Journal of Applied Pharmaceutical Science Vol. 5 (02), pp. 017-021, February, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.50204
ISSN 2231-3354

In vitro antimicrobial and antioxidant activities of bioactive compounds (secondary metabolites) extracted from *Streptomyces lydicus* A2

Monthon Lertcanawanichakul*, Kanchanat Pondet, Jutaporn Kwantep

School of Allied Health Sciences and Public Health, Walailak University, Thasala 80161, Thailand.

ARTICLE INFO

Article history:
Received on: 23/11/2014
Revised on: 09/12/2014
Accepted on: 18/01/2015
Available online: 27/02/2015

Key words:
Agar well diffusion, DPPH, *Streptomyces lydicus*, Walailak University.

ABSTRACT

In the present study, we report antibacterial and antioxidant activities of bioactive compounds extracts (ethyl acetate extract, methanolic extract, n-hexane extract) of *Streptomyces lydicus* A2 isolated from air in Sciencetific and Technological Equipments Building, Walailak University, Thailand. The *S. lydicus* A2 is potential to produce bioactive compounds showed various biological activities. In previous research, its culture broth or extract shows antimicrobial activity. In this study, extract prepared from *S. lydicus* A2 stored at -80 ^{o}C was re-evaluated the antimicrobial activity when grew in various medium and incubation time to find out storage effect towards the antimicrobial activity. The extract (fractions) from *S. lydicus* A2 still inhibit test bacteria even though it decreases 50% than the extract prepared from *S. lydicus* A2 without -80 ^{o}C storage. Antimicrobial activity against *Staphylococcus aureus* and *Bacillus cereus* was only exhibited by the methanolic fraction *S. lydicus* A2. Only, the ethyl acetate extract was found to possess dose dependent DPPH free radical scavenging. The isolate A2 could be a potential candidate for the development of novel therapeutic agents active against pathogens and free radicals. Further studies on genomic characterization of isolate and structure determination of the bioactive compounds are under progress.

INTRODUCTION

Family Actinomycetaceae are the group of microorganisms as the source of secondary metabolites, pivotal compounds, for drug-based recovery due to biological activities of those compounds. From all the genera of actinomycetes, the genus *Streptomyces* is represented in nature by the largest number of species and varieties. These species can produce a large number of antibiotic and active secondary metabolites (Devi *et al*., 2006). The genus *Streptomyces* which shows the highest contribution in production bioactive compounds especially antimicrobial activity. Apart from antimicrobial activity, *Streptomyces* also exhibit other activities. *Streptomyces* spp. KR-5 exhibited cytotoxic activity against the growth of human breast cancer cell line (Sateesh *et al.,* 2011). Whereas, *Streptomyces* spp. SRDP-H03(Rakesh *et al*., 2013) and BI244 exhibited antioxidant activity (Kiruthika *et al*.,2013). The secondary metabolites were generally produced at stationary phase of growth of microorganisms. It may be induced by depletion of nutrient in their growth medium so that microorganisms compete with other microorganisms to get nutrients which it creates a stressful condition for them. Differences in the composition of growth mediums also affect type of secondary metabolites produced by microorganisms. For instance, in the previous study, antimicrobial activity of *Streptomyces lydicus* A2 cultivated half-formula Luria-Bertani (LB/2) broth medium is better than that of *Streptomyces lydicus* A2 cultivated Yeast Extract Malt Extract (YM) broth medium. Some secondary metabolites may be not biosynthesized all time even though their cultivations have conducted with similar condition. Biosynthesis of secondary metabolites of microorganisms are affected many factors. For instance, sponge-associated microorganisms can produce a specific secondary metabolite compound but it probably cannot re-biosynthesized while microorganisms cannot associate with sponge.

* *Corresponding Author*
Email: lmonthon@wu.ac.th

In continued search in our laboratory for novel microbial secondary metabolites, a number of *Streptomyces* strains were screened. This has resulted in isolation of a promising strain of *Streptomyces* species from air samples collected at Walailak University campus. *S. lydicus* A2 has showed antimicrobial activity which inhibit growth 6 of tested methicillin resistant *Staphylococcus aureus* (MRSA). In this study, we re-evaluated the antimicrobial activity on LB/2 and incubation time to find out storage effect toward the antimicrobial activity. The crude ethyl acetate, methanolic, n-hexane extracts were evaluated toward antimicrobial, and antioxidant activities.

MATERIALS AND METHODS

Streptomycete

Streptomyces lydicus A2 was isolated from air at Sciencetific and Technological Equipments Building 7th, Walailak University, Thailand by the Biosampler, Microflow90 (Aquaria), at flow rate 100 l/min for 30 min according to manufacture's instruction. The basic local alignment search tool (BLAST) was used to compare the 16S rRNA gene sequence of A2 with 16S rRNA gene sequences available in the EzTaxon server10 to identify the species of strain A2. *Streptomyces* sp. A2 exhibited a sequence similarity of 99.58% with *S. lydicus* NRBC 13058^{T}, such it expected to be *S. lydicus*. Pure culture was kept in 15% glycerol at -80^{o}C storage.

Cultivation and Extraction of *S. lydicus* A2

Medium and incubation time for *S.lydicus* A2production was carried out by various medium (LB, LB/2, YM, YM/2) and incubation (4, 8 and 12 days). The seed culture was prepared by inoculation of 1 colony in 10 ml of liquid media was carried out by various medium as described above and incubated in shaker incubator (Cocono, Taiwan) at 200 rpm, 30 ^{o}C for 3 days. Furthermore, the 1% of seed culture was inoculated into 100 ml of liquid media in 500-ml Duran bottle and incubated on shaker incubator at 200 rpm for certain time. Finally, the culture broth was harvested by centrifuged at 12,000 rpm, 4 ^{o}C for 20 min and separately extracted the secondary metabolites using ethyl acetate or hexane as solvent as described in the next step.

Extraction of bioactive compounds from culture broth

The centrifuged culture broth (supernatant) was subjected solvent extraction using ethyl acetate equal volume (1:1) of supernatant and ethyl acetate were taken in a separation funnel and agitated for about 30 minutes. The solvent layer was separated and the supernatant was again extracted with ethyl acetate. The solvent layers were pooled and evaporated to dryness at 40^{o}C (Alimuddin *et al.*, 2011). The crude solvent extract thus obtained was screened for antibacterial and antioxidant activities.

Partition of the extract

The crude extract re-dissolved with methanol then was added the same volume with n-hexane. The mixture was shaked and let it form 2 layers. The layers were separated and each layer was concentrated using rotary evaporator so that found 2 extract namely n-hexane and methanolic extracts.

Total phenolic content of ethyl acetate extract of *S. lydicus* A2

The total phenolic content of ethyl acetate extract was determined by employing Folin-Ciocalteu Reagent (FCR) method. Herein, a dilute concentration of the extract (0.5 ml) was mixed with 0.5 ml of diluted Folin-Ciocalteu reagent (1:1) and 4 ml of sodium carbonate (1 M). The tubes were allowed to stand for 15 minutes and the absorbance was read colorimetrically at 765 nm. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml) and the total phenolic content of extract was estimated as µg Gallic acid equivalents (GAE)/mg of extract (Junaid *et al*.**,** 2013)

Antimicrobial activity of ethyl acetate extract of *S. lydicus* A2

Agar well diffusion method was performed to screen antibacterial activity of ethyl acetate extract of strain A2 against eight Gram positive bacteria ie. *S.aureus* TISTR 517, *B. cereus* TISTR 11778, including MRSAs (142, 1096, 2499, 2559, 7645, 1424). Three Gram negative bacteria i.e., *Escherichia coli* TISTR 887, *Salmonella typhimurium* TISTR 292, *Pseudomonas aeruginosa* TISTR 1467. The test bacteria were inoculated into sterile Mueller Hinton broth (MH; HiMedia, Mumbai) tubes and incubated for 24 hours at 37^{o}C. Using sterile swabs, the broth cultures of test bacteria were swabbed on sterile MH agar (HiMedia, Mumbai) plates followed by punching wells of 6mm diameter using sterile cork borer. 100µl of ethyl acetate extract (3 mg/ml of 20% DMSO), standard (Streptomycin, 1mg/ml) and DMSO (20%) were transferred into labeled wells and the plates were incubated at 37^{o}C for 24 hours. The plates were observed for zone of inhibition formed, if any, and measured using a ruler (Kekuda *et al*., 2012). This procedure was also determined antimicrobial activity using fungal test, *C. albicans* but the medium assay was potato dextrose (PDA; HiMedia, Mumbai) agar. The experiment was carried in replicate and the average value was recorded.

DPPH free radical scavenging activity

DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay was performed to evaluate radical scavenging nature of extract of strain A2 (Kekuda *et al*., 2010). Briefly, 2ml of DPPH solution (0.002% in methanol) was mixed with 2ml of different concentrations (5-200µg/ml) of ethyl acetate extract and reference standard (ascorbic acid) in separate tubes. The tubes were incubated in dark at room temperature for 30 minutes and the optical density was measured at 515 nm using ELISA micotiter plate reader. The absorbance of the DPPH control (without extract/standard) was noted. The scavenging activity was calculated using the formula:

Scavenging activity (%) = [(A – B) / A] x 100, where A is absorbance of DPPH control and B is absorbance of DPPH in the presence of extract/standard.

RESULTS AND DISCUSSION

Streptomycetes and preliminary test for antimicrobial activity of isolates

The life cycle of Streptomycetes provides 3 distinct features for microscopic characterization namely vegetative mycelium, aerial mycelium baring chains of spores and the characteristic arrangement of spores and the spore ornamentation. The latter two features produce most diagnostic information (Anderson *et al*., 2001; Taddei *et al*., 2006). Details on cultural and microscopic characteristics together assisted the researchers to classify actinomycetes as members of the genus *Streptomyces*. Many studies have been carried out where the actinomycetes isolates were identified as species of *Streptomyces* based these properties or characteristics (Kekuda *et al*., 2010; Kekuda *et al*., 2012).A total of 8actinomycetes isolates (A2, AB, AG, AH, AL10, AL15, AL18, S2) were recovered from the air of Scientific and Technological Equipments Building 7th on LB/2 agar plate. The details for identification of those of strains will be reported elsewhere.

All the isolates were subjected for cross streak method in order to assess antagonistic property against a panel of 6 bacteria. Presence of reduced growth of test bacteria near the growth of actinomycetes was considered as positive for antagonistic activity. All the isolates were potent enough to inhibit at least one of the test bacteria.

The *S. lydicus* A2showed prominent inhibitory activity against all test bacteria**(Table 1)**.There are many techniques for detecting antimicrobial activity; most of them are based on methods involving diffusion through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms (Lertcanawanichakul and Sawangnop, 2008). The cross-streaking is an easy and relatively rapid method for screening cultures in search for new antibiotics and thus establish a spectrum of inhibiting properties of any bacterium, mold, or actinobacteria which will grow discretely on an agar plate (Williston *et al*., 1947). As a result, the inhibitory activity on tested bacteria by cross streak method as preliminary screening for antimicrobial activity is seen as better **(Table 1)** than those obtained by agar well diffusion method (Lertcanawanichakul and Sawangnop, 2008). Similar findings earlier study that reported lactic acid bacteria could be showed better antimicrobial activity when tested by spot-on-lawn method, because of all metabolites; lactic acid, acetic acid, diacetyl, bacteriocin etc., are present and being produced during the assay period (Con and Gökalp, 2000).

In addition, in antimicrobial activity research the spot-on-lawn method (Con and Gökalp, 2000) or cross streak method (Lertcanawanichakul and Sawangnop, 2008) in this study is a practical and suitable technique. However, in bacteriocin or secondary metabolites investigations, the spot-on-lawn method or cross streak method should be controlled with the disc diffusion method or agar well diffusion method, it could be caused of the major drawback of the 'cross streak method' was difficulty in obtaining quantitative data, since the margins of the zone of inhibition were usually very fuzzy and indistinct (Williston *et al*., 1947).

Antimicrobial activity of culture broth

The culture broth of *S. lydicus* A2was prepared by cultivation the A2 in various media and incubation time to evaluate this effect toward antimicrobial activity according to the number of inhibited microorganism tests.

The result is all of the culture broth that cultivated in 4 media did not inhibit growth *P. aeruginosa* but inhibit *S. aureus*, *B. subtilis* and *S. typhimurium*. Interestingly, the best antimicrobial activity can be seen only on *S. lydicus* A2 cultivated in LB/2 broth with 12 days of incubation time. Medium and incubation time plays a pivotal role in secondary metabolites productions especially antimicrobial activity. Decrease of nutritional value from LB to LB/2 did not increase the number of inhibited test microorganism although is not for YM/2 medium. The crude extract produced in poor nutritional YM medium (YM/2) with incubation more than 14 days showed it can inhibit growth more test microorganisms compared with that of rich nutritional YM medium (YM broth). Based on the result, limited nutrition on cultivation of *Streptomyces* is not guaranteed to get the best antimicrobial compounds from its extract. It could be depended upon the composition of media that there is article explained the negative effects of carbon catabolites on antibiotic production (Sa´nchez *et al*., 2010).

Antimicrobialactivity ofextracted fractions

The efficacy of ethyl acetate extract (crude extract) of strain *S. lydicus* A2to inhibit bacteria was tested against a panel of 3 test bacteria. It was observed that the inhibition of Gram positive bacteria by the extract was highest when compared to Gram negative bacteria. Among Gram positive and Gram negative bacteria, high susceptibility to extract was shown by *S. aureus* and *E. coli*, respectively. However, the inhibitory effect of extract was lesser than that of standard antibiotic. DMSO (20%) did not show any inhibition of test bacteria **(Table 2)**.Similar findings were obtained in earlier studies (Hassan *et al*. 2001; Anansiriwattana *et al*. 2006; Al-Hulu *et al*. 2011; Kekuda *et al*. 2012) which report high susceptibility of Gram positive bacteria than Gram negative bacteria. The low antibacterial activity of ethyl acetate extract against the Gram negative bacteria could be ascribed to the presence of an outer membrane that possess hydrophilic polysaccharides chains and forms an additional barrier for the entry of extract as well as antibiotics into the cells (Lodhia *et al*., 2009; Nalubega *et al*., 2011)

The crude extract was partitioned using n-hexane as asolvent so that was found two fractions namely n-hexane andmethanol. Both fractions were tested antibacterial activity against 3 test microorganisms (*S. aureus* TISTR 517, *B. cereus* TISTR 11778 and *E. coli* TISTR 887). On the basis antimicrobial activity, the fraction exhibiting antibacterial activity is only methanol **(Table 2)** so antibacterial compounds is probably polar compounds.

Table 1: Preliminary screening for antibacterial activity of actinomycetes isolates.

species	Extent of inhibition of test bacteria					
	S. aureus	***B. cereus***	**MRSA 142**	***E. coli***	***P. aeruginosa***	***S. typhimurium***
S. lydicus A2	+	+	+	+	-	+
S. vinaceusdrappus AB	+	-	+	+	-	-
*S. gancidicus*AG	+	-	+	+	-	-
S. longispororuber AH	+	-	+	-	-	-
*S. coerulescens*AL10	+	-	+	-	-	-
*S. rochei*AL15	+	-	+	-	-	-
S. plicatus AL18	+	-	+	-	-	-
S. angustmycinicus S2	+	-	+	-	-	-

+, has antimicrobial activity ; -, has no antimicrobial activity

Table 2: Antibacterial activity of fractions of *S. lydicus* A2.

Test bacteria	Zone of inhibition (mm)				
	Ethyl acetate Fraction	**Methanolic Fraction**	**n- Hexane Fraction**	**Streptomycin**	**DMSO**
S. aureus	16	17	0	35	0
B. cereus	14	13	0	31	0
E. coli	0	0	0	29	0

S. lydicus A2 used in this study was activated from -80^{o}C storage for 14 months. Generally, the extract*S. lydicus* A2 prepared by various media and incubation time can only active against around 3 of 11 test microorganisms (*S. aureus*, *B. cereus*, MRSA 142). Whereas, the extract prepared from *S. lydicus* A2 without -80 ^{o}C storage can active 6 of 11 test microorganisms [*S. aureus*, *B. cereus*, MRSAs(142, 1096, 1424, 2559)]. On the basis of the results, there are decrease significantly in the inhibition of test microorganisms compared with the extract prepared from *S. lydicus* A2 without -80 ^{o}C storage. It means that the extract prepared from *S. lydicus* A2 isolated from its habitat and use directly to make an extract will give a better antibacterial activity, especially anti-MRSAs activity.

Antioxidant activities of fractions

Actinomycete produce various secondary metabolites which are probably showing various biological activities. To find out other biological activities, All fractions were conducted antioxidant test. DPPH is a stable, organic and nitrogen centered free radical having the absorption maximum band around 515-528nm (517nm) in alcoholic solution. It accepts an electron or hydrogen atom and becomes a stable diamagnetic molecule. Though a number of *in vitro* assays have been developed to evaluate radical scavenging activity of compounds. The effect of antioxidants on scavenging DPPH radical is due to their hydrogen donating ability. In this assay, the antioxidants reduce the purple colored DPPH radical to a yellow colored compound diphenylpicrylhydrazine, and the extent of reaction will depend on the hydrogen donating ability of the antioxidants (Bondent *et al.*, 1997). In the present study, a decrease in the absorption of DPPH solution in the presence of various concentrations of ethyl acetate extract was measured at 515 nm. It was observed that the radical scavenging activities of extract and ascorbic acid increased on increasing concentration. The absorbance of reaction mixture at 515 nm increased with the increase in DPPH* (free radical) and converting it into DPPHH, the scavenging effect of the extract was lesser than that of ascorbic acid. The radical scavenging effect of extract was >50% at concentration of 3.4 mg/ml and higher concentration of extract indicating reducing potential of extract. However, the reducing potential of extract was lesser than that of reference standard. Although the scavenging abilities of extract was lesser, it was evident that the extracts showed hydrogen donating ability and therefore the extract could serve as free radical scavengers, acting possibly as primary antioxidants (Chung *et al*., 2006). Unfortunately, two fractions, methanolic fraction and n-Hexane fraction showed no antioxidant activity in the DPPH free radical scavenging activity. It cannot detect the phenolic contents in both fractions, whereas it could be estimated the phenolic contents in ethyl acetate fraction was to be 0.18$\pm$0.01 µg Gallic acid equivalents (GAE)/mg of extract.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Hsu *et al*., 2006). In the present study, it was found that the reducing power of the extract and ascorbic acid increased with the increase of their concentrations. Moreover, the DPPH radical scavenging activity will increase depend upon the increasing concentration of total phenolic compounds. However, though reducing power of extract was lesser than that of ascorbic acid, it is evident that the extract possesses reductive potential and could serve as electron donors, terminating the radical chain reactions (Chung *et al*., 2006).

CONCLUSION

The study was successful in the isolation and investigation of antagonistic actinomycetes from air sample collected at Walailak University, Thailand. The isolate A2 was identified as a species of the genus *Streptomyces* based on cultural, microscopic characteristics and 16S rDNA analysis (unpublished data), identified as *S. lydicus* A2. It was stored at -80 ^{o}C, exhibited instability of antimicrobial activity especially for test microorganisms which it decreases activity than *S. lydicus* A2 without stored at -80 ^{o}C. However, the fractionated extract (ethyl acetate, methanolic and n-hexane fraction), especially ethyl acetate fraction, has various biological activities such as antimicrobial and antioxidant activities. The extract was found to contain bioactive

principles which are to be separated and subjected for activity determinations. The *S. lydicus* A2 can be a potential candidate for the development of therapeutic agents. Further studies on characterization of the isolate and the purified bioactive components in the solvent extract are under progress.

ACKNOWLEDGEMENTS

The research was grant by Walailak University (WU58113). The authors are sincerely thankful to School of Allied Health Sciences and Public Health for providing facilities to conduct work and moral support.

CONFLICT OF INTERESTS

The author(s) have no declared any conflict of interests.

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How to cite this article:

Monthon Lertcanawanichakul, Kanchanat Pondet, Jutaporn Kwantep. *In vitro* Antimicrobial and Antioxidant Activities of Bioactive Compounds (Secondary Metabolites) Extractsfrom *Streptomyces lydicus* A2. J App Pharm Sci, 2015; 5 (02): 017-021.