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Abstract. Soil *Streptomyces* are filamentous Gram-positive bacteria which were the biggest producer of remarkable bioactive compounds with multiple biological roles. This study aimed to assess the antioxidant and cytotoxic activities of crude extract derived from 3 soil *Streptomyces* strains, namely APM-7, APM-11, and APM-21, which was isolated from Muna Islands, Southeast Sulawesi as well as profiling its compounds using gas chromatography and mass spectrometry (GC-MS). The results indicated that the ethyl acetate extract of APM-7 strain showed the most antioxidant potential with an IC_{50} value for both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) of 31.61 and 57.91 μg/mL, respectively. Interestingly, this corresponding extract exhibited the highest total phenolic content (TPC) and total flavonoid content (TFC) values of 41.74 mg GAE/g extract and 32.51 mg QE/g extract. The cytotoxic effect of APM-7 extract (100 μg/mL) against human breast carcinoma cells (MCF-7) was found as having the best with the inhibition value of 81.31%. GC-MS analysis of APM-7 extract revealed 12 peaks which included some dominant compounds, including isophorone and Bis (2-ethylhexyl) phthalate which might be responsible for the antioxidant and cytotoxic properties. Our results indicate that the *Streptomyces* sp. strain APM-7 could be developed as medically useful compounds.
Keywords: antioxidant, APM-7 strain, cytotoxic, GC-MS, *Streptomyces* spp.

1. Introduction

In the present era, high attention has been paid to investigate natural products potential for pharmacological purposes. Therefore, it is of interest to highlight for discovery of new active compounds, including as antioxidant and anticancer agents, which showed different structure, sources, and modes of action along with minimal side effects [1]. Notably, cancer is one of the most life-threatening illnesses affecting human life. Several cancer types are contributed in the high mortality number of around a million patients each year [2]. One of several types of cancer that is deadly is breast cancer (5th primary cause of cancer mortality worldwide), which is the most frequently occurring in women, with an estimated cases of 2.3 million in 2020 [3]. It has also been reported that there is a complex and strong correlation between oxidative stress and the inflammatory response of cancer cells. In line with that, antioxidant compounds could scavenge the free radicals that induce oxidative stress in cells. Therefore, antioxidant agents could provide one of the mechanisms of its anticancer system as reducing oxidative stress response [4].

Actinomycetes are noteworthy as bioactive compound producers with numerous bioactivities. Approximately 23,000 microbial active compounds have been identified, and more than 10,000 compounds are produced by actinomycetes strain. Interestingly, 76% of those active compounds are exerted by *Streptomyces* spp. species which are noted as a primary source of promising compounds [5]. Moreover, *Streptomyces* spp. and other related actinomycetes remain to be useful sources of new active compound with a wide range of pharmaceutical activities which is ultimately used as an antioxidant, anticancer, or other medically valuable constituents [6]. In relation with antioxidant and cytotoxic bioactivities, some previous strains including *S. pluripotens* MUSC 137, *S. carpaticus* MK-01, and *Streptomyces* sp. act-2h have been reported as having those potential activities [7-9]. Of note, those *Streptomyces* sp. strains were isolated from outside Indonesia region.

In this study, three strains of *Streptomyces* spp. from Muna Island, Southeast Sulawesi, Indonesia were used. This region is characterized with natural views of karst hills, dry soil, and calcareous rock [10]. Such extreme, and harsh environmental characteristics allow microbial colonization with the ability to synthesize a unique secondary metabolite [11]. The studies on soil *Streptomyces* spp. with respect to antioxidant and cytotoxic activity against carcinoma breast cancer cells (MCF-7) are very limited in Indonesia and most of *Streptomyces* spp. isolates were yet to be investigated for bioactivity of their secondary metabolites. Therefore, recent study was carried out to extract the bioactive compound from soil *Streptomyces* spp. and to demonstrate its antioxidant, and cytotoxic activities against MCF-7 cells along with chemical profiling of the most potential extract from *Streptomyces* spp. isolates.

2. Materials and methods

2.1. Actinomycetes isolates

In this study, three actinomycetes isolates namely APM-7, APM-11, and APM-21 were used which had been isolated from the soil of the Muna Islands, Southeast Sulawesi derived from our previous research (Collection of Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University). Of note, these three isolates have been identified by molecular approach as belonging to the genus of *Streptomyces* spp. which the sequence of these isolates have been deposited into GenBank (https://ncbi.nlm.nih.gov) under accession number of OR066165, OR066165, and OR066165. Those three actinomycetes isolates were routinely cultured on International *Streptomyces* Project-2 (ISP-2) medium (composition: 10 g/L malt extract, 4 g/L yeast extract, and 4 g/L glucose) prior to be used in this study. In addition, macroscopic and microscopic cells observations of three actinomycetes isolates were also conducted using a light microscope.

2.2. Fermentation and extraction of bioactive compounds

Fermentation was carried out on ISP-2 liquid medium with an incubation period of 10 days with 120 rpm shaking at 28-30 °C. subsequently, an amount of 1 L of culture was added with 1:1 (v/v) ethyl

acetate and shaken at 150 rpm for 2 h. Soon thereafter, the solvent layer was separated and evaporated at 45 °C. The crude extract obtained was stored for further analysis [12].

2.3. Assessment of antioxidant activity

The antioxidant activity was determined by DPPH and ABTS scavenging assay [13]. As for DPPH radicals, several concentrations (2500 – 19.5 μg/mL) of extract was made in serially dilution of 96 well plates. Nearly 100 μL DPPH (125 μM) in ethanol was added to each well containing various concentrations of extracts (total volume of 200 μL). The samples were incubated under dark condition at 37 °C for 20 min and the absorbance was read at 515 nm using an ELISA microplate reader (Thermo Scientific Varioskan Flash-Thermo Fischer). On other hand, potassium persulfate (2.45 mM) solution was reacted with ABTS solution (7 mM) and kept for overnight in the dark to yield ABTS radical cations. Further, the ABTS radical was diluted with ethanol until an absorbance of about 0.70±0.02 at 745 nm. Free radical scavenging property was conducted by adding 50 μL of extract (various concentration of 2500 – 19.5 μg/mL) to 150 μL of ABTS radicals followed by incubation for 30 min at the dark prior to be observed at 734 nm. The percentage inhibition of radical scavenging activity was then measured using the formula: 1–[(A–C)/(B–C)] ×100, whereas A is absorbance of radicals in the presence of samples, B is absorbance of DPPH with ethanol and C is absorbance of ethanol. Ascorbic acid was used as positive controls. The data are calculated using regression linear approach from inhibition values and presented as the inhibitory concentration of 50% (IC_{50}).

2.4. Measurements of total phenolic contents (TPC) and total flavonoid contents (TFC)

Total phenolic and flavonoid contents were conducted by the spectrophotometric analysis using an ELISA microplate reader (Thermo Scientific Varioskan Flash-Thermo Fischer) following a method by Prastya *et al*. [12]. In short, a 500 μL of sample (1 mg/mL) was mixed with 250 μL of Folin-Ciocalteu's phenol reagent and 3.5 mL deionized distilled water. After 8 min incubation at 29 °C, 750 μL of a 20% Na_2CO_3 solution was added to the mixture and mixed thoroughly. The mixture was kept in the dark for 120 min at 29°C, after which the absorbance was detected at 765 nm. The TPC was measured from extrapolation of calibration curve from gallic acid standard. The TPC was expressed as milligrams of gallic acid equivalents per g extract (GAE/g extract). As for total flavonoid content was performed in a 10 mL test tube which contained 500 μL of extract (1 mg/mL), 2.45 mL of deionized distilled water, and 150 μL of $NaNO_2$ (5%) following incubation for 2 min at 29 °C. Soon thereafter 150 μL of $AlCl_3$ (10%) were mixed gently. After 8 min incubation at 29 °C, 2 mL of NaOH (1 M) was added. The solution was mixed well, and the absorbance was read at 510 nm. The standard curve was made using quercetin standard solution and the total flavonoids were expressed as milligrams of quercetin equivalents per g extract (QE/g extract).

2.5. Cytotoxic assay on human breast carcinoma cells (MCF-7)

For evaluation of cytotoxic activity, human breast carcinoma cells, MCF-7 (ATCC HTB-22; Rockville, MD, USA) were used for testing following previous method [14]. Cells were grown in Dulbecco's-modified Eagle's medium (DMEM) in humidified incubator (5% CO_2 in air at 37 ℃) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin-streptomycin; Sigma). Further, cells were seeded into a sterile flat bottom 96-well plate at a density of 1×10^4 cells/well and allowed to adhere by overnight incubation. Subsequently, 100 μL extract (100 μg/mL) was added to the culture medium containing MCF-7 cells and further incubated for 24 h. Then, 10 μL of 3- (4, 5-dimethylthiazol-2-2,5 diphenyl tetrazolium bromide) (MTT; Roche) (0.5 mg/mL) was added to each well and the plates were incubated at 37 ℃ for 3 h in a humid atmosphere with 5% CO_2. An amount of 100 μL of Dimethyl sulfoxide (DMSO) was then added to dissolve the formazan crystals and the absorbance was measured at 570 nm using ELISA microplate reader, a Varioskan Flash multimode reader (Thermo-Fisher Scientific, USA). Doxorubicin (100 μg/mL) was included as positive control. The data was reflected as percentage inhibition at concentration of 100 μg/mL from sample and positive controls.

2.6. Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis of extracts was carried out with a column of HP-5MS 5% Phenyl Methyl Silox 0 °C-325 °C (325 °C): 30 m x 250 µm x 0.25 µm on an Agilent 19091S-433: 93.92873 GC-MS System fitted. Ultra-high purity helium was utilized as carrier gas at a constant flow rate of 1 mL/min. The ionizing energy was 70 eV. The oven temperature was set from 40 °C (hold for 2 min) to 300 °C. The extracts were mixed with the solvent (1/100, v/v) and filtered. Pressure was set to 7.0699 psi with an average velocity of 36.262 cm/sec and hold time of 1.3789. The characterization and identification of chemical compounds was according to the retention time. The mass spectra were matched with computer data standards available in mass libraries. The interpretation on Mass-Spectrum GC-MC was analyzed using the database of National Institute Standard and Technology (NIST).

2.7. Statistical analysis

Experimental data are showed as mean ± standard deviation from three replications. For TPC, TFC, antioxidant, and cytotoxic test, one way ANOVA test followed by Multiple Duncan's Range Test was used to analyze the differences in the same experiment. A probability of $p < 0.05$ was considered as significantly different.

3. Results

3.1. Macroscopic and microscopic characters of Streptomyces spp.

Three actinomycetes isolates, APM-7, APM-11, and APM-21 were observed based on colony and cell morphology of the 10-day-old culture grown on ISP-2 solid medium followed by Gram staining (figure 1). These three actinomycetes isolates had different colony morphology, both in terms of diameter, shape, color, elevation, and colony boundaries (table 1). In addition, microscopic observations showed that all three isolates had a filamentous cell shape and were stained purple which indicated Gram positive bacteria.

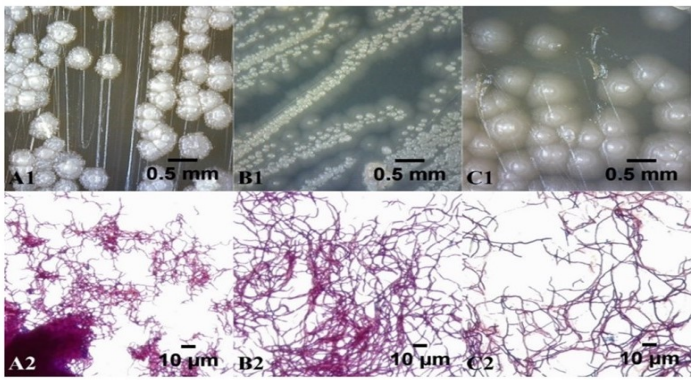

Figure 1. Colony morphology of *Streptomyces* spp. APM-7 (A1), APM-11 (B1), and APM-21 (C1) grown on ISP-2 medium after 10 days incubation. Cell morphology of isolates APM-7 (A2), APM-11 (B2), and APM-21 (C2), observed under a light microscope with a magnification of 400x

Table 1. Morphological characters of three *Streptomyces* spp. isolates

Morphological characters	Isolates		
	APM-7	APM-11	APM-21
Colony diameter	± 0.5 mm	± 0.4 mm	± 0.7 mm
Shape	Circular	Irregular	Circular
Colony colour	White	Yellow orange	Light brown
Elevation	Umbonate	Umbonate	Umbonate
Border	Lobate	Filamentous	Entire

3.2. APM-7 extract exhibited the best antioxidant activity, TPC, and TFC

The results of antioxidant assay from the *Streptomyces* spp. extracts are summarized in table 2. The results revealed that APM-7 extract showed the lowest IC_{50} value of 31.61±2.81, and 57.91±5.31 for DPPH and ABTS radicals, respectively. Of note, the lower IC_{50} value indicated stronger activity, therefore APM-7 extract possesses the most active extract. Even though compared with positive control, those activities are still lower. Furthermore, in line with antioxidant activity, we identified that APM-7 extract also showed the highest value for TPC and TFC of 41.74±4.71 mg GAE/g extract and 32.51±4.38 mg QE/g extract, respectively (table 3).

Table 2. Antioxidant activity of three *Streptomyces* spp. extracts

Extracts	IC_{50} (μg/mL)	
	DPPH	ABTS
APM-7	31.61±2.81[b]	57.91±5.31[b]
APM-11	683.29±17.12[d]	2529.84±31.96[d]
APM-21	114.73±8.37[c]	523.62±29.31[c]
Ascorbic acid	4.21±1.36[a]	6.27±1.22[a]

Different superscript letters in the same columns indicate the data were significantly different

Table 3. Total phenolic and flavonoid contents of three *Streptomyces* spp. extracts

Extracts	Total phenolic (mg. GAE/g extract)	Total flavonoid (mg. QE/g extract)
APM-7	41.74±4.71[c]	32.51±4.38[b]
APM-11	8.29±2.78[a]	4.41±1.47[a]
APM-21	12.47±1.81[b]	5.31±2.41[1]

Different superscript letters in the same column indicate the data were significantly different

3.3. APM-7 extract showed the highest inhibition value on MCF-7 cells line

The APM-7 extract (at concentration of 100 μg/mL) showed the strongest cytotoxic property against human breast carcinoma (MCF-7) cell lines with the inhibition value of 81.31±6.47% (table 4). This extract treatment seems to damage cancer cells so that the cells appear to shrink and burst as shown in figure 2. However, this corresponding extract treatment still has lower activity than positive control, doxorubicin.

Table 4. Cytotoxic activity of three *Streptomyces* spp. extracts

Sample	Cell growth inhibition against MCF-7 cells (%)
APM-7	81.31±2.47[d]
APM-11	57.21±9.12[c]
APM-21	38.21±4.19[b]
Doxorubicin	90.38±1.21[e]
DMSO	0±0[a]

Different superscript letters in the same column indicate the data were significantly different

3.4. GC-MS analysis of APM-7 extract

The presence of volatile compounds of the most potential extract, APM-7 was analyzed by the GC-MS analysis. The chromatogram patterns exhibited 12 peaks, indicating the presence of different types of

chemical constituents (figure 3). Those detected compounds included two dominant compounds namely isophorone and Bis (2-ethylhexyl) phthalate which has several important bioactivities (table 5).

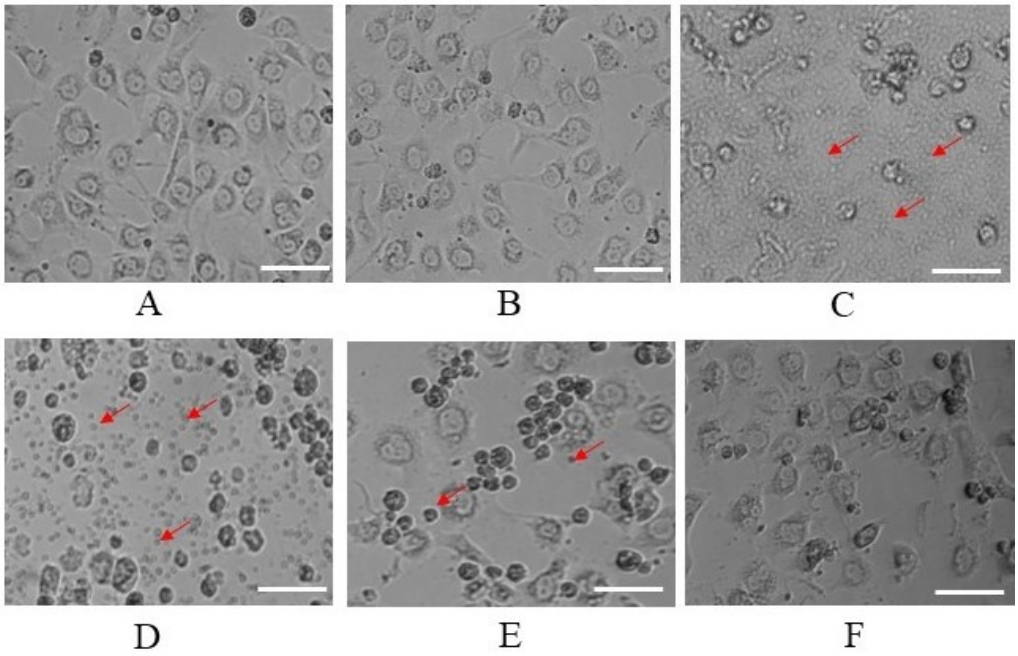

Figure 2. MCF-7 cell lines on (A) DMEM, treatment with (B) 1% DMSO, (C) Doxorubicin, (D) APM-7 extract, (E) APM-11 extract, (F) APM-21 extract. Each extract and doxorubicin were applied at concentration of 100 µg/ml. 1%DMSO used for negative control. Bars represent 30 µm. Red arrows show apoptotic cells

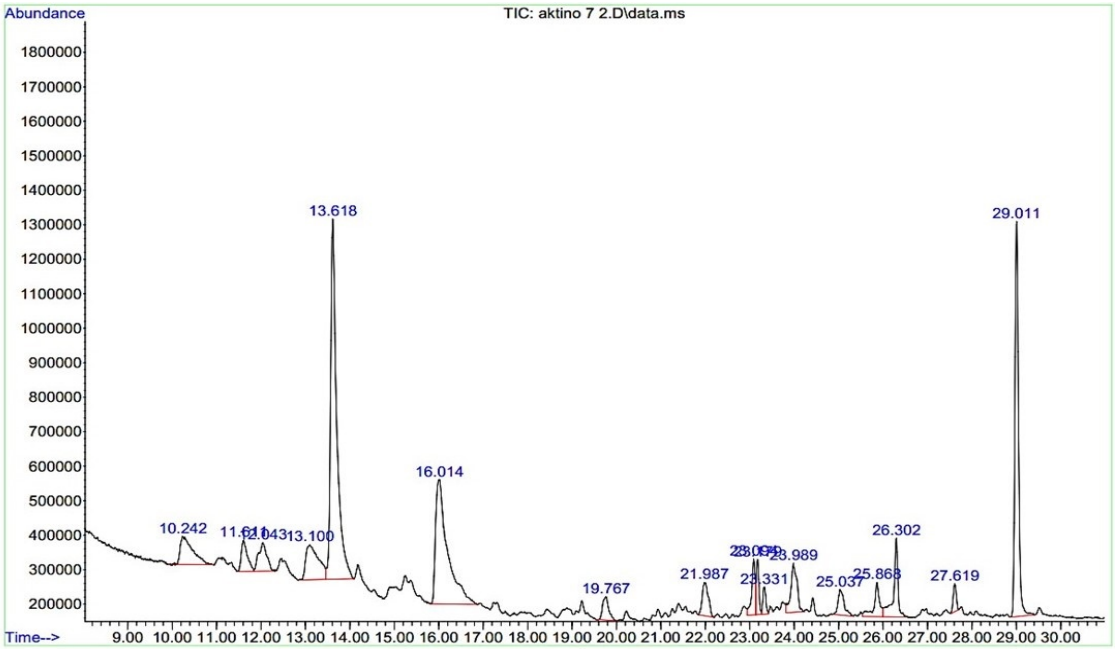

Figure 3. Chromatogram profile of APM-7 extract analysed by GC-MS instrument

Table 5. Chemical constituents detected from APM-7 extract

Retention time (Minutes)	Compounds detected	Abundance (%)	Similarity (%)	Other sources of compounds	Bioactivities	References
13.627	Isophorone	27.12	81	Endophytic fungal *Chaetomium globosum; Streptomyces* sp. VITGV	Cytotoxic, antibacterial	[15, 16]
16.018	Mevalonic acid lactone	18.32	55	-	Unknown	-
19.761	Hexadecane	1.73	60	Fungal *Photorhabdus* sp.;	Antimicrobial	[17]
21.992	1-Octadecene	2.46	96	Fungal *Tirmanea nivea*	Antimicrobial	[18]
23.089	Pentadecanoic acid	2.31	97	-	Unkown	-
23.328	Hexadecanoic acid	0.97	97	Endophytic fungal *Penicillium janthinellum*	Antioxidant, antiandrogenic, nematicide	[19]
23.983	3-Eicosene	3.67	93	Endophytic fungal *Chaetomium globosum*	Antimutagenic, antioxidant, antiproliferative	[20]
25.042	Heptadecanoic acid	1.89	95	Yeast *Pseudozyma flocculosa*	Antifungal	[21]
25.874	1-Docosene	2.33	96	Endophytic fungal *Talaromyces purpureogenus*	Antioxidant and antiproliferative	[22]
26.302	Tributyl acetyl citrate	4.26	90	-	Unknown	-
27.613	Nonacos-1-ene	1.03	50	*Cissampelos mucronata*	Antibacterial and antihelmintic	[23]
29.012	Bis (2-ethylhexyl) phthalate	17.23	98	*Streptomyces* sp. TN17; *S. mirabilis* NSQu-25; *Streptomyces* sp. G60	Antibacterial, antifungal; Antibacterial, cytotoxic; antibacterial	[24-26]

4. Discussion

Three actinomycetes isolates, APM-7, APM-11, and APM-21 were characterized based on colony, cell morphology and identified through molecular approach as belonging to *Streptomyces* spp. These three isolates have primary characteristics including non-mucous and filamentous colonies along with Gram positive staining (figure 1). These morphological and cells characters were consistent with its assignment to the genus of *Streptomyces* spp. [27]. Of note, *Streptomyces* spp. are well known as a rich source for pharmacologically useful active compounds due to their ability to synthesis diverse metabolites with numerous properties including antimicrobial, antioxidant, cytotoxic, etc. [7].

Therefore, further investigation was performed to analyze the potential active compound produced by these three *Streptomyces* spp. acting for antioxidant and cytotoxic properties.

Reactive Oxygen Species (ROS), including hydroxyl radicals and superoxide radicals, are continuously being synthesized in cellular level during metabolic reactions and could come from toxic environments. The uncontrolled accumulation of ROS is known to modulate several diseases such as cancer, alzheimer's, inflammation, and others. Therefore, such ROS needs to be scavenged with active compound to enhance the host defense system by antioxidant mechanism [28]. Previous reports have shown that *Streptomyces* spp. genus can exert secondary metabolites including flavonoid, phenols, steroids, and others which correspond to the antioxidant potential [29]. Interestingly, the most potential *Streptomyces* spp. isolate namely APM-7 strain could produce active compound with high antioxidants along with TPC and TFC (table 2-3). In agreement with our findings, it has been previously known that the higher content of phenolic compounds of *Streptomyces* spp. extract could be linked to their enhanced pharmacological properties, including antioxidant, cytotoxic and others [9]. Some *Streptomyces* spp. species such as *S. lavendulae* isolated from India soil and *Streptomyces* sp. strain E23-4 from Morocco soil have been shown to exhibit high levels of phenolic compound along with antioxidant activities [30, 31]. Moreover, our findings also exhibited stronger antioxidant activity than some previous studies which analyze the antioxidant activity of *Streptomyces* spp. strain isolated from Indonesia region. Our results showed stronger activity as compared to *S. avermitillis* A18TE-8 isolated from North Sumatra soil with an IC_{50} DPPH value of 122.96 μg/mL [32]. In addition, another *Streptomyces* sp. strain SAE4034 has slightly lower activity with an IC_{50} DPPH value of 32.14 μg/mL [33]. These results indicated that our potential *Streptomyces* sp. APM-7 could produce promising active compound to have strong antioxidants activity. Therefore, investigation for its potential properties to inhibit the development of breast carcinoma cell lines (MCF-7).

Cancer cells initiation and progression process is known to associate with accumulation of ROS which further promotes oxidative stress [34]. ROS could cause various modifications to biomolecules such as DNA, lipids, and protein [35]. Notably, antioxidants constituents are reported to scavenge ROS, and activate the cell signaling pathways which are important for survival against cancer cells. Therefore, the antioxidant has a close relation to anticancer mechanism by reducing ROS which is one of the causes of cancer cells [36]. Interestingly, our results indicated that APM-7 extract showed the strongest cytotoxic activity against MCF-7 (table 4). Of note, this corresponding extract also exhibited strong antioxidant properties, hence indicating that antioxidants might play a vital role in the cytotoxic by scavenging ROS molecules. In agreement with our findings, it was well known that the higher activity of cytotoxicity of *Streptomyces* spp. extract likely related to their antioxidant properties. Similar *Streptomyces* spp. such as *S. pluripotens* MUSC 137, *S. carpaticus* MK-01, and *Streptomyces* sp. act-2 have been shown to produce active compounds with strong antioxidant, and cytotoxic activities along with remarkable phenolic content [7-9]. These findings have provided new insight into the cytotoxic activity against MCF-7 cancer cells derived from *Streptomyces* sp. APM-7 which isolated from Indonesia region. Further investigation of the target action of the corresponding active compounds in inhibiting cancer cells growth should be investigated. Ultimately, our study of both antioxidant, and cytotoxic assessment indicates the presence of potential active compounds derived from APM-7 extract, which then promote the subsequent analysis using GC-MS instrument to investigate the chemical profile of the potential extract.

GC-MS is a valuable instrument and plays a vital use in natural product discovery, including active compounds isolated from actinomycetes group [37]. Our results detected twelve compounds in the extract of APM-7 with most dominant of compounds namely isophorone, and Bis (2-ethylhexyl) phthalate. As for other detected compounds were summarized in table 5 with their identity and bioactivities. Our highlighted were suspected to two dominant compounds including isophorone, and bis (2-ethylhexyl) phthalate which has the highest proportion in the APM-7 extract. Of note, isophorone (3,5,5-trimethyl–2-cyclohexen–1-one), a monoterpene, and the structurally related 1,8-cineole and camphor, have exhibited some potential bioactivities including a protective effect against cancer cells, antimicrobials, and antioxidant [38]. Previous studies reported this potential compound produced by

some microorganism such as fungal *Chaetomium globosum*, and *Streptomyces* sp. VITGV with cytotoxic and antibacterial properties derived from its crude extracts [15, 16]. On the other hand, bis (2-ethylhexyl) phthalate is an active compound belonging to the family of phthalates which main characters contain aromatic benzene [39]. Interestingly, this corresponding compound was already produced by some *Streptomyces* spp. with their promising bioactivities including *Streptomyces* sp. TN17 (antibacterial and antifungal), *S. mirabilis* NSQu-25 (antibacterial, cytotoxic), and *Streptomyces* sp. G60 (antibacterial) [24-26]. Based on previous reports, it is suspected that the highest antioxidant and cytotoxic activities of APM-7 extract might be associated with the presence of these two potential compounds. The present findings support the view that the active compound profile of *Streptomyces* sp. APM-7 is dominated by both isophorone and bis (2-ethylhexyl) phthalate compounds as it includes a main activity for cytotoxic, and antioxidant agents.

5. Conclusion

Based on the results, it could be concluded that *Streptomyces* sp. APM7 isolate is considered as valuable sources of active compounds. This corresponding isolate could produce active constituents including phenolic, and flavonoid compounds, which may be correlated to their antioxidant, and cytotoxic activities. Based on GC-MS analysis, such active isolates proved to have both active dominant compounds namely isophorone, and bis (2-ethylhexyl) phthalate which suggested as the active constituents correspond to its valuable activity. However, this is still an initial step and therefore required further investigation on extraction, purification and production of active compound represent a promising approach to develop an alternative therapeutic application towards cancer and antioxidant agents.

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