotics.[2] Antibiotic resistance in bacteria may be due to the genetic changes, i.e. mutation of genes in bacteria resulting in drug inactivation/ alteration, modification of drug binding sites/ targets, change in cellular drug permeability[3]. Almost all resistance genes are located on the same cluster of the antibiotic biosynthesis genes. The resistance genes may be carried on the chromosomes, plasmids or transposons. The infections caused by multidrug-resistant bacteria (MDR) are one of the most serious problems of human health. Several MDR pathogens, including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. are the leading cause of nosocomial infections affect human public health and are the major problem in global public health throughout the world, and are usually caused by excessive drug usage, inapplicable use of antimicrobials, and substandard pharmaceuticals.[4]

For these reasons, this work has focused on investigating a highly potent antibiotics against these pathogens from our new bacterial isolate (actinomycete strain SMC256). By this mean, antibiotic was extracted and partially purified from the strain SMC256 cultured in suitable liquid medium and tested its antimicrobial activity against *A.baumannii* which is a gram-negative opportunistic pathogen causing a major problem in primarily associated with hospital-acquired infections.[5]

Methodology

Fermentation of actinomycetes strain SMC256 to produce antibacterial

Stock of actinomycetes strain SMC256 was streaked on the oatmeal agar (one litre: 30 g oatmeal and 15 g agar) and incubated at 28 ^{o}C for 3 days. Single colony was streaked on the surface of entire plate and incubated for 7 days. The mycelia were inoculated into 15 ml of seed culture broth (one litre: 3 g yeast extract, 5 g bacto peptone, 3 g malt extract, 10 g glucose and 170 g sucrose). After 3 days of incubation, the seed culture was transferred to 100 ml of production medium (as the same ingredient of seed culture) broth and incubated at 28 ^{o}C on a rotary shaker for 7 and 14 days.[6]

Extraction of antibacterial from actinomycetes strain SMC256 with organic solvent

After 14 days of incubation, biomass was separated from fermentation broth by centrifugation at 5,000 rpm for 15 min. Supernatant was extracted twice with an equal volume of ethyl acetate.[7] The extract was dehydrated by Na_2SO_4, concentrated under vaccuum to give a residue, and dissolved with methanol.[8]

Antibacterial activity assay against Acinetobacter baumannii by the disc diffusion method

A.baumannii was grown at 37 °C on a tryptic soy agar (TSA) overnight to produce a single colony for making a seed culture. A tryptic soy broth (TSB) inoculated with the 1% seed culture was incubated at 37 °C until its turbidity reached to OD_{625nm} of 0.1, then the culture was used to swab on Mueller Hinton Agar (MHA) plate.[9] Antibacterial activity test of the extract was performed by placing a disc paper containing the extract dissolved in methanol on the surface of MHA as described previously, and the antibacterial activity was determined after incubate at 37 °C for 24 h by measuring the inhibition zones related to degree of the activity.

Purification of antibacterial using chromatography technique

The ethyl acetate extract dissolved in methanol was subjected to purification by silica gel chromatography.[10] The column was equilibrated with dichloromethane, followed by loading the extract and eluting with 20-100% dichloromethane in hexane and 10-100% methanol in dichloromethane respectively. Thirty fractions (1 ml each) were collected and measured the absorbance at wavelength of 254 nm (A_{254nm}). All fractions were concentrated under reduced pressure to obtain residues, which were dissolved in small volume of methanol. Finally, the concentrated fractions were tested the antibacterial activity against *A. baumannii* as mentioned earlier.[11]

Results and Discussion

The liquid culture broths of actinomycetes strain SMC256 that were cultivated at 28 ºC for 7 and 14 days showed no antibacterial activity against *Acinetobacter baumannii*. After extraction of the liquid media with ethyl acetate, the extracts derived from 7- and 14-day actinomycetes cultures exhibited the antibacterial activity against *A. baumannii* at different degrees of inhibition. The extract of 14-day culture yielded higher anti- *A. baumannii* activity (15 mm in diameter) than that of 7-day culture (10 mm in diameter) (**Figure 1.**).

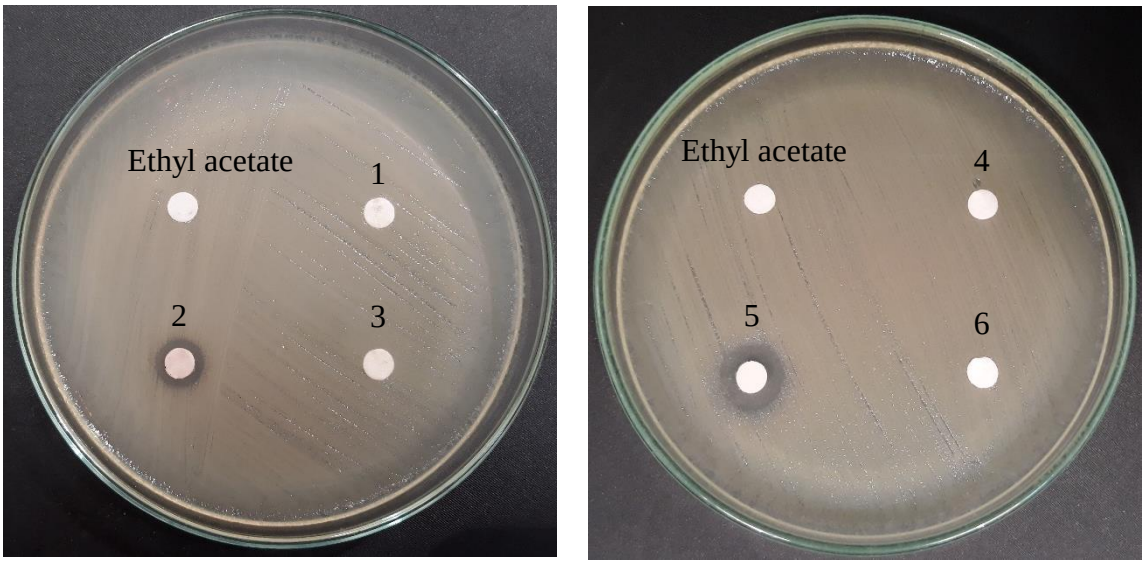

Figure 1. Antibacterial activity of the ethyl acetate extract from actinomycetes strain SMC256 by disc diffusion method. 1: broth before extraction of 7-day culture, 2: ethyl acetate extract from 7-day culture, 3: broth after extraction of 7-day culture, 4: broth before extraction of 14-day culture, 5: ethyl acetate extract from 14-day culture and 6: broth after extraction of 14-day cultures

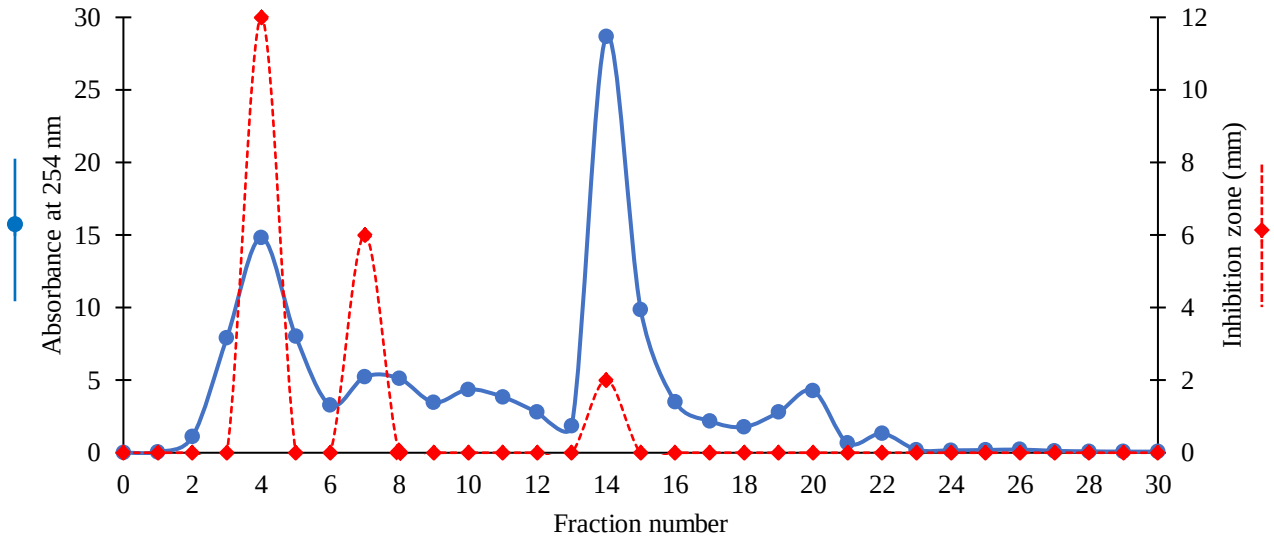

Figure 2. Chromatographic separation of the ethyl acetate extract from actinomycetes strain SMC256 by silica gel column chromatography

The crude ethyl acetate extract from the 14-day actinomycetes culture was further purified by a silica gel column chromatography, and revealed the profile of chromatographic separation as shown in **Figure 2**. The fractions with highest anti- *A. baumannii* activity were obtained at the first step of the elution with 20% dichloromethane in hexane (fraction 4). Moreover, a few fractions with lower activity were also eluted at higher polarity solvents (100% dichloromethane [fraction 7] and 10% methanol [fraction 14] respectively). However, the high values of A_{254nm} corresponding to total organic matters, including organic impurities remained in the active fractions (fraction 14 and fraction 4) are required to be removed in the next steps of chromatographic purification with different stationary and mobile phase systems.

Conclusion

The anti- *A. baumannii* compound could be isolated by ethyl acetate extraction from actinomycetes strain SMC256. The strain SMC256 produces the highest antibacterial activity against *A. baumannii* after 14-day incubation at 28 ^{o}C in defined liquid medium. The main antibacterial compound was able to be purified and eluted with low polarity solvent (20% dichloromethane in hexane) in contrast to the majority of impurity which was eluted with the higher polarity solvent (10% methanol in dichloromethane).

References:

1. Hwang K-S, Kim HU. Biotechnol. Adv. 2014;32(2):255-68.
2. De Lima Procópio RE, da Silva IR. Braz J Infect Dis. 2012;16(5):466-71.
3. Santajit S, Indrawattana N. Biomed Res Int. 2016. doi:10.1155/2016/2475067
4. Zhu H, Swierstra J. Microbiology. 2014;160(8):1714-25.
5. Howard A, O'Donoghue M. Virulence. 2012;3(3):243-50.
6. Saravana Kumar P, Duraipandiyan V. Kaohsiung J Med Sci. 2014;30(9):435-46.
7. Kavitha A, Prabhakar P. Res Microbiol. 2010;161(5):335-45.
8. Sanghvi GV, Ghevariya D. Biotechnology Reports. 2014;1–2:2-7.
9. Sathish KSR, Kokati VBR. Asian Pac J Trop Biomed. 2012;2(10):787-92.
10. Park SB, Lee IA. J Microbiol Biotechn. 2011;21(12):1236-42.
11. Nakashima T, Boonsnongcheep P. J. Biosci. Bioeng. 2015;120(5):596-600.

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