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Quantitation of Total Phenolic Contents of Bioactive Compounds Fractions *Streptomyces* species

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Abstract: *Streptomyces* species, KB1 and KB3, were isolated from air samples collected from Aonang, Krabi province, Southern Thailand. The strain KB3 was found to be an efficient pigment (dark brown) producer and has been selected for further studies. Both strains were used to study for antioxidant activity. The fraction extracts with ethyl acetate (EA), hexane (HE) and methanol (MT) possessed DPPH free radical scavenging activity, especially pigment extracted from KB3 with MT (KB3-MT). The results of antioxidantactivity produced by the *Streptomyces* species clearly indicated that the actinomycetes are source of bioactive compounds for usefulness in pharmacological strategies.
Keywords: Bioactive compounds,DPPH free radical, Folin-Ciocalteu reagent, Pigment, *Streptomyces* sp., total phenolic contents.

Introduction

Actinomycetes are prokaryotes with commonly recognized for their ability toproduce chemically diverse and pharmaceutically usefulcompounds[1]. These are Gram positive bacteria frequently filamentous and sporulating organisms, belongs to actinomycetales. The filamentous actinomycetales produce over 10,000 bioactive compounds are of high significance as antimicrobial agents on a wide range of pathogenic microorganisms[2,3].However, in recent years, the rate of discovery ofnovel compounds from these bacteria has significantlydecreased[4,5].One approach to reverse this trend is to isolateactinomycetes from a wide variety of environmental sourcesand to employ diverse isolation methods to obtain newbioactive compoundsand extra cellular enzymes[6]. Members of the actinomycetes genus, especially *Streptomyces* sp. have been recognized as prolific producer of useful bioactive compounds with broad spectrum of activities that produce about 75% of commercially and medically useful antibiotics. The bioactive compounds have many clinical valuable drugs such as vancomycin and erythromycin (antibacterial), cyclosporin (immune suppressant) and antitumor drug such as antracyclines, aureolic acids, enediynes and other[7]. Meanwhile, previous studies showed that the *Streptomyces* could metabolize the compounds with antioxidant activity such as isoflavonoids[8], phenolic contents[9,10], melanin pigment[11].

We have preliminarydata that the isolation of actinomycetes from air were characterized and identified as *Streptomyces* sp. by partial sequence of 16SrDNA gene analysis. The analysis data of isolates, KB1 and KB3, were submitted in the gene bank (NCBI, USA) with accession number KF939581.1 andKF939582.1, respectively. The extracellular bioactive compounds of both strains have anti-methicillin resistant *Staphylococcus aureus*activity (unpublished data). The objective of this research was quantitative analysis of total phenolic contents of the fractions frombioactive compound produced by *Streptomyces* sp., KB1 and KB3,and to evaluate the antioxidant of the metabolites.

Materials and Methods

Chemicals

The media were purchases from Hi Media, Mumbai.Chemicals were purchased from Merck, Germany and Sigma-Aldrich, USA.

Microorganisms

Streptomyces sp.,KB1 and KB3, were isolated from air samples collected at Krabi province, Thailand by theBiosampler, Microflow90 (Aquaria), at flowrate 100 l/min for 30 min according to manufacture's instruction. The basic local alignmentsearch tool (BLAST) was used to compare the 16S rRNA genesequence of KB1 and KB3 with 16S rRNA genesequences available in the EzTaxon server10 to identify the species ofstrain KB1 and KB3. Both*Streptomyces* sp.were expected to be a newspecies of the genus *Streptomyces*, and theirs 16S rRNA gene sequenceexhibited a low sequence similarity of98.98% with *Streptomycesvarsoviensis* for KB1 and 99.51% with S. *hiroshimensis* for KB3. The detailed identification of this strain will be reportedelsewhere.

Extraction of the pigment

Only, the pigmented strain KB3 was inoculated to50 ml test tubes containing 10 mlhalf-formula Luria Bertani (LB/2) medium broth (2.5 g/l yeast extract, 5 g/lTryptone, 5 g/l NaCl), followed by incubation at30^{o}C for 4 days on a rotary shaker (200 rpm).The culture was transferred as 5% (v/v) inoculum into 200 ml of the LB/2 contained in 500 mlconical Duran bottles. TheBottles were then incubated on an incubatorshaker (200 rpm) at 30^{o}C for 12 days. Afterincubation the broth was filtered throughWhatmann No.1 filter paper to remove themycelial mat. The filtrate was further centrifugedat 8,000 rpm for 30 min at 4^{o}C the crudebioactive compound was recovered from thesupernatant by solvent extraction with thrice thevolume of methanol. The mixture was rotate shakenovernight at 4 ^{o}C and then allowed to stand for 60 minfor complete separation of the aqueous phaseand organic phase. The organic phase wasevaporated to dryness in room temperature andpowdered pigment residues were collected andweighed. The crude extract was used further foranalysis of antioxidant activity.

Extraction of bioactive compounds

Fermentation of bioactive compounds

Streptomyces sp. KB1 was cultivated in 50 ml test tubes containing 10 mL of LB/2medium broth.The test tubes were shaken on a reciprocal shaker at 30°C for 12 days (200 rpm). Aliquots (5 ml) of the cell suspensions were then transferred to 500 ml Conical Duran Bottle, added with 40 glass beads, containing 200 mL of the LB/2 medium broth and incubated at the same condition as describe above. After 12th day the culture broth was centrifuged at 12,000 rpm, 4 ^{o}C for 20 min to remove the bacterial cells/spores. The culture broths were used to extracted the active fractions by liquid-liquid extraction with organic solvents in the next step, ie., ethyl acetate (EA), hexane (HE) and methanol (MT) as follow;

A. Ethyl acetate extraction (EA)

The clear supernatant (culture broth) was extracted thrice with equal volume of ethyl acetate in separation funnel and the solvent layers were pooled. The solvent was evaporated in vacuum evaporatorto yield pale yellow colored extract. The extract was dried in dessicator, dissolved with DMSO and used to screen antioxidant activity. The extract was stored in -20^{o}C and it was used for further analysis.

B. Hexane extraction(HE)

In laboratory, the clear supernatant (culture broth) was extracted thrice with equal volume of exane in separation funnel and the solvent layers were pooled. The solvent was evaporated in vacuum evaporatorto yield extract. The extract was dried in dessicator, dissolved with DMSO and used to screen antioxidant activity. The extract was stored in -20^{o}C and it was used for further analysis.

C. Methanol extraction(MT)

Methanolic extract was prepared according to extraction of pigment as described above. The extract was then filtered using Whatman filter paper No.1 and the filtrate obtained wascollected for further studies.

Total Phenolic Content of Solvent Extract

Folin-Ciocalteu (FC) method was used to determine total phenolic content of the extracts. The Colorimetric method described by Kekuda*et al*., 2011[12] was modified using in laboratory. Thereaction mixture consisted of a dilute concentration ofextract (0.1 ml) mixed with 2 ml of 20 mg/ml Na_2CO_3 for 2 min**,** and then 0.9 ml of FC reagent (previously diluted 2-fold with distilled water) was added. The tubes were allowed to stand for 30min and the absorbance was measured at 765nmin Spectrophotometer,micotiter plate reader. The Folin-Ciocalteu reagent issensitive to reducing compounds includingpolyphenols, thereby producing a blue colour uponreaction. This blue colour is measuredspectrophotometrically. Thus total phenolic contentcan be determined[13,14].A standard curve wasprepared by using increasing concentrations of Gallicacid in methanol. All determination wasperformed in triplicate. Total phenol contents were calculated as Gallic acid from a calibration curve: $Y = 6.307X + 0.601$, $R^2 = 0.996$, where Y was the absorbance and X was total phenolic content in the different extracts of fractions expressed in mg/gthe Gallic acid equivalent (mg Gallic acid(GAE)/g extract).

Antioxidant activity

The antioxidant activities were assessed by scavenging of synthetic radical in polar organic solvent Herein, common synthetic radical used in the present study included total phenol content and melanin pigment.

DPPH Radical-Scavenging Activity

A 96-well plate was used for the 1,1-diphenyl-2-picrylhydrazyl radical-scavenging assay, antioxidant activity[15,16].Briefly, the extracts and ascorbic acid as a positive control were dissolved in DMSO. Next, 10 µl of 400 µM DPPH dissolved in absolute ethanol (EtOH) and 10 µl of each extracts at different concentrationswere separately mixed in a 96-well microplate,then vortexed. After 30 min of incubation at room temperature in the dark, the absorbance was measured at 515 witha spectrophotometer and is calculated as DPPHScavenging activity % = [(Control OD – sampleOD)/Control OD] × 100. The control wasprepared without any extract containing 10 µl ofmethanol and 10 µl of 400 µMDPPHand the rest of the proceduresremaining the same.

Results and Discussion

Total Phenolic and flavonoid content

Phenolic contents have been considered as the major antioxidant in scavenge reactive oxygen species. It is due to their redox properties, electron donating properties and singlet oxygen quenching properties[17]. **Table 1**shows the contents of total phenols that were measuredby FC reagent in terms of Gallic acidequivalent. The total phenol varied from 0.00±0.00 to 0.24±0.02 mg/g in the extracts.The maximum phenolic content was only found in the ethyl acetate extract (0.24±0.02 mg/g) of culture broth of strain KB1. Similar to earlier studythat reported that *Streptomyces* sp. R56-07 could be produced antioxidative phenolic contents or melanin pigments excreted into the culture broth and then could be extracted with EA or MT[9-11].

Table 1. Total Phenolic Contents of *Streptomyces*species's culture broth in Different fraction Extracts

Extracts	Concentration (µg/ml)	Mean±SD
KB1-ET	63.33	0.24±0.02
KB1-HE	30.00	0.00±0.00
KB1-MT	7.33	0.00±0.00
KB3-MT	0.83	0.00±0.00

Each value in the table was obtained by calculating the average of three experiments ± standard deviation KB1-ET, fraction of KB1 extracted with ethyl acetate; KB1-HE, fraction of KB1 extracted with hexane; KB1-MT, fraction of KB1 extracted with methanol; KB3-MT; fraction of KB3 extracted with methanol

Antioxidant activity

The free radical scavenging activity of fraction extracts used may be attributed to the presence ofphenolic compounds as these fraction extracts exhibit important mechanism of antioxidant activity[11,17]. Moreover, the soluble dark brown-black pigment probablyrefers to melanin[18]was also exhibited antioxidant activity[19]. Melanins are pigments of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and usually are dark brown or black[20-22]. In this study, strain KB3 appeared the color of substrate mycelium is brown after 7 days of incubation time for at least. Diffusible dark brown pigmentation was observed in LB/2 medium or Yeast Extract Malt Extract medium (YM). Fermentation of the strain was carried out at 30°C for 12 days with shaking conditions at 200 rpm/min. The fermented broth was centrifuged and methanol was added to cell free supernatant and mixed well. The solvents werethen evaporated and the powdered pigment residues were collected. This procedure yielded 0.83 mg of crude pigment per 200 ml of fermented broth.

In the present study the DPPH free radical scavenging activity of the pigment extract (KB3-MT)and of total phenolic contents from fraction extracts(KB1-EA, KB1-HE, KB1-MT) were found to increase in concentration dependent manner. The addition of an antioxidant resulted in decrease of absorbance proportional to the concentration and free radical scavenging activity of the extracts and it indicated an increase of the DPPH radical scavenging activity[23]. The EC50 value of thepigment extract from KB3-MT was found to be 64.24$\pm$3.5 µg/ml. Whereas, the EC50 value of the fractions from KB1-ET and KB1-HE were found to be >49.65$\pm$6.53 µg/ml, 74.90$\pm$5.69 µg/ml, respectively. Unfortunately, fraction KB1-MT did not show the EC50 value. However, interestingly, the KB1-HE fractiondid not found the phenolic contents (Table 1) but showed the antioxidant activity. It may be from other molecules that produced by *Streptomyces* spp. in the stage of growth.The purification and characterization of fractions should be concerned in the further study.

Conclusion

The amount of total phenols were determined with the FC reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalents. The maximum phenolic content was found in the KB1-EA (0.24±0.02 mg/g). The result of the present study showed that the fraction extracts which contain amount of phenolic contents and brown pigment which exhibited the antioxidant activity. A fraction extract which did not contain phenolic contents (KB1-HE) which also exhibited the antioxidant activity. However, the scavenging property of fraction extracts from KB1 and KB3 may be due to hydroxyl groups existing in the phenolic compounds. These findingcan be concluded that the metabolite from the*Strepromyces*species, KB1 and KB3, are a potent antioxidantagent which suggests its beneficiary role as apharmacological agent. Further research has to be carriedout to characterize the active principle.

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