

## 2, 4-Di-tert-butylphenol, the bioactive compound produced by *Streptomyces* sp. KB1

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### ARTICLE INFO

#### Article history:

Received on: 20/08/2015

Revised on: 23/09/2015

Accepted on: 10/10/2015

Available online: 12/11/2015

#### Key words:

*Streptomyces* sp. KB1, Anti-methicillin resistant *Staphylococcus aureus* activity, 2, 4-Di-tert-butylphenol.

### ABSTRACT

*Streptomyces* sp. KB1(TISTR 2304), identified by 16S rDNA gene, was collected from the air sample at Aonang, Krabi province, Thailand and deposited in the GeneBank database (accession number KF939581.1). It could produce bioactive compounds and excrete into liquid culture medium within 4 days of incubation period at 30 °C, 200 rounds per minute in shaking incubator. Bioactive compounds in culture broth supernatant were extracted with 3-times of ethyl acetate (EA), evaporated by rotary evaporator at 45 °C under reduced pressure, purified by silica gel column chromatography and eluted by gradient solvent system of ethyl acetate: hexane at ratio of 1:9, 3:7, 4:6, 6:4 and 10:0, v/v. Eight pooled fractions were obtained, name PF1 - PF8, from basis of their TLC profile. Only PF4 displayed as purified active compound which showed anti-methicillin resistant *Staphylococcus aureus* (MRSA) activity. The PF4 was predicted as 2, 4-Di-tert-butylphenol (C<sub>14</sub>H<sub>22</sub>O) with molecular weight 206.2.

### INTRODUCTION

Actinomycetes are gram positive, aerobic, branching and unicellular microorganisms having high Guanine-Cytosine percent (about 69-75%) in their DNA and ubiquitous in nature (Ventura *et al.*, 2007). They have significant capability for degradation of complex organic compounds and they are also invaluable bacteria as they are well known for production of wide range of active secondary metabolites (Solanki *et al.*, 2008). From 22,500 of total bioactive compounds that have been reported. It was found that about 45% ( $\approx$  10,125) have been produced by actinomycetes, 38% ( $\approx$  8,550) by fungi, and 17% ( $\approx$  3,825) by unicellular bacteria (Berdy, 2005). Among actinomycetes, *Streptomyces* species alone produce

around 8,100 of bioactive compounds and contributing majorly towards the reservoir of important bioactive compound with less contribution from other genera (Renu *et al.*, 2015). They were well known that bioactive compounds produced by *Streptomyces* were used to develop as many important substrates in medical field such as antibiotics, immunomodulators, anticancer drugs, antiviral drugs, herbicides, and insecticides (Watve *et al.*, 2001; Rahman *et al.*, 2010). Today around 11,900 antibiotics had been discovered by 1994 of which around 7,140 (60%) were produced by these bacteria (Procopio, 2012; Safey *et al.*, 2013). From the problem of old antibiotics for infectious disease treatment is failure leads to infectious diseases are increase (Garcia, 2009; Oancea and Stoia, 2010). Therefore, novel antibiotics had been continuously invented and developed (Raja and Prabakarana, 2011). The interesting specie of *Streptomyces* used in the present study was collected from the air sample at Aonang, Krabi province, Thailand, identified by means of partial 16S rDNA gene sequence analysis at National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani province, Thailand, submitted to GenBank (accession number KF939581.1) and finally named as

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*Streptomyces* sp. KB1. An unknown species, *Streptomyces* sp. KB1, is one of an important bioactive compound-producing strain. In our previous studies, it showed broad spectrum of antimicrobial activity against representative of Gram-positive bacteria (*Staphylococcus aureus* TISTR 517, *Micrococcus luteus* TISTR 884 and *Bacillus cereus* TISTR 11778), Gram-negative bacteria (*Escherichia coli* TISTR 887, *Salmonella typhimurium* TISTR 292 and *Pseudomonas aeruginosa* TISTR 1467) and yeasts (clinical isolate of *Candida albicans* and *Cryptococcus neoformans*). Therefore, the aim of this present study was to extract, separate, purify the bioactive compounds produced by *Streptomyces* sp. KB1 and elucidate the chemical structure after obtain the pure active compound.

## MATERIALS AND METHODS

### Microorganisms, Media and Cultural conditions

**Producing strain:** *Streptomyces* sp. KB1 was collected from air sample at Ao-nang, Krabi province, Thailand, by using Biosampler, Microflow 90 (Aquaria, Italy), at flow rate 100 l/min for 30 min according to the manufacture's instruction and isolated as pure culture on half-formula of Luria Bertani (LB/2; Himedia, India) agar medium (5 g/l Tryptone, 2.5 g/l Yeast extract, 5 g/l NaCl and 15 g/l Agar powder). The isolate was identified by means of 16S rDNA gene sequence analysis at BIOTEC, Pathum Thani province, Thailand, and published in GenBank database (NCBI, USA). Firstly, it was cultured on LB/2 agar medium at 30 °C in static incubator (WTB binder, Germany) for 5 days. Later, isolated colony was inoculated into 10 ml of LB/2 broth medium in 25 × 150 mm of screw cap test tube, incubated at 30 °C, 200 rpm in shaking incubator (Thermo scientific, USA) for 2 days and the cells were stored in 15% glycerol at -80 °C until use.

### Indicator strains

*Staphylococcus aureus* TISTR 517 and clinical isolate of methicillin-resistant *Staphylococcus aureus* 142 (MRSA 142) were streaked on Muller Hinton (MH) agar medium (Himedia, India), incubated at 37 °C in static incubator for 24 h. Single colony was inoculated into 10 ml of LB broth medium in 25 × 150 mm of screw cap test tube, incubated at 37 °C, 200 rpm in shaking incubator for 24 h. The cells were stored in 15% glycerol at -80 °C until use.

### Cultivation of producing strain

#### Starter preparation

1 × 1 cm<sup>2</sup> of initial streak of 5-days culture of *Streptomyces* sp. KB1 was inoculated to 10 ml of LB/2 broth medium in 25 × 150 mm of screw cap test tube. Inoculum was incubated at 30 °C, 200 rpm in shaking incubator for 2 days and assigned as starter.

#### Fermentation

12 l of supernatant was prepared by 6-times of cultivation of *Streptomyces* sp. KB1. Briefly, 2 l of LB/2 broth

medium was prepared in a large container and divided as 200 ml to ten flasks of 1,000 ml baffled flasks. All flasks were autoclaved at 121 °C for 15 min. Then, 1% starter of *Streptomyces* sp. KB1 was inoculated to all flasks, incubated at 30 °C, 200 rpm in shaking incubator for 4 days. After incubation, all of the culture broth was centrifuged to separate the cell sediment at 4 °C, 10,000 rpm for 30 min and harvested the supernatant. 12 l of supernatant was filtered through Whatman No. 1 filter paper by using vacuum pump (Gast, U.S.A). The clear supernatant was tested for the activity against *S. aureus* TISTR 517.

### Extraction of bioactive compounds from clear supernatant

The clear supernatant (12 l) was extracted 3-times with ethyl acetate (EA) at ratio 1:1 (v/v), harvested and pooled the solvent layer. The pooled EA extracts were evaporated by using rotary evaporator at 45 °C under reduced pressure. EA extract slag was weighed, dissolved in the least amount of 100% acetone and named as crude EA extract.

### Separation of the bioactive compounds

Separation of the bioactive compounds into their components was conducted by thin layer chromatography (TLC). The dissolved crude EA extract was spotted on 1 × 5 cm of silica TLC plate and developed in the gradient solvent system of EA: hexane in ratio of 1: 9 - 10: 0 (v/v). The separated compound(s) were visualized under UV at 254 nm (absorbance) and 365 nm (fluorescence) or detected by spraying with sulfuric acid and heating at 150 °C for 20 sec.

### Purification of the bioactive compound

The bioactive compounds were purified by using silica gel column (4.5 × 60 cm) chromatography. EA : hexane (1:9, v/v) was used as an eluting solvent. The column was left for overnight until the silica gel 60 - 120 mesh size of chromatography grade (Merck, Germany) was completely settled. 10 ml of dissolved crude EA extract was intermixed with 5 g of silica gel, poured into the silica gel column surface and gradually added a suitable solvent systems. Fractions (each of 10 ml) were collected as regular intervals at flow rate 2.5 ml/min, loaded on silica TLC plate, developed with same solvent system and observed the pattern of spot(s). The fractions that exhibited same R<sub>f</sub> values were pooled. The pooled fractions (PF) were evaporated to remove the solvents at appropriate condition. The compounds sediment was weighed, dissolved with 100% acetone for final concentration of 10 mg/ml and investigated the anti-MRSA activity by applying from bioautography method.

### Anti-MRSA activity-guided isolation

Bioautography is a method to localize antimicrobial activity on a chromatogram (Patil *et al.*, 2013). In present study, it was applied to investigate the anti-MRSA 142 activity. Briefly, 2, 4, 6 and 8 µl of 10 mg/ml of each dissolved compounds were spotted on 1 × 5 cm of silica TLC strip. The silica TLC strip was

air dried to remove the residue acetone in fume hood at room temperature for 20 min and then placed on Muller Hinton agar (MHA) plate. Finally, the MHA plate were poured with semi-solid MHA medium (0.7% agar) which had  $1.5 \times 10^8$  CFU/ml of MRSA 142, placed at room temperature for 15 min and incubated in static incubator at 37 °C for 24 h. The plates were observed the clearance formed around the spot on silica TLC strip.

### Spectroscopy of bioactive compounds

#### *Proton and carbon nuclear magnetic resonance (NMR) spectra*

<sup>1</sup>H and <sup>13</sup>C NMR, HMBC, HMQC, DEPT 90, DEPT 135, and COSY spectra were obtained from FT-NMR spectrometer (Bruker, model AVANCE DPX-300, Germany) at center for scientific and technological equipments, Walailak University, Thailand. In this process was operated at 300 MHz for proton and 75 MHz for carbon. Chloroform (CDCl<sub>3</sub>) was used as deuterated solvents in NMR experiments. Referent signals were the signals of residual undeuterated solvents at  $\delta$  7.24(<sup>1</sup>H) and 77.0 (<sup>13</sup>C) ppm for CDCl<sub>3</sub>.

#### *Fourier transforms infrared (FT-IR) absorption spectra*

FT-IR absorption spectrum of bioactive compound was obtained from FT-IR spectrophotometer (Bruker, model TENSOR 27, Germany) at center for scientific and technological equipments, Walailak University, Thailand. The compounds were examined as dry film on NaCl cell.

#### *Ultraviolet (UV) absorption spectra*

UV absorption spectrum of bioactive compound was obtained from UV/VIS spectrophotometer (JASCO, model V-630, Japan) at center for scientific and technological equipments, Walailak University, Thailand.

#### *Mass Spectra*

Mass spectrum of bioactive compound was obtained from Mass spectrometer (Thermo, model MAT 95 XL, Germany) at scientific equipment center, Prince of Songkla University, Thailand.

### Anti- MRSA activity of bioactive compounds

Anti- MRSA efficacy of bioactive compounds was determined by agar well diffusion method (Kekuda *et al.*, 2012). Single colony of 24 hours-culture of MRSA 142 was inoculated to 5 ml of LB broth medium in 15 × 150 mm of screw cap test tube and then incubated at 37 °C, 200 rpm in shaking incubator for 24 hours. Cell suspension was approximately adjusted to a 0.5 McFarland standard turbidity by 0.85% NaCl and swabbed on sterilized MHA plates using sterile cotton swab and drilled well by a 5 mm diameter of sterile cork borer. 50  $\mu$ l of bioactive compounds (1mg/ml of 100% DMSO), Oxacillin (200  $\mu$ g/ml), Vancomycin (200  $\mu$ g/ml) and DMSO (100%) were transferred into labeled wells. The plates were incubated at 37 °C in static

incubator for 24 hours. The plates were observed the zone of inhibition formed around the each well.

## RESULTS AND DISCUSSION

### Cultivation of producing strain

Medium and incubation period plays a pivotal role on bioactive compounds production and their antimicrobial activity (Reddy *et al.*, 2011). This study, *Streptomyces* sp. KB1 was cultivated in LB/2 broth medium for 4 days. The cultivation in decrease of nutritional value from LB to LB/2 did not impact on bioactive compounds production (data not shown). This phenomenon was supported by research which reported that the best antimicrobial activity of *Streptomyces lydicus* A2 was seen by cultivating it in LB/2 broth medium (Lertcanawanichakul *et al.*, 2015).

### Extraction, Separation and Purification of bioactive compound

#### *Extraction*

EA is solvent that was used to extract the most of bioactive compounds or antibiotic because maximum bioactive compounds or antibiotic yield was observed in residue extracted by using EA (Parthasarathi *et al.*, 2012). In this study, 2.82 g of crude EA extract was extracted from 12 l of clear supernatant. Crude EA extract, prepared as 50 mg dry weight/ml with 100% DMSO, showed inhibition zone of  $20.67 \pm 0.58$  mm against MRSA142.

#### *Separation*

TLC experiment was performed to investigate the suitable solvent system for using as eluted solvent system in purification process. After TLC plates were developed with ten gradients elution of EA: hexane, sprayed with sulfuric acid and heated at 150 °C for 20 sec. Different spots of compounds on each TLC plate were showed. From this process, solvent systems of EA: hexane in ratio of 1:9, 3:7, 4:6, 6:4 and 10:0 were selected to use for separating the each compound.

#### *Purification*

Bioactive compounds were purified by silica gel column chromatography techniques. This techniques used in the isolation depend on the amount and metabolite profiles of the crude EA extracts. Silica gel could strongly attach with high polar compound, therefore, low polar compounds were separated from column before high polar compounds which were sequentially eluted by increasing polarity of developing solvent (1: 9, 3: 7, 4: 6, 6: 4 and 10: 0, v/v of EA: hexane). Two hundred and three fractions of total were collected and pooled on the basis of their TLC profile after investigated by silica gel TLC technique. The pooled-PF were marked as fractions 1 - 35 (PF1), 36 - 59 (PF2), 60 - 89 (PF3), 90 - 114 (PF4), 115 - 136 (PF5), 137 -164 (PF6), 165 - 188 (PF7) and 189 - 203 (PF8). The dry weight and R<sub>f</sub> value of each PF was showed in **Table 1**.

**Table 1:** Illustrate various data of each PF.

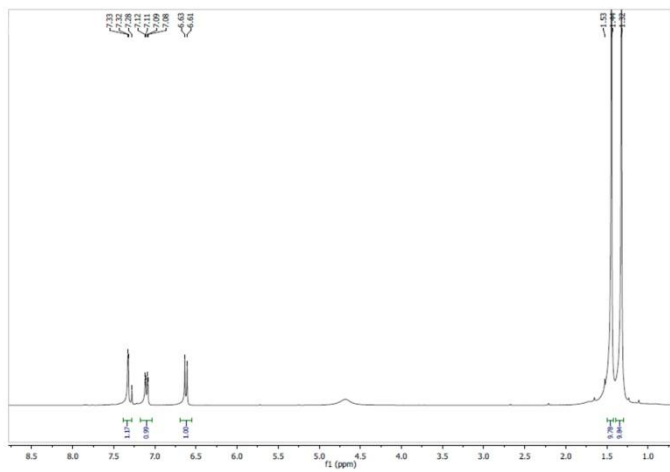
The pooled fraction (PF)	Ratio of Ethyl acetate: Hexane	Formation from fraction	No. of fraction	Dry weight of each pooled fraction	Rf values
1	1 : 9	1 - 35	35	1.6	0.94
2	1 : 9	36 - 59	24	10.4	0.69
3	3 : 7	60 - 89	30	67.3	0.46
4	4 : 6	90 - 114	25	51.0	0.36
5	6 : 4	115 - 136	22	10.8	0.38
6	6 : 4	137 - 164	28	2.1	0.27
7	10 : 0	165 - 188	24	2.8	0.25
8	10 : 0	189 - 203	15	2.6	0.14

### Anti-MRSA activity-guided isolation

After 8 PFs were investigated anti-MRSA activity. It was found that PF3, PF4 and PF5 showed activity against MRSA 142. They were selected, investigated the purity from spot feature on silica gel TLC plate when developed with many solvent systems including 2:8 and 4:6 (v/v) of dichloromethane: hexane, 0.5:9.5 (v/v) of EA: hexane and 4:6 (v/v) of EA: dichloromethane. From spot feature on silica gel TLC plate, only PF4 was selected to investigate the purity of compound in the later time.

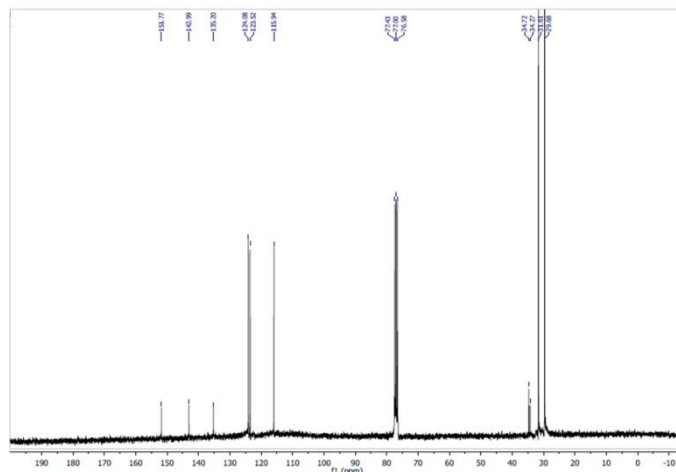
### Spectroscopy

After PF4 in CDCl<sub>3</sub> was investigated the purity by screening <sup>1</sup>H NMR, the result showed that this compound has high purity (Figure 1).

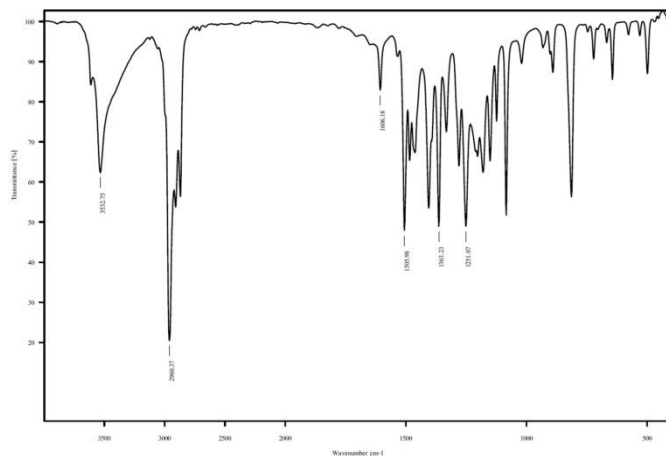
**Fig. 1:** <sup>1</sup>H NMR spectrum of pure active compound.

The <sup>1</sup>H NMR spectra of this compound showed the presence of a total 21 protons, of which 18 protons were present as six tert-methyls at  $\delta$  1.34 (9H, s, 3  $\times$  CH<sub>3</sub>) and 1.46 (9H, s, 3  $\times$  CH<sub>3</sub>). The remaining three protons were present in the downfield aromatic region at 6.63 (1H, d, J = 8.2 Hz), 7.11 (1H, dd, J = 8.2, 2.4 Hz), and 7.34 (1H, d, J = 2.4 Hz), suggesting that this compound is a trisubstituted aromatic compound. This suggestion was supported by <sup>13</sup>C NMR spectra which showed the presence of 10 carbon signals, of which six downfield carbon signals between 115.1 and 151.8 ppm were in the aromatic region, whereas the remaining four carbon signals were in the upfield region between

29.7 and 34.7 ppm. Of six aromatic carbon signals, three were quaternary carbons (135.2, 143.0, and 151.8 ppm), suggesting that the aromatic substitutions were at these carbons. The most downfield quaternary carbon at 151.8 ppm indicated the presence of a phenolic OH group at this carbon in the molecule. Among the four upfield carbon signals, the two at 34.3 and 34.7 ppm were tertiary carbons and the remaining two at 29.7 and 31.6 ppm were methyl signals. This clearly suggested the presence of two tert-butyl substitutions on the two remaining quaternary carbons at 135.2 and 143.0 ppm in the aromatic ring (Figure 2).

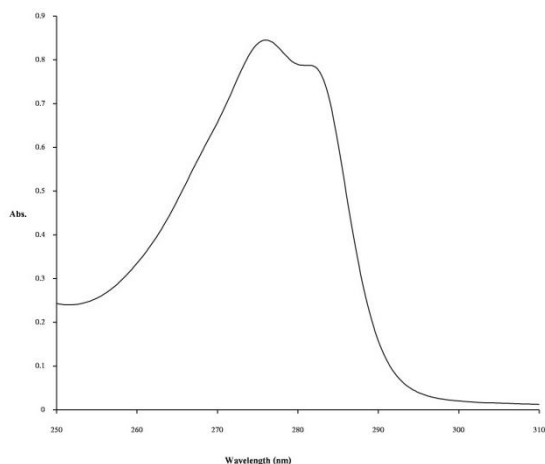
**Fig. 2:** <sup>13</sup>C NMR spectrum of pure active compound.

Finally, on the basis of the splitting patterns and coupling constants of the three aromatic protons, the chemical structure of bioactive compound or PF4 was deduced as 2, 4-Di-tert-butylphenol.

**Fig. 3:** FTIR spectrum of pure active compound.

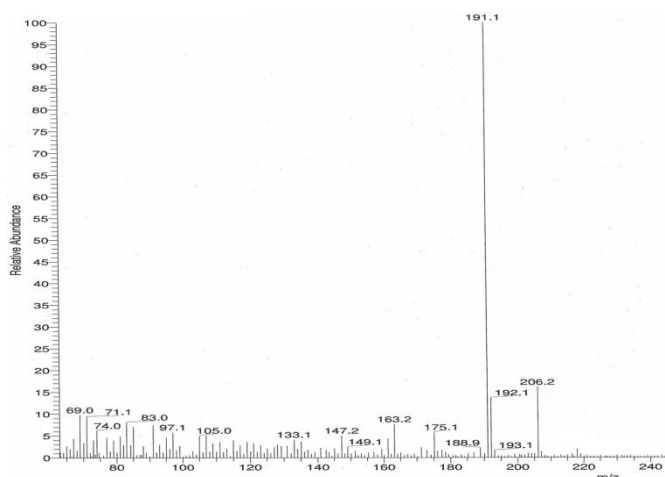
The FT-IR spectrum (Figure 3) of bioactive compound showed various peaks following at 3532 cm<sup>-1</sup> indicates the presence of O-H, at 2960 cm<sup>-1</sup> indicates the presence of C-H stretching in -CH<sub>3</sub> or -CH<sub>2</sub>, at 1506 - 1606 cm<sup>-1</sup> indicates the presence of C=C stretching in aromatic compounds, at 1363 cm<sup>-1</sup> indicates the presence of C-H bending in tert-butyl group and at

1251  $\text{cm}^{-1}$  indicates the presence of C-O stretching in phenol group. The ultraviolet (UV) absorption spectrum of bioactive compound was recorded a maximum absorption peak at 276 and 282 nm (**Figure 4**).



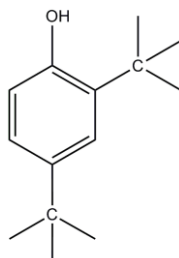
**Fig. 4:** UV-VIS spectrum of pure active compound.

The Mass spectrum revealed that molecular weight and formula were 206.2 g/mol and  $\text{C}_{14}\text{H}_{22}\text{O}$ , respectively (**Figure 5**).

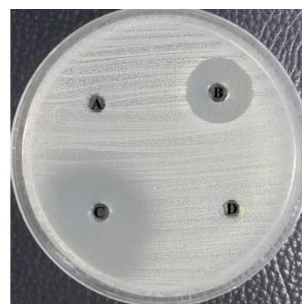


**Fig. 5:** Mass spectrum of pure active compound.

Based on the various spectral data, it can be predicted that the molecule structure of bioactive compound or 2, 4-Di-tert-butylphenol as **Figure 6** and its anti-MRSA 142 efficacy was showed in **Figure 7**.



**Fig. 6:** Molecule structure of pure active compound or 2, 4-Di-tert-butylphenol produced by *Streptomyces* sp. KB1.



**Fig. 7:** Illustrate zone of inhibition formed around the well of oxacillin (A), vancomycin (B), 2, 4-Di-tert-butylphenol (C) and DMSO (D) against MRSA142.

## CONCLUSION

Bioactive compounds produced from *Streptomyces* sp. KB1 (TISTR 2304) showed anti-MRSA 142 activity. After purification step, based on the spectral data, the bioactive compound was deduced as 2, 4-Di-tert-butylphenol which had molecular weight, molecular formula and UV visible maximum absorbance were 206.2 g/mol,  $\text{C}_{14}\text{H}_{22}\text{O}$  and 276 and 282 nm, respectively. It still showed the anti-MRSA activity.

## ACKNOWLEDGEMENT

The authors are sincerely thankful to Ms. Chotika Jeerapong and Mr. Imron Meechai for kindly helping and the laboratory officer of Center for Scientific and Technological Equipments and faculty of Medical Technology, School of Allied Health Sciences and Public Health, Walailak University, Nakhon Si Thammarat, Thailand, for well recommending of some instrument and facilities during this study. The research was grant by Walailak University (WU 56606) to Kittisak Chawawisit. Authors also acknowledge the partially support provided by The Higher Education Research Promotion (HERP).

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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**How to cite this article:**

Kittisak Chawawisit, Phuangthip Bhoopong, Worrarong Phupong and Monthon Lertcanawanichakul. 2, 4-Di-tert-butylphenol, the bioactive compound produced by *Streptomyces* sp. KB1. J App Pharm Sci, 2015; 5 (Suppl 3): 007-012.