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Caryophyllene-Type Sesquiterpenoids from the Stembark of *Aglalia harmsiana* and Their Cytotoxic Activity Against MCF-7 Breast Cancer Cells

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ABSTRACT. Sesquiterpenoid is a class of terpenoid compounds that have the most abundant diversity of structures and biological activities that can be found in natural resources. Tropical plants are main source of sesquiterpenoid compounds such as *Aglaia* genus belong to Meliaceae family. A. *harmsiana* is a species from *Aglaia* that only has few previous researchs. Therefore, the purpose of this study was to isolate and determine the structure of sesquiterpenoid compounds from stem bark A. *harmsiana* along with their cytotoxic activity against MCF-7 breast cancer cells. The isolation process begins by extracting powder from A. *harmsiana* stembark using *n*-hexane, ethyl acetate and methanol. All extracts were evaluated for their cytotoxic activity against MCF-7 breast cancer cells, and *n*-hexane extracts showed significant cytotoxic activity with IC_{50} values of 117.86 µg/mL. Therefore, *n*-hexane extracts were further separated and purified by various chromatographic techniques to obtain compounds **1** and **2**. Compounds **1** and **2** were elucidated their chemical structures by spectroscopic methods including IR, NMR, and MS as well as by comparison of data with literatures and identified as caryophyllene-type sesquiterpenoids, *β*-caryophyllene oxide (**1**) and senecrassidiol (**2**). Compounds **1** and **2** were submitted for cytotoxic eveluation on MCF-7 breast cancer cells and as a result, *β*-caryophyllene oxide (**1**) showed the stronger activity compared to senecrassidiol (**2**). These finding indicated that the cytotoxic activity of caryophyllene-type sesquiterpenoid are influenced by the presence of double bonds and configuration of methyl groups.

Key words: *Aglaia harmsiana*, *β*-caryophyllene oxide, senecrassidiol, sesquiterpenoid, cytotoxic activity

INTRODUCTION

The genus *Aglaia* is the largest genus of the family Meliaceae (Farabi et al., 2017). *Aglaia* comprises more than 100 species distributed mainly in India, Indonesia, Malaysia, and part of the Western Pacific region (Hidayat et al., 2018). In Indonesia, the flowers of A. *odorata* tradionally used as an insects repellent (Joycharat et al., 2010). Some of *Aglaia* species have been phytochemically observed previously. Its chemical constituent include diterpenoids (Yodsaue et al 2012), rocaglamides (Pan, Woodard, Lucas, Fuch, & Kinghorn, 2014), lignans (Sianturi et al., 2016), triterpenoids (Zhang, Wang, Gu, & Kong, 2010; Harneti et al., 2012; Awang et al., 2012), and sesquiterpenoids (Liu et al., 2014). In total, less than twenty sesquiterpenoids were isolated previously from genus *Aglaia* species because this compound did not attract considerable attention.

Sesquiterpenoid can be classified based on the number of cyclics formed due to the modification of the cyclization reaction and rearrangement of the carbon skeleton. Based on the cyclic number, sesquiterpenoids are divided into four groups, namely sesquiterpenoid acyclic, monocyclic, bicyclic and tricyclic (Ludwiczuk, Skalcka-Wozniak &

Georgiev, 2017). Each sesquiterpenoid cyclic structure can consist of cyclopropane, cyclobutane, cyclopentane, cyclohexane to cycloheptane (Chappell and Coates, 2010). Therefore, more than 10000 types of sesquiterpenoids have been successfully isolated to date (Celik, Togar, Turkez & Taspinar, 2014). The more abundant variety of cyclic sesquiterpenoid skeletons compared to other terpenoids is due to the presence of farnesil pyrophosphate (FPP) as precursors that have three double bonds with more flexible carbon chains (Chappell and Coates, 2010).

Besides its structural diversity, sesquiterpenoids are known for their abundant application and biological activity. Sesquiterpenoid has a stronger aroma than other terpenoids (Buckle, 2015). Sesquiterpenoids are widely used as raw materials in fragrances such as patchoulol compounds. Sesquiterpenoid has also been shown to be pharmacological activities such as antimalarial, cytotoxic, antifungal, antibacterial, antiviral, anti-inflammatory, anti-inflammatory, and antinociceptive. Even some sesquiterpenoids have been used as medicine, one of which is artemisinin. Artemisinin is a sesquiterpenoid lactone isolated from *Artemisia annu*a and has been used universally as a malaria drug (Awouafack, Tane, Kuete & Eloff., 2013).

Aglaia species which have been shown to contain sesquiterpenoid compounds are *A. minahassae* (Kurniasih et al., 2018), *A. foveolata* (Roux et al., 1998), *A. odorata* (Liu and Xu, 2016), *A. silvestris* (Poitinger et al., 2008), *A. grandis* (Inada et al., 2000), *A. perviridis* (Pan et al., 2013) and *A. leucophylla* (Benosman, Richomme, Sevenet, Hadi & Bruneton, 1994) with six sesquiterpenoid groups, namely sesquiterpenoid eudesmane, guaiane, aromadendrane, cadinene, isodaucane, and caryophyllene. Caryophyllene-type sesquiterpenoids, particularly caryophyllene oxide, has showed cytotoxic activity against various cancer cell lines, such as HepG2, AGS, HeLa, SNU-1, and SNU-16 (Jun et al., 2011)

Although sesquiterpenoids of other *Aglaia* species have been investigated previously, the sesquiterpenoid compounds of the stem bark of *A. harmsiana* is yet to be reported. The chemical constituents that have been reported previously from the leaves of *A. harmsiana* are cycloartane triterpenoids (Inada et al., 1997) and rocaglaol (Nugroho et al., 1997). In this paper, the isolation and structure identification of caryophyllene-type sesquiterpenoid along with their cytotoxic activity are described.

EXPERIMENTAL SECTION

General Experiment Procedure

The IR spectra were recorded on a SHIMADZU IR Prestige-21 in KBr. The mass spectra were obtained with a Waters Xevo QTOF MS. NMR spectral data were recorded on JEOL ECZ-600 spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts are given on a δ (ppm) scale with tetramethyl silane (TMS) as an internal standard. Column chromatography was conducted on silica gel 60 (70–230 and 230–400 mesh, Merck). TLC analysis was carried out on silica gel 60 GF_{254} (Merck, 0.25 mm) using various solvent systems, and spots were detected by irradiating under ultraviolet-visible light (257 and 364 nm) and heating the silica gel plates sprayed with 10% vanillin sulfat in ethanol.

Plant Material

The stembark of *A. harmsiana* were collected in Bogor Botanical Garden, Bogor, West Java Province, Indonesia in January 2016. The plant was identified by Mr. Ismail, the staff of Bogoriense Herbarium, Research Center for Biology, Indonesian Institute of Science, Bogor, Indonesia and a voucher specimen has been deposited at the herbarium.

Extraction and Isolation

The dried ground stembark (1.70 kg) of *A. harmsiana* was successively extracted with *n*-hexane (10 L), ethyl acetate (10 L) and methanol (14 L) at room temperature. Evaporation of the extracts in reduced pressure resulted the crude extracts of *n*-hexane (43.97 g), EtOAc (43.25 g), and methanol (170.3 g), respectively. All of the extract were evaluated for their cytotoxic activity against MCF-7 breast cancer lines and the *n*-hexane and ethyl acetate extracts showed significant cytotoxic activity with IC_{50} values of 117.8 and 235.5 μg/mL, respectively.

The *n*-hexane soluble fraction (40.0 g) was separated by vacuum liquid chromatography (VLC) on silica gel G60 with *n*-hexane-ethyl acetate-methanol (100:0 - 0:100) as a solvent with a gradient of 10% to produced four combined subfractions (A-D). Subfraction B (911.4 mg) was further separated by vacuum liquid chromatography eluted with *n*-hexane - dichloromethane (100:0-0:100) in a gradient of 10%, resulting in nine combined subfractions (B1-B9). Subfraction B6 (664.8 mg) was separated by column

chromatography on silica G60 (70-230 Mesh) eluted with *n*-hexane:dichloromethane:ethyl acetate (9:0.8:0.2) to produced twelve combined subfractions (B6A-B6L). Subfraction B6C (158.8 mg) was separated by column chromatography on silica RP-18 eluted with methanol: acetonitrile:water (6:2:2) to produce two combined subfractions (B6C1 and B6C2). Finally, subtractions B6C1 (32.2 mg) was separated using column chromatography on silica G60 (230-400 Mesh) eluted with *n*-hexan: dichloromethane:ethyl acetate (1:1.4:0.6) to give compound **1** (7.9 mg). While, subtraction B6C2 (58.3 mg) was separated using column chromatography on silica G60 (230-400 Mesh) eluted with *n*-hexane : dichloromethane (7:3) to give compound **2** (26.0 mg).

β-Caryophyllene Oxide (1)

Colourless oil; IR (KBr) ν_{max} 3069, 2930, 1633, 1368, 1384 and 1075 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) and ^{13}C-NMR ($CDCl_3$, 150 MHz) see Table 1; HR-TOFMS *m/z* 221.2001 $[M+H]^+$, (calcd. $C_{15}H_{25}O$ *m/z* 221.1905).

Senecrassidiol (2)

Colourless oil; IR (KBr) ν_{max} 3421, 2949, 2861, 1620, 1462, 1361, 1384, 1359 and 1078 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) and ^{13}C-NMR ($CDCl_3$, 150 MHz) see Table 1; HR-TOFMS *m/z* 220.1825 $[M+H]^+$, (calcd. $C_{15}H_{24}O$ *m/z* 221.1827).

Bioassays for Cytotoxic Activity

The MCF-7 cells were seeded into 96-well plates at an initial cell density of approximately 3 x 10^4 cells cm^{-3}. After 24 h of incubation for cell attachment and growth, varying concentrations of samples were added. The compounds added were first dissolved in DMSO at the required concentration. Subsequent six desirable concentrations were prepared using PBS (phosphoric buffer solution, pH = 7.30 - 7.65). Control wells received only DMSO. The assay was terminated after a 48 h incubation period by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named as thiazol blue] and the incubation was continued for another 4 h, in which the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added and another 24 h incubation was conducted. Optical density was read by using a micro plate reader at 550 nm. IC_{50} values were taken from the plotted graph of percentage live cells compared to control (%), receiving only PBS and DMSO, versus the tested concentration of compounds (*μ*M). The IC_{50} value is the concentration required for 50% growth inhibition. Each assay and analysis was run in triplicate and averaged.

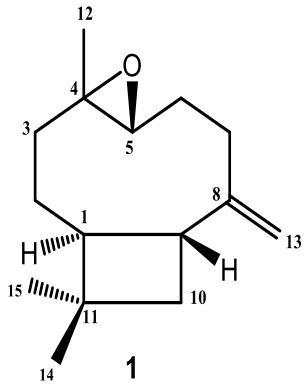
RESULTS AND DISCUSSION

The *n*-hexane extract of the stembark of *A. harmsiana* was chromatographed over a vacuum-liquid chromatographed (VLC) column packed with silica gel 60 by gradient elution. The VLC fractions were repeatedly subjected to silica gel and silica RP-18 column chromatography afforded a compounds **1** and **2** (**Figure 1**).

Compound **1** was obtained as a colorless oil which dissolves in chloroform and gives an orange color after being sprayed with 10% sulfuric acid in ethanol, indicated the presence of terpenoid structure. Compound **1** has the characteristic of not glowing under UV light at wavelengths of 254 nm and 365 nm, indicated there is no conjugated double bond in compound **1**. The molecular formula of compound **1** was identified as $C_{15}H_{24}O$ based on the HR-TOFMS *m/z* $[M+H]^-$ 221.2001, with calculations for $C_{15}H_{25}O$ *m/z* 221.1905, thus obtaining four hydrogen deficiency index. The IR spectra showed absorption peaks due to of an isolated double bond (1633 cm^{-1}), an aliphatic (2930 cm^{-1}), *gem*-dimethyl (1368 and 1384 cm^{-1}) and an ether group (1074 cm^{-1}). Based on the ^{1}H-NMR spectrum of compound **1**, displayed the presence of three tertiary methyl signals at δ_H 0.97, 0.98 and 1.20, respectively. Then, the presence of a typical signal for olefinic protons at δ_H 4.96 and 4.83 with a $J^{1,2}$ 1.8 Hz, which is the pairing constant for the geminal proton of terminal double bond. In addition, the signal for the oxygenated proton at δ_H 2.87 was also observed in the ^{1}H-NMR spectrum. Based on the ^{13}C-NMR spectrum with detailed analysis of Distortionless Enhancement by Polarization Transfer (DEPT) and Heteronuclear Multiple-Quantum Correlation) experiment (HMQC) measurements showed the presence of fifteen carbon signals consisting of three methyl tertiary which resonance at δ_C 17.0 (Me-12), 21.5 (Me-14) and 29.9 (Me-15), five methylene at δ_C 27.0 (C-2), 39.0 (C-3), 30.2 (C-6), 29.7 (C-7)) and 39.7 (C-10), two methines at δ_C 50.9 (C-1) and 48.9 ppm (C-9), one oxygenated methine δ_C 64 (C-5), one quaternary carbon at δ_C 34.0 (C-11), one oxygenated quaternary carbon at δ_C 59.8 (C-4), one sp^2 quaternary carbon at δ_C 151.9 (C-8) and one sp^2 methylene carbon at δ_C 113.0 (C-13). These functionalities accounted for one out of the total four hydrogen deficiency index. Three remaining hydrogen deficiency index was corresponded to the

tricyclic sesquiterpenoid structure and one cyclic comes from an epoxide group (Kurniasih et al., 2018; Roux et al., 1998; Liu et al., 2014).

In order to determine the position of functional group in compound **1**, (^{1}H-^{1}H-Correlated Spectroscopy (^{1}H–^{1}H COSY) and Heteronuclear Multiple-Bond Correlation spectroscopy (HMBC) spectra (**Figure 2**). Correlations in vicinal protons in C_3-C_2-C_1-C_9-C_{10} and C_5-C_6-C_7, supporting the presence of caryophyllene-type sesquiterpenoid skeleton in compound **1**. Two methyl tertiary at δ_H 0.98 (Me-14) and 0.97 (Me-15) were correlated to sp^{3}quaternary carbon at 34.1 (C-11), indicated that *gem*-dimethyl was attached at C-11. Another methyl tertiary at δ_H 1.20 and oxygenated methine at δ_H 2.87



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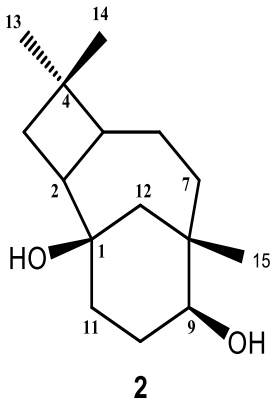

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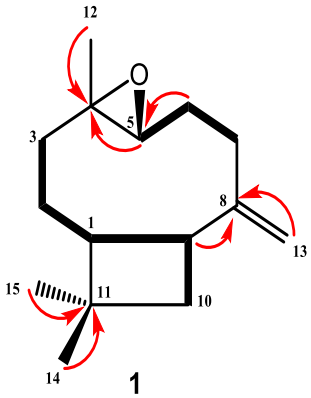
Figure 1. Chemical Structures of Compounds **1** and **2**



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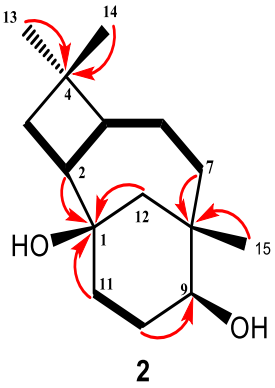

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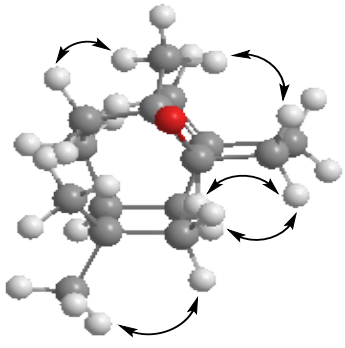
Figure 2. Selected ^{1}H-^{1}H COSY and HMBC Correlations for Compounds **1** and **2**

were correlated to oxygenated carbon at δ_C 59.7, suggested that the remaining methyl was attached at C-4 and an epoxy ring was located at C-4 and C-5. An olefinic protons at δ_H 4.83 and 4.96 were mutually coupled and correlated to sp^2 quaternary carbon at δ_C 151.9, suggested that double bond terminal was located at C-8.

The relative stereochemistry of compound **1** was supported by nuclear Overhauser effect spectroscopy (NOESY) spectrum (**Figure 3**) and based on a biogenetic point of view the occurrence of caryophyllene-type sesquiterpenoid in *Aglaia* genus. Based on the biosynthesis of β-caryophyllene, the position of hydrogen in C-1 has an α-orientation, whereas hydrogen in C-9 has an β-orientation (Dewick, 2009). In the NOESY spectrum of compound **1**, there is no cross peak between H-1 and H-9, H-1 and H-9, indicated those proton located in different sides. In the NOESY spectrum also showed correlation between H_5-H_1 and H_{14}, indicated that the hydrogen in C-5 and methyl at C-14 are α-oriented, consequently, the oxygen in C-5 is β-oriented. A detailed comparison of compound **1** to those of β-caryophyllene oxide was isolated from *Caesalpinia pulcherrima* (Ragasa, Ganzon, Hofilena, Tamboong, & Rideout, 2003), revealed that both compounds were very similar, consequently compound **1** was identified as a β-caryophyllene oxide. That compound was isolated also from *Caesalpinia pulcherrima* belong to Fabaceae family (Ragasa et al., 2003), but was isolated from *Aglaia* species for first time.



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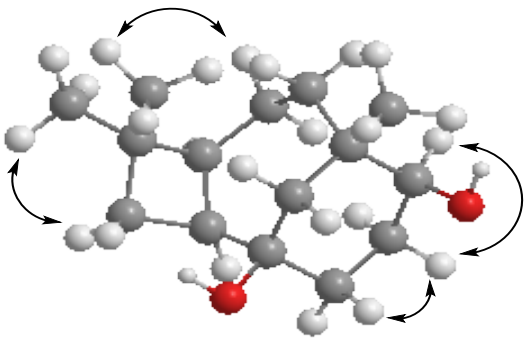

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Figure 3. Selected NOE correlations for **1** and **2**

Table 1. NMR data for Compounds **1** and **2** ($CDCl_3$, 600 MHz for ^1H and 150 for ^{13}C)

Position	**1**		**2**	
Carbon	δ_H [(ΣH, mult., *J* (Hz)]	δ_C (mult.)	δ_H [(ΣH, mult., *J* (Hz)]	δ_C (mult.)
1	1.75 (1H, t, 10.2)	50.7 (d)	-	69.7 (s)
2	1.65 (1H, m)	27.3 (t)	2.13 (1H, t, 11.5)	37.1 (d)
	1.60 (1H, m)			
3	2.05 (1H, m)	39.2 (t)	1.08 (1H, d, 3.1)	33.0 (t)
	2.09 (1H, m)		1.06 (1H, d, 3.8)	
4	-	59.7 (s)	-	34.0 (s)
5	2.87 (1H, dd, 4.2, 9.8)	63.9 (d)	1.80 (1H, m)	42.9 (d)
6	2.08 (1H, m)	29.8 (t)	1.45 (1H, m)	19.4 (t)
	2.12 (1H, m)		1.32 (1H, m)	
7	2.21 (1H, m)	30.3 (t)	1.39 (1H, m)	41.1 (t)
	2.28 (1H, m)		1.34 (1H, m)	
8	-	151.9 (s)	-	38.3 (s)
9	2.60 (1H, q, 10.2)	48.8 (d)	3.36 (1H, t, 2.65)	71.2 (d)
10	1.68 (1H, m)	39.8 (t)	1.96 (1H, m)	27.1 (t)
	1.59 (1H, m)		1.68 (1H, m)	
11	-	34.1 (s)	1.57 (1H, d, 4.6)	32.4 (t)
			1.43 (1H, d, 2.7)	
12	1.20 (3H, s)	17.1 (q)	1.32 (1H, d, 13.0)	34.4 (t)
			1.07 (1H, d, 13.0)	
13	4.96 (1H, d, 1.8)	112.9 (t)	0.94 (3H, s)	19.8 (q)
	4.83 (1H, d, 1.8)			
14	0.98 (3H, s)	21.7 (q)	0.92 (3H, s)	29.5 (q)
15	0.97 (3H, s)	29.9 (q)	0.85 (3H, s)	25.6 (q)

Compound **2** is obtained as colourless oil. The molecular formula of **2** is determined as $C_{15}H_{26}O_2$ based on HRTOFMS spectra and NMR spectral data (**Table 1**). With the negative ion mode, the molecular ion peak [M-H_2O] is obtained at *m/z* 220.1825, calculated as *m/z* 220.1827 as $C_{15}H_{24}O$, thus obtaining three hydrogen deficiency index. The IR spectrum of **2**, showed the presence the typical absorbance of a hydroxy group was observed at ν_{max} 3421 cm^{-1}, stretch of aliphatic at ν_{max} 2980 and 2870 cm^{-1} and stretch of C–O at ν_{max} 1078 cm^{-1}.

The ^1H-NMR spectrum of **2** displayed the presence of three tertiary methyl signals at δ_H 0.94 (3H, s, Me-12), 0.92 (3H, s, Me-14) and 0.85 (3H, s, Me-13), indicated the characteristics of caryophyllene-type sesquiterpenoid (Fraga et al., 2014). The presence of an oxygenated methine signal at δ_H 3.36 (1H, d, *J*=2.65 Hz, H-9) and other aliphatic signals in the upfield region were also observed in the ^1H NMR spectrum. The ^{13}C-NMR spectrum of **2** along with DEPT experiments indicated the presence of fifteen carbon signals consisting of three tertiary methyl at δ_C [29.5 (Me-13), 19.8 (Me-14) and 25.6 (Me-15)], six methylene signals at δ_C [33.0 (C-3), 19.4 (C-6), 41.1 (C-7), 21.1 (C-10) and 32.3 (C-11)], three sp^3 methine at δ_C [37.1 (C-2) and 42.9 (C-5)], one oxygenated sp^3 methine at δ_C [71.2 (C-3)] and one oxygenated sp^3 quaternary carbon at δ_C [69.7 (C-3)]. There is no functionalities based on NMR spectra, consequently, three degrees of unsaturation were consistent with tricyclic caryophyllene-type sesquiterpenoid (Fraga et al., 2014). Furthermore, the chemical structure of **2** was deduced from the ^1H–^1H COSY and HMBC spectra (**Figure 2**). ^1H-^1H-COSY corelations at C_5-C_2-C_3-C_6-C_7 and C_9-C_{10}-C_{11}, supporting the presence of caryophyllene-type sesquiterpenoid in **2**. Two methyl signals at δ_H 0.92 and 0.94 were correlated to sp^3 quaternary carbon at δ_C 34.0, suggested that *gem*-dimethyl were attached at C-4. A methyl signal at δ_H 0.85 was correlated to sp^3 quaternary carbon at δ_C 38.3 and oxygenated carbon at δ_C 71.2, indicated that another tertiary methyl located at C-8. An oxygenated methine at δ_H 3.36 was correlated to quaternary carbon at δ_C 38.3 (C-8) and methylene carbon at δ_C 27.1 (C-10), suggested that a secondary hydroxy was located at C-9. A methine proton at δ_H 2.13, methylene proton at δ_H 1.32 and

1.57 were correlated to oxygenated carbon at δ_C 69.7 (C-1), indicated that a tertiary alcohol was located at C-1. A detailed comparison of NMR spectra of compound **2** to those of tricylic caryophyllene-type sesquiterpenoid, senecrassidiol (Fraga et al., 2014), revealed that both compounds were very similar, consequently compound **2** was identified as a senecrassidiol. The relative stereochemistry was determined as a senecrassidiol based on the similirity of coupling constant (^3J) values in the ^{1}H NMR spectrum and others similarity. Consequently compound **1** was identified as a senecrassidiol. That compound was isolated also from Root Cultures and Aerial Parts of *Bethencourtia hermosae* (Fraga et al., 2014), but was isolated from *A. harmsiana* for first time.

Compounds **1** and **2** were evaluated for their cytotoxicity against MCF-7 breast cancer cell line according to a method previously described (Supratman et al., 2019; Supriatno et al., 2018), using cisplatin as positive control (Hadisaputri et al., 2012; Chavoshi et al., 2017) and the results showed that β-caryophyllene oxide (**1**) stronger cytotoxic activity than senecrassidiol (**2**) with IC_{50} values of 0.62 and 1.32 μM, respectively. These results indicated that the cytotoxic activity of caryophyllene-type sesquiterpenoid are affected by the presence of double bonds and configuration of methyl groups.

CONCLUSIONS

Two known caryophyllene-type sesquiterpenoid, β-caryophyllene oxide (**1**) and senecrassidiol (**2**) have been isolated from the stembark of *A. hamrsiana*. The discovery of two sesquiterpene compounds from the stembark of *A. minahassae*also reinforces previous phytochemical studies of the occurance of sesquiterpenoid in the *Aglaia* genus. β-caryophyllene oxide (**1**) showed the stronger cytotoxic activity than senecrassidiol (**2**) against MCF-7 breast cancer lines, indicated that the cytotoxic activity of caryophyllene-type sesquiterpenoid are influenced by the presence of double bonds and configuration of methyl groups.

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