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Original Article

ISOLATION, IDENTIFICATION OF PHENYLACETIC ACID FROM STREPTOMYCES GALBUSTP2 STRAIN AND ITS TOXICITY

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ABSTRACT

Streptomyces, which constitutes one genus of Actinomycetes class usually found in the soil, has specific characteristics that can be utilized as a producer of antibiotics. The Objective of this research wasto find out the safety of compounds resulting from the broth of *Streptomycesgalbus*TP2 strain on test animals and normal cells. The fermentation liquid from *Streptomyces galbus*TP2 strainwas extracted by ethyl acetate, then purified through vacuum liquid chromatography and radial chromatography. The identification of pure compound was done bya UV spectrophotometer, (^{1}H, ^{13}C, Dept, HMBC and HMQC) NMR, and LCMS. The result of structural elucidation showed that the pure molecule was Phenylacetic acid. Theacute toxicity test was LD_{50}> 15 g/kg of body weight(subsequently abbreviated into bw) against mice and cytotoxic test was IC_{50} =1228 µg/ml against the normal cell, this indicating that it was safe to be a raw material medicine.

Keyword: Streptomyces sp., Structural Elucidation, Acute Toxicity, Cytotoxic.

INTRODUCTION

Streptomyces commonly produces antibiotics in the form of secondary metabolites that industrially have an economic value in the fields of medicine, pharmacology, agriculture, and biotechnology due to its wide roles as an agent of antibacteria, antifungi, anticancer, antiparasite, immunomodulator, herbicide, and some important enzymes in foods and other important industries [1, 2, 3, 4].

In the last decade, research related to cancer has made amazing progress. Although many anticancer drugs have been invented, most of these drugs are known to be toxic to cells and normal tissues; and they are selective towards limited cancer cells, too. As a result, for therapeutic purposes they are limited to only some certain anticancer types. Besides the identification of new target drugs that can destroy cancer when exposed to chemotherapy, to overcome the limit of and increase the effectiveness of chemotherapy for cancer it is deemed urgent that new anticancer drugs with toxicity specific to cancer cells be found soon[5, 6, 7].A variety of anticancer antibiotics originating either from plants[8, 9] and microorganisms or through synthetic ways are continuously invented [10]. According to data available, in the realm of microbes, Streptomyces is a dominant producer of anticancer antibiotics [11]. The objective of this research is to isolate secondary metabolites and evaluate their toxicity from *Streptomyces galbus*TP2 strain. This strain was isolated from the soil around Tangkubanperahu mountain, West Java, Indonesia.

MATERIALS AND METHODS

Purification by extraction, fractionation, and isolation of pure molecules

The liquid resulting from thefermentation of *Streptomycesgalbus* TP2 strainwas separated from its biomass by centrifugating (Eppendorf 5804R) it at 4,500 rpm for 15 minutes. The fermentation liquid was extracted using ethyl acetate at pH 3 utilizing a funnel with a volume ratio being 1:1. The purification of ethyl acetate extract was done in silica gel (merck) vacuum liquid chromatography (particle size: 35-70 micrometer) by elution with the organic solvent thatwas 80% hexane/20% ethyl acetate to 100% ethyl acetate. This was then continued with radial chromatography (Chromatotron 8924) by elution with the organic solvent thatwas 60% hexane/40% ethyl acetate to methanol 100%. Each result of purification was analyzed using thin layer chromatographyand visualization with ultraviolet light 254 nm-long wave and vapor of H_2SO_4 or cerium spraying solution. Components having the same retention factor (Rf) were pooled and concentrated (free of solvent) using the Rotavapor (BuchiRotavapor R-125) [12].

Identification and characterization of pure molecules

Structural elucidation was done by aUV-Visible spectrophotometer (Hewlet Packard 8453), proton magnetic resonance (^{1}H NMR),carbonmagnetic resonance (^{13}C NMR). Distortionlessenhancement by polarization transfer (Dept NMR), HMBC NMR, and HMQC NMR of compound in CD_3OD (Methanol-D4) was recorded on a Jeol spectrometer operating at 500 MHz.Mass spectrum was recorded on finnigan LQC spectrometer equipped with an electro spray ion source (positive ion mode) “ESI”. The scan range was from 100-1500 m/z[13].

Safety test of compound 1 by acute toxicity and cytotoxic

This acute toxicity test was done to determine the value of LD_{50}andcytotoxic test was carried out to determine the value of IC_{50}. The value of LD_{50} was determined by performing a test on the animals applying 6 doses (g/kg bw) of compound that were 0, 15, 5, 2.5, 1.25, and 0.625 respectively. The compound was fed to the animals orally and observation was done at the 0.5th, 1st, 2nd, 4th, and 24th hour. The observation was next conducted again for 14 days to find out if there occurred any death to the test animals. During the daily observation, the test animals’ body weight was measured. The test animals used for this research were mice. If the highest dose applied didn’t cause any death—this indicated by LD_{50}> 15 g/kg bw, this would mean that the compound test was safe [14, 15].

The value of IC_{50} was fixed by plotting a variety of six concentrates (µg/ml) of compound that were500, 250, 125, 62.5, 31.25, and 15.625respectively with a total of (%) living cells. The result of linear regression rendered the value of IC_{50}. The higher the value of IC_{50} is, the safer the test compound is. In this research, the pure compound resulting from the research was tested against the normal cell Vero taken to represent human normal cells[16].

RESULTS AND DISCUSSION

Isolation of pure molecules

From 6.25 l of fermentation liquid, as much as 5 g of ethyl acetate dried extract was produced. The purification of ethyl acetate extract using silica gel vacuum liquid chromatography and chromatotronradialchromatographyled to one pure molecule, namely, compound 1. It issoluble in methanol. The weight of compound 1 was 10.1 mg.

Structural elucidation of compound 1by UV- Spectrophotometer (^{1}H, ^{13}C, Dept, HMBC, HMQC) NMR, and LCMS

Compound 1 was obtained from a fraction of hexane, ethyl acetate (5:5). The compound appears in the form of white-colored crystals. The UV spectrum of compound 1 shows maximum absorption at a wavelength (λ) of 283 nm (absorbance of 0.8). The presence of a peak at λ > 214 indicated that the compound had a conjugated double bond (Fig. 1).

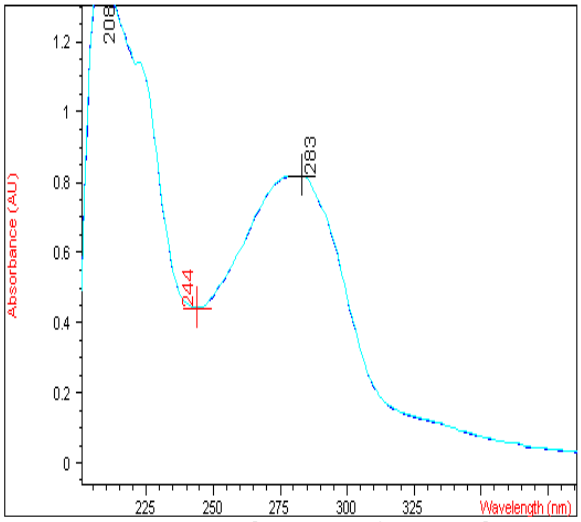

Fig. 1: UV spectrophotometry of compound 1

The chemical shift in protons (^{1}H)was shown bythe signals in the aliphatic area at 3.5911 ppm, five signals in the aromatic area at (7.234; 7.275; 7.276; 7.280; 7.300) ppm respectively. OH bonded at carbonyl at 10 – 12 ppm (Fig.2).

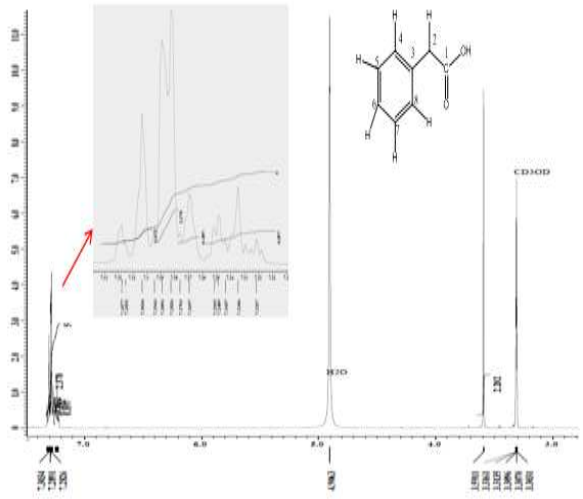

Fig. 2: ^{1}H NMRspectrumofcompound 1

The chemical shift in carbons (^{13}CNMR+DEPT^{135})was shown bythe signals in the aliphatic or alkyl area that were a straight chain at 42.1664 ppm. Five signals in the aromatic area at (127.973; 129.556; 130.472; 136.271) ppm showed the presence of benzene, carbonyl, aldehyde and ketone, and ester-amide-carboxylate respectively. C quartenershowedat 175.864 ppm (Fig.3).

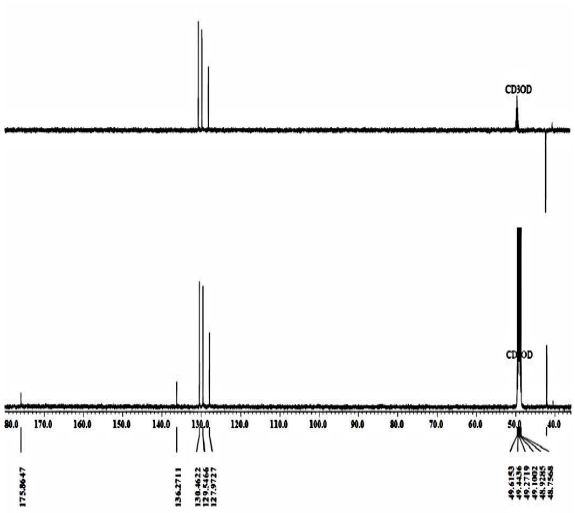

Fig. 3: ^{13}C RMI and DEPT spectrums of compound 1

Characterization of HMQC and HMBC 2D-NMRexplains the relationship between H–C in accordance with the shift, namely, the relationship between aromatic H and aliphatic H (the relationship between aromatic H–C and the relationship between aliphatic H and aromatic and carbonyl) (Fig. 4 and 5).

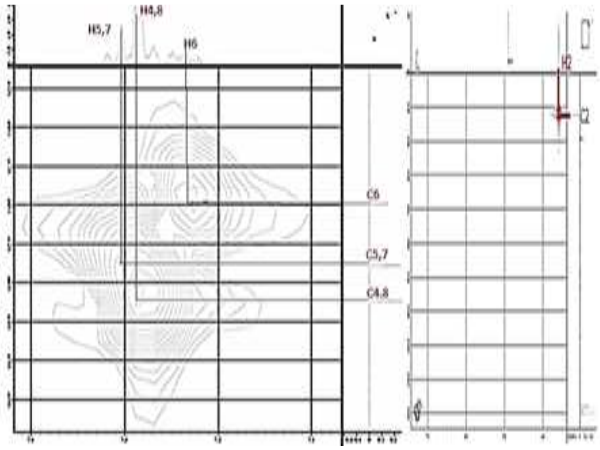

Fig. 4: HMQC spectrum of compound 1

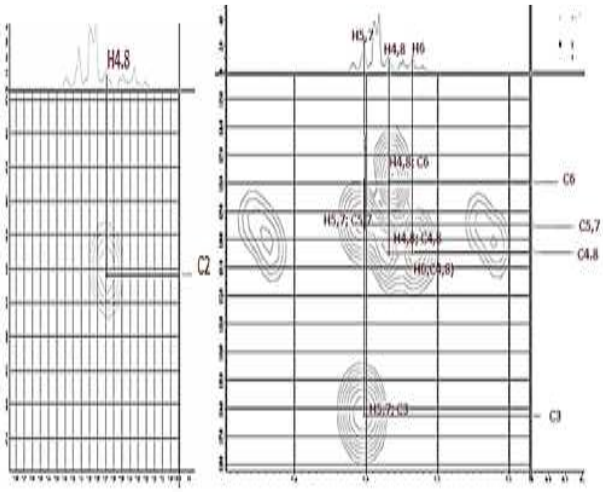

Fig. 5: HMBC spectrumofcompound

Table 1: Data of ^{1}H RMI, ^{13}C RMI, Dept, HMQC,and HMBC spectrums of compound 1

Positionof Carbon	^{1}H-NMR	^{13}C-NMR	Dept	HMQC	HMBC
1	-	175.86	C	-	-
2	3.60	42.16	CH2	3.60	C4,8; C5,7; C3, C1
3	-	136.27	C	-	-
4,8	7.28	130.46	CH	7.28	C4,8;C6
5,7	7.31	129.54	CH	7.31	C3;C5,7
6	7.23	127.97	CH	7.23	C4,8

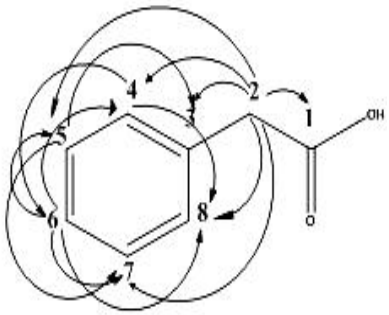

Fig. 6: Specific correlation of HMQC and HMBCspectrumsofcompound 1

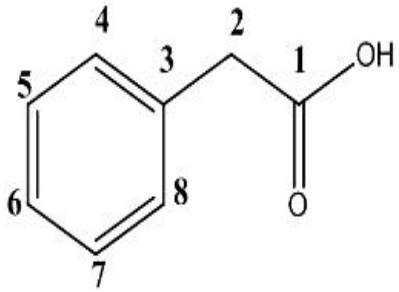

Fig. 7: Chemical structure of compound 1

Carbon (C) at position 2 has a chemical shift value (δ) of 42 ppm; it has proton (H) at a chemical shift of 3.600 ppm. The shift of proton is related to the carbon at positions 4, 8, 5, 7, 3, and 1. Carbon at positions 4 and 8 has a chemical shift value (δ) of 130.460 ppm; it has proton at a chemical shift of 7.280 ppm. The shift is related to the carbon at positions 4, 8, and 6. Carbon at positions 5 and 7 has a chemical shift value (δ) of 129.540 ppm; it has proton at a chemical shift of 7.310 ppm. The shift is related to the carbon at positions 3, 5, and 7. Carbon at position 6 has a chemical shift value (δ) of 127.970 ppm; it has proton at a chemical shift of 7.230 ppm. The shift is related to the carbon at positions 4, and 8.

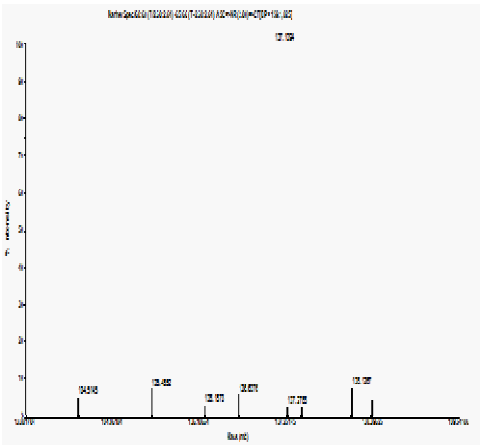

Fig. 8: Mass spectrum (LCMS)of compound 1

The pattern of mass spectrum fragmentation from compound 1 shows a strong molecular ion (M^{+}, m/e 137). Next ions were formed directly from ionic molecules through the loss of H+. m/e137 released H^{+} to become 136. m/e136 released H^{+} to become 135. m/e135 released H^{+} to become 134. Therefore, a total of 3 H^{+} were released (Fig. 8).

The result of structure elucidation and identification using a UV Spectrophotometer, (^{1}H, ^{13}C, Dept, HMBC and HMQC) NMR, and LCMSindicated that compound1wasPhenylacetic Acid (Fig. 1, 2, 3, 4, 5, 6, 7, 8, and Table 1).

Safety test on compound 1 against mice

At the 0.5th through 1st hour, there were some mice that were weak at a dose of 15 g/kg bw; but the result of observation of behaviors for 4 hours and 24 hours after the treatment with the test substance indicated that all mice were normal and equal to those in the control group. At the platform condition, the number decreased at the 0.5th through the 2nd hour at a dose of 15 and 5 g/kg bw, but at the 24th hour all mice at all doses showed a normal condition again. Motoric activities were generally normal; only at the 0.5th through the 2nd hour at a dose of 15 g/kg bw the mice turned quiet. At the application of dose of 15 g and 5 g the mice showed a decrease in swinging responses and indicated reestablishment at the 0.5th through the 2nd hour, but at the 4th hour all mice returned normal. The mice in the state of defecation and urination didn't show different effects from those in the control group.

There was not found any straub, ptosis, phyloerection, lacrimation, catalepsy, salivation, vocalization, tremor, convulsion, and muscle-stretching. All mice could swing and reestablish their muscle-related activities. They showed pineal reflex and cornea state that indicated a normal nerve system. All mice experienced grooming, this equal to those in the control group. The same was also true of their defecation and urination.

Until the dose of 15 g/kg bw there was encountered no death to the mice. This test has been approved by the animal ethics committee of InstitutTeknologi Bandung (ITB)

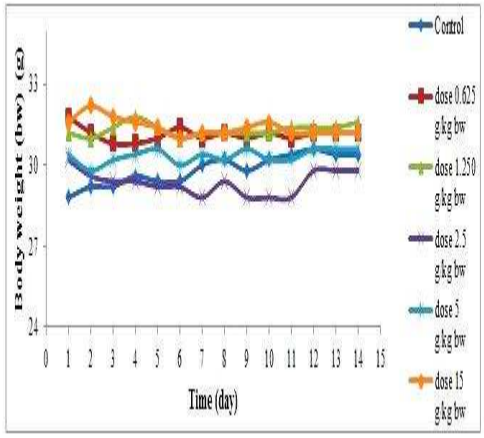

Fig. 9: The mice's body weight for 2 weeks by the compound 1 treatment

The profile of increase in body weight was equal in all groups, this suggesting that the mice were healthy (Fig. 9).

Safety test on compound 1against the normal cell

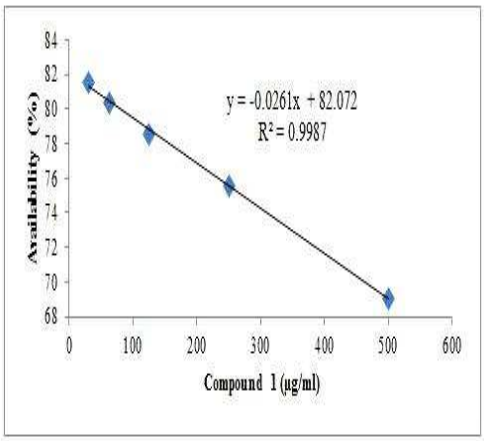

Fig. 10: Cytotoxic test of compound 1(µg/ml)against normal cell

ina 24-hours incubation

The result of test on compound 1 against the normal cell showed that IC_{50}compound 1 was 1228 (µg/ml) (Fig 10).

CONCLUSION

The product of secondary metabolite of *Streptomyces galbus*TP2 strain was phenylacetic acid with the LD_{50}> 15 g/kg bw and IC_{50}= 1228 µg/ml, this indicating that it was safe to be a raw material medicine.

ACKNOWLEDGEMENT

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REFERENCES

1. DesakGede Sri Andayani, UkanSukandar, ElinYulinahSukandar, I KetutAdnyana.Antibacterial, antifungal and anticancer activity of five strains of soil microorganism isolated from Tangkubanperahu mountain by fermentation process, submitted (under review) in Journal of Mathematical and Fundamental Sciences2014.
2. Sukandar EY, Taguchi H. Studies on antibiotics produced by *Streptomyces sp.*isolated from Indonesian soil.Int. CME 1980: 3.
3. Hwang BK, Lim SW, Kim BS, Lee JY, Moon SS.isolation and in vivo and in vitro antifungal activity of phenylacetic acid and sodium phenylacetate from *Streptomyces humidus*.Applied and Environmental Microbiology 2001; 67: 3739-3745.
4. Kim Y, Cho JY, Kuk JH, Moon JH, Cho JI, Kim YC, Park KH. Identification and antimicrobial activity of phenylacetic acid produced by *Bacillus licheniformis* isolated from fermented soybean, chungkook-Jang.Current Microbiology 2004; 48: 312-317.
5. Lowe SW, Lin AW. Apoptosis in cancer.Carcinogenesis 2000; 21: 485-495.
6. Johnson DE, Ochieng J, Evans SL. Phenylacetic acid halides inhibit estrogen receptor (ER)-positive MCF-7 cells, but not ER-negative human breast cells or normal breast epithelial cells.Anti-cancer Drugs1996; 7: 288-292.
7. Pineau T, Hudgins WR, Liu L, Chen LC, Sher T, Gonzales FJ, Samid D. Activation of human peroxisome proliferator-activated receptor by the antitumor agent phenylacetate and its analogs.Biochemical Pharmacology 1996; 52:659-667.
8. Sampath M, Vasanthi M. Isolation, structural elucidation of flavonoids from *Polyalthialongifolia*(Sonn.) Thawaites and evaluation of antibacterial, antioxidant and anticancer potential.Int J of Pharmacy and Pharmaceutical Sciences 2013; 5: 336-341.
9. Nehete J, Bhatia M. Correlation of antioxidant activity with phenolic content and isolation of antioxidant compound from *Lygodiumflexuosum* (L.) sw. extracts.Int J of Pharmacy and Pharmaceutical Sciences 2011; 3: 48-52.
10. Giddings LA, Newman DJ.Microbial natural products: Molecular blueprints for antitumor drugs.J. Ind. Microbiol. Biotechnol 2013, 40: 1181-1210.
11. Berdy J.Bioactive microbial metabolites: a personal view.J. Antibiotic2005;58: 1-26.
12. Satyajit DS, Zahid L, Alexander IG.Natural products isolation.2nded. New Jersey: Humana Press; 2006.
13. Shriner RL, Hermann CKF, Morrill TC, Curtain DY, Fuson DY. The Systematic identification of organic compounds. 8th ed. Danvers: John Wiley & Sons Inc; 2004.
14. Omori Y. OECD guidelines for testing of chemicals. Kagaku Kogyo Nipposha, Tokyo; 1981. p.492-497.
15. Sukandar EY, Andrajati R, Sigit JI, Adnyana IK, Setiadi AP, Kusnandar. ISO pharmacotherapy. 2nd ed.Jakarta: PT. IAI Press; 2011.
16. Mosmann F,Rapid calorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assay.J. Immunology Methods1983; 65: 55-63.