

VOLUME 52
ISSUE 6, 2017



ISSN 1314-7471 (print)
ISSN 1314-7978 (on line)
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JOURNAL

OF CHEMICAL TECHNOLOGY AND METALLURGY



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Volume 52, Iss. 6, 2017

Journal of Chemical Technology and Metallurgy

52, Iss. 6, 2017

ISSN 1314-7471 (print)

ISSN 1314-7978 (on line)

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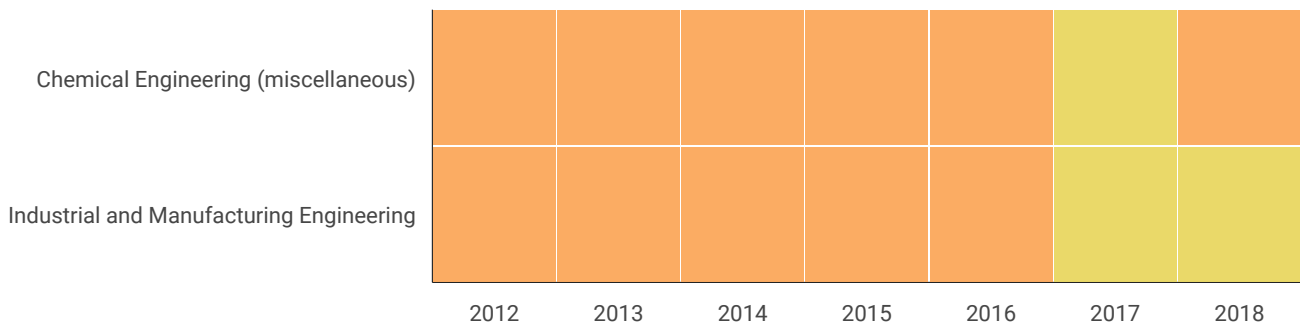
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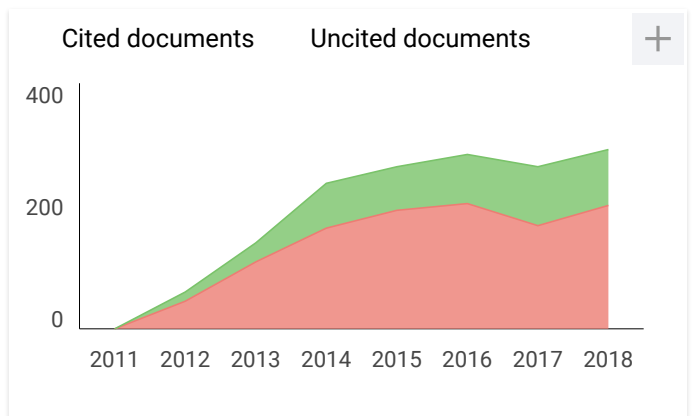
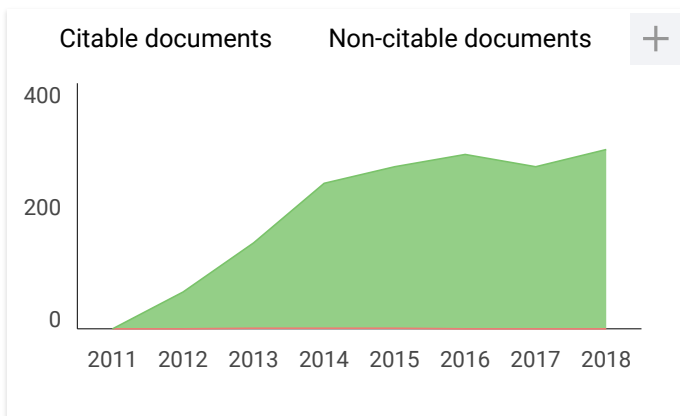
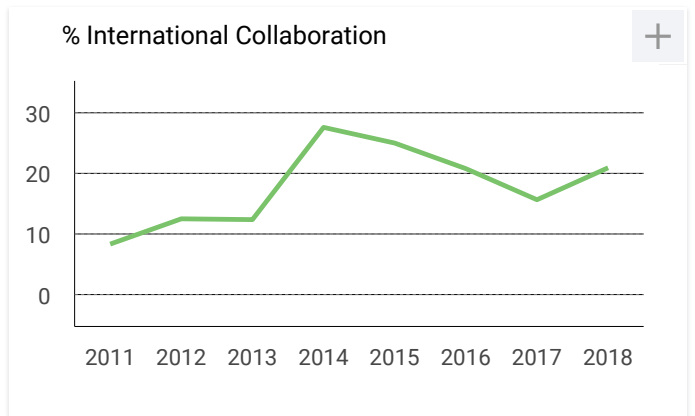
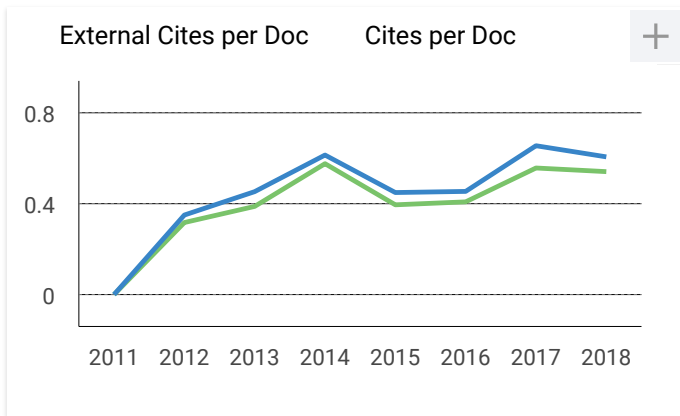
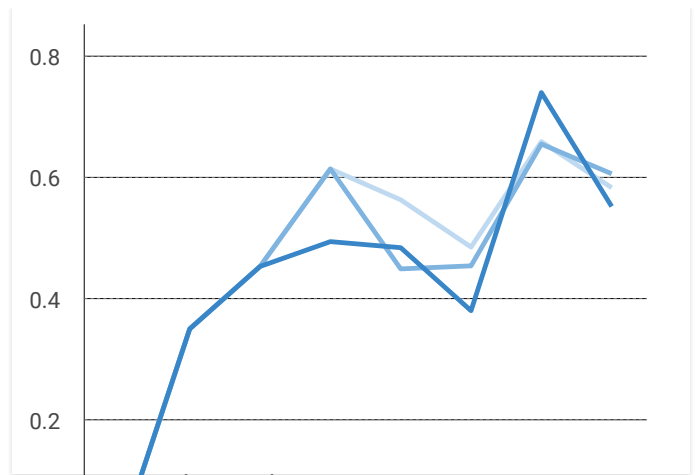
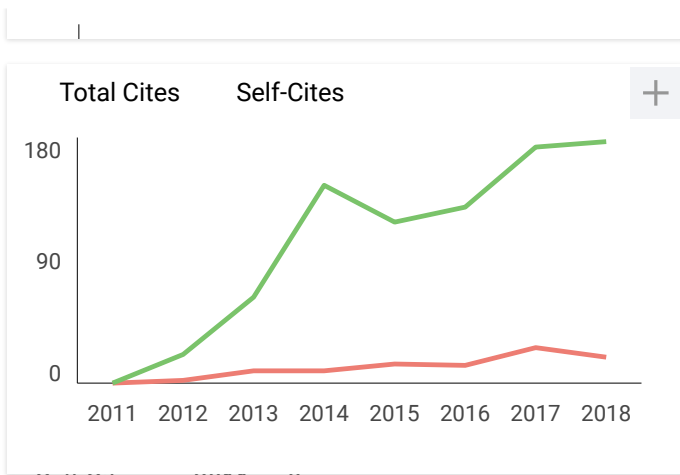
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PHENOLIC COMPOUNDS FROM *Aquilaria microcarpa* STEM BARK

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Received 05 January 2017
Accepted 20 July 2017

ABSTRACT

This paper reports a study of secondary metabolites contained in *Aquilaria microcarpa*, a species belonging to Thymelaceae. This species has not been investigated so far. *Aquilaria microcarpa* is one of *Aquilaria* species that grows in Indonesia. The sample plant used is taken from east Kalimantan. Extraction of stem bark is done using methanol. Two phenolic compounds, namely 5,3',4'-trihydroxy-7-methoxyflavon or known as 3'-hydroxy genkwanin and 6-hydroxy-2-(2-phenylethyl)chromone are isolated and identified. The chemical structure of these compounds is determined based on spectroscopic data, as well as HR-ESI-MS and NMR spectra. Both compounds were reported previously but they were extracted from another species of *Aquilaria*.

Keywords: *Aquilaria microcarpa*, Thymelaeaceae, chromone, flavonoid.

INTRODUCTION

Aquilaria is a genus belonging to Thymelaeaceae family. The species of *Aquilaria* are widely distributed in Asia. Certain trees of *Aquilaria* species produce the fragrant resinous wood, known in the different regions as agarwood, eaglewood, gaharu, kanankoh, jinkoh, chen xiang or tram. Other people also call it aloeswood or agalloch [1, 2]. The *Aquilaria* genus is rich in a variety of different classes of natural products, especially sesquiterpenes and chromones. Flavonoid, benzophenone, diterpenoid, triterpenoid and lignin compounds are also present.

Agarwood preparations are used in Kampo medicine in Japan because of their sedative, analgesic or digestive properties [3]. *Aquilaria* leaves are applied in China topically to treat injuries such as fractures and bruises [4], while in Korea agarwood is used for the treatment of cough, asthma, and as a sedative among others [5]. In Saudi Arabia and other Arabic countries, the wood of *Aquilaria* trees is used as incense at important religious occasions [6, 7].

Some species of *Aquilaria* which are widely studied

are *A. sinensis*, *A. malaccensis*, *A. crassna* and *A. agallocha*, whereas *A. beccariana*, *A. hirta*, *A. cumingiana*, *A. filaria* dan *A. microcarpa* are *Aquilaria* species that grow in Indonesia and are not studied so far.

This paper reports research results referring to *A. microcarpa* as one of these species. Two phenolic compounds are isolated from stem bark of *A. microcarpa*. They are identified as 5,3',4'-trihydroxy-7-methoxyflavon or known as 3'-hydroxy genkwanin (**1**) and 6-hydroxy-2-(2-phenylethyl)chromone (**2**). Their structure of is determined spectroscopically. It is presented in Fig. 1.

EXPERIMENTAL

Non infected stem bark of *A. microcarpa* obtained from Bukit Bangkirai forest conservation, Samboja, Samarinda, Kalimantan Timur was used.

Methanol, *n*-hexane, ethyl acetate, diisopropyl ether, chloroform, cerium sulfate, acetone, silica gel 60 GF₂₅₄ (Merck), silica gel 60 PF₂₅₄ (Merck), silica gel 60 GF₂₅₄ 0.25 mm (Merck) were the chemical reagents applied.

A rotary vacuum evaporator, column and radial

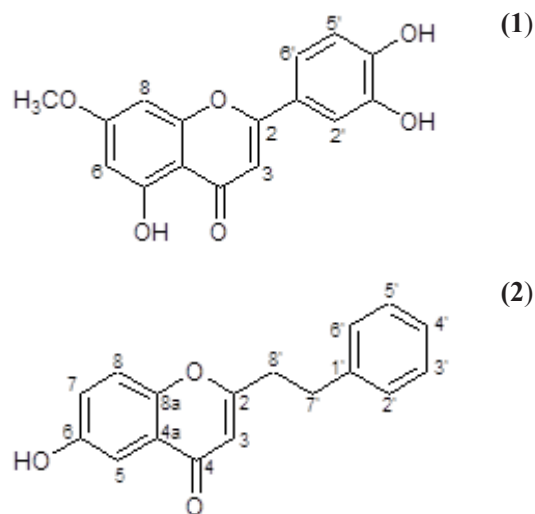


Fig. 1. Structure of isolated compounds.

chromatography were used in the course of the experimental procedure applied. The latter was as follows. 1.69 kg of stem bark was cut up and then subjected to extraction with n-hexane. The residue was separated from the filtrate and then macerated again using methanol. The extract methanol was fractionated with ethyl acetate. The ethyl acetate fraction was then separated using a column chromatography on silica gel. The fractions of interest were subsequently separated again using several methods of chromatography until a pure product was obtained. Compound **1** was obtained after a double separation using gravity column chromatography followed by a double separation using radial chromatography. Mixtures of hexane - ethyl acetate, hexane - acetone, hexane - chloroform and hexane - diisopropyl ether whose polarity was gradually increased were used as an eluent in those separations. Meanwhile, compound **2** was obtained after a double separation using flash chromatography and a single separation using radial chromatography. A mixture of hexane - ethyl acetate whose polarity was gradually increased was the eluent used in those separations. Compounds **1** and **2** were obtained as much as 3 mg each.

The structure of each compound was elucidated using UV-Vis spectroscopy, HRESI-MS and NMR. UV-Vis Shimadzu 1800, HR-ESI-MS Waters LCT XE ESI-TOF (*electro spray ionization-time of flight*), and NMR JEOL ECA 400 ($^1\text{H-NMR}$, 400 MHz) and ($^{13}\text{C-NMR}$, 100 MHz) were used.

Tabel 1. ^1H and ^{13}C -NMR data of compound **1** and **2**.

No	Compound 1 (CDCl_3)		Compound 2 (acetone- d_6)	
	δ_{H} (m, J Hz)	δ_{C}	δ_{H} (m, J Hz)	δ_{C}
2	-	158,0	-	169,1
3	6,18 (s)	103,9	6,04 (s)	109,7
4	-	183,2	-	182,7
4a	-	102,0	-	124,6
5	-	163,6	7,48 (d, J = 2,1 Hz)	108,9
6	6,32 (d, J = 2,2 Hz)	101,0	-	156,6
7	-	166,7	7,24 (dd, J = 8,3 & 2,1 Hz)	120,2
8	6,46 (d, J = 2,2 Hz)	100,1	6,88 (d, J = 8,3 Hz)	115,8
8a	-	160,5	-	151,2
1'	-	122,9	-	141,3
2'	7,36 (d, J = 2,2 Hz)	119,1	7,27 (m)	129,2
3'	-	147,0	7,27 (m)	129,2
4'	-	139,4	7,27 (m)	129,2
5'	7,54 (d, J = 8,6 Hz)	124,4	7,27 (m)	129,2
6'	7,13 (dd, J = 8,6 & 2,2 Hz)	124,0	7,27 (m)	129,2
7'	-	-	3,08 (m)	33,4
8'	-	-	2,96 (m)	36,4
5-OH	11,14 (s)	-		
7-OCH ₃	3,67 (s)	55,7		

RESULTS AND DISCUSSION

5,3',4'-Trihydroxy-7-methoxyflavon (1) is a pale yellow solid; λ_{max} (MeOH) nm (log ϵ): 278 (4.09), 287 sh (3.97), and 326 (3.97); HRESI-MS: $[\text{M-H}]^-$ at m/z 301.0718 in accordance with the molecular formula $\text{C}_{16}\text{H}_{13}\text{O}_6$; the NMR data is showed in Table 1.

6-Hydroxy-2-(2-phenylethyl)chromone (2) is a pale yellow solid; λ_{max} (MeOH) nm (log ϵ): 242 (4.33), 314 (3.58), and 369 (3.39); HRESI-MS: $[\text{M-H}]^-$ at m/z 265.0805 in accordance with the molecular formula $\text{C}_{17}\text{H}_{14}\text{O}_3$; NMR data is shown in Table 1.

The $^1\text{H-NMR}$ spectrum of the isolated flavonoid compounds **1** in CDCl_3 shows two units of aromatic proton signals that refer to three aromatic proton signals of ABX system and two aromatic proton signals of AX system. The three aromatic proton signals of ABX system are attributed to the protons in the chemical shifts

Table 2. Correlation one bond from HMQC analysis.

Compound 1		Compound 2	
δ_H (mult, J Hz)	δ_C	δ_H (mult, J Hz)	δ_C
7,54 (1H, d, J= 8,6 Hz)	119,1	7,48 (d, J= 2,1, 1H)	108,9
7,36 (1H, d, J= 2,2 Hz)	124,4	7,27 (m, 5H)	129,3 129,2 127,1
7,13 (1H, dd, J= 8,6 & 2,2 Hz)	124,0	7,24 (dd, J= 8,3 dan 2,1, 1H)	120,2
6,46 (1H, d, J= 2,2 Hz)	100,1	6,88 (d, J= 8,3, 1H)	115,8
6,32 (1H, d, J= 2,2 Hz)	101,0	6,04 (s, 1H)	109,7
6,18 (1H, s)	103,9	3,08 (m, 2H)	33,4
3,67 (3H, s)	55,7	2,96 (m, 2H)	36,4

δ_H (ppm) of 7.54 (*d*, *J* = 8.6 Hz), 7.36 (*d*, *J* = 2.2 Hz) and 7.13 (*dd*, *J* = 8.6 and 2.2 Hz). The pair of doublet signals in AX system with *meta* coupling constants (*J* = 2.2 Hz) is attributed to the proton in the chemical δ_H (ppm) shifts of 6.46 and 6.32. Those units' signals indicate that the isolated flavonoid compounds are substituted at C-5, C-7, C-3 and C-4'. The singlet signal at δ_H 6.18 ppm shows that the isolated compound is derived of flavones (Zhao *et al.*, 2005). The ¹H-NMR spectrum gives also one singlet signal attributed to a methoxy group that appears at δ_H (ppm) 3.67. The deshielding proton signal at δ_H 11.14 ppm characterizes the hydroxyl group at C-5 which is capable of forming a hydrogen bond with the carbonyl group at C-4.

The ¹³C-NMR spectrum of (**1**) shows 16 signals attributed to six methine carbons CH (δ_C 124.4; 124.0; 119.1; 103.9; 101.0 and 100.1 ppm), one methoxy carbon (δ_C 55,7 ppm), and nine quaternary carbons (δ_C 183.2; 166.7; 163.6; 160.5; 158.0; 147.0; 139.4; 122.9 and 102.0 ppm). Six of the latter are aryloxy carbons (δ_C 166.7; 163.6; 160.5; 158.0; 147.0; and 139.4 ppm). The presence of a carbonyl carbon atom is evidenced by a signal of δ_C 183.2 ppm. It follows that there is only one methoxy group. The three other substituents refer to hydroxy groups. ¹H and ¹³C-NMR data of compound **1** is listed in Table 1.

The ¹H NMR spectrum of isolated compound (**2**) in acetone-*d*₆ shows two units of aromatic proton signals referring to three ABX system aromatic proton signals and five monosubstituted aromatic proton signals. The

three ABX system aromatic proton signals appear in the chemical shifts δ_H of 6.88 (*d*, *J* = 8.3 Hz), 7.24 (*dd*, *J* = 8.3 and 2.1 Hz), and 7.48 (*d*, *J* = 2.1 Hz). The proton signals of the other aromatic unit (five protons) appear as a multiplet at δ_H 7.27 ppm. The proton singlet signal at δ_H 6.04 ppm and the two proton multiplet signals at δ_H 3.08 and 2.96 show a characteristic chromone class of compounds that have a hydroxy substituent at C-6 or C-7.

The ¹³C NMR spectrum analysis shows fifteen carbons that are perfectly separated. Based on HR - ESI - MS analysis there should be seventeen carbons. This suggests the existence of two symmetric carbons. Two symmetric carbons are located in ring B at C-2' / 6' and C-3' / 5'. Fifteen carbon signals refer to six quaternary carbons (δ_C (ppm) 182.7; 168.9; 156.6; 151.2; 141.2; 124.6), seven methine carbons CH (δ_C (ppm) 109.7; 108.9; 120.2; 115.8; 129.2; 127.1; 129.3) and two methylene carbons CH₂ (δ_C (ppm) 33.4 and 36.4). Four of these six quaternary carbon signals consist of three aryloxy carbon signals (δ_C (ppm) 169.2; 156.6; 151.2) and one of carbonyl carbon signal (δ_C 182.7 ppm). This indicates that compound **2** has the structure of 2-(2-phenylethyl)chromone with one hydroxyl substituent. The ¹H and ¹³C-NMR data of compound **2** is presented in Table 1. A correlation of one bond in the HMQC spectrum analysis of compound **1** shows seven correlations as presented in Table 2. The same correlation occurs also in compound **2**.

The presence of a methoxy and hydroxyl groups in the structure of compound (**1**) is evidenced by the HMBC spectrum. It is confirmed that the two hydroxyl groups in ring B are positioned at 3' and 4'. The position of the methoxy group and the remaining hydroxyl group in ring A has to be determined. The positions to be filled refer to numbers 5 and 7. The proton signal of the hydroxyl group (δ_H of 11.14 ppm) shows correlations with two quaternary carbon signals (δ_C of 163.6 ppm and 102.0 ppm) and one methine carbon signal at δ_C of 101.0 ppm. It has been stated above that a deshielding proton signal at δ_H of 11.14 ppm is characteristic for the hydroxyl group at C-5 which is capable of forming a hydrogen bond with the carbonyl group at C-4. Hence it follows that the signal at 163.6 ppm refers to C-5, that at 102.0

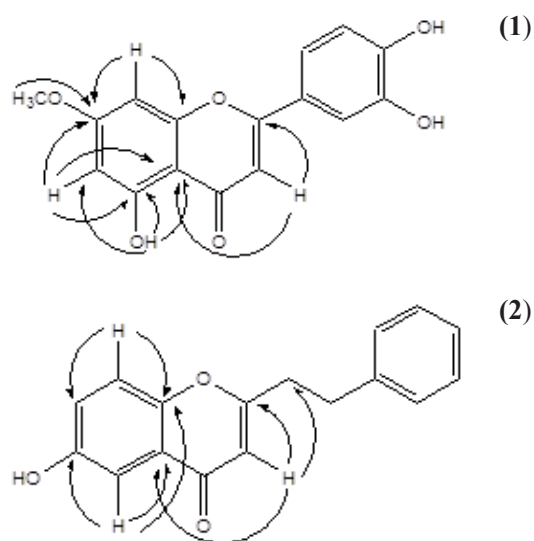


Fig. 2. HMBC correlation of compound **1** and compound **2**.

ppm refers to C-4a, while that at 101.0 ppm refers to C-6. The singlet proton signal of the methoxy group (δ_{H} of 3.67 ppm) gives one correlation with the quaternary carbon signal at δ_{C} of 166.7 ppm. The latter is a signal referring to C-7. The results of HMBC spectrum analysis of compound **1** are illustrated in Fig. 2.

HMBC analysis is required to verify the hydroxy group position (at C-6 or C-7) in compound **2** structure. A singlet proton signal at δ_{H} of 6.04 ppm (H-3) shows correlations with two quaternary carbon signals at δ_{C} of 169.1 ppm (C-2) and 124.6 ppm (C-4a) and one methine carbon at δ_{C} of 36.4 ppm (C-8'). Another correlation is revealed between the aromatic proton in ABX system and the two aryloxy carbons and one quaternary carbon. The proton considered appears as a doublet with a coupling constant of 2.1 Hz at δ_{H} of 7.48 ppm (H-5). The two aryloxy carbons mentioned above show signals at δ_{C} of 156.6 (C-6), 151.2 (C-8a) ppm respectively, while the quaternary carbon appears at δ_{C} of 124.6 ppm (C-4a). A correlation is also observed between the doublet ($J = 8.3$ Hz) proton signal at δ_{H} of 6.88 ppm (H-8) with one aryloxy carbon at δ_{C} of 151.2 ppm (C-8a) and one methine carbon at δ_{C} of 120.2 (C-7). The overall correlations data indicates that the hydroxyl group is attached at C-6. The results of HMBC spectrum analysis of compound **2** are shown in Fig. 2.

All data obtained is compared to that found in the

literature. Compound **1** is reported by Qi [8] who succeeds isolating this compound from the leaves of *A. sinensis*. In that report, Qi mentions also that this 3'-hydroxy genkwanin has a significant inhibitory activity in respect to neutrophils respiratory burst stimulated by PMA with IC_{50} value $0.80 \pm 0.13 \mu\text{mol/l}$. Likewise, compound **2** is compared to the same compound isolated [9] from an ether extract of powdered agarwood from Kalimantan. In 1989 Yang [10] is the first to obtain this compound from the ether soluble fraction of an alcoholic extract of *A. sinensis* (Lour.) Gilg. The same compound is also successfully isolated from a 70 % MeOH extract of *A. malaccensis* agarwood chips [11] and from an EtOAc extract of Chinese agarwood induced by artificial holing from *A. sinensis* [12]. The latter reference mentions also that this compound exhibits acetylcholinesterase inhibitory activity with percentage of inhibition of $19.3 \pm 0.8 \%$. Bioactivity tests referring to compound **1** are not performed in the course of the present investigation because of the small quantity isolated. However an anticancer activity test against T47d cell line is carried out with the participation of compound **2**. The result indicated that the IC_{50} is 2884.03 $\mu\text{g/mL}$. The same test is also carried out with the ethyl acetate fraction used for the isolation of compound **1** and compound **2**. The result shows that this fraction has a weak activity with IC_{50} value of $26.48 \pm 0.02 \mu\text{g/mL}$.

CONCLUSIONS

A plant sample of *Aquilaria microcarpa* was used to isolate two compounds. Their structure was determined based on analyses, including UV, HR-ESI-MS and NMR. The compounds obtained were identified as 5,3',4'-trihydroxy-7-methoxyflavon or known as 3'-hydroxy genkwanin **1** and 6-hydroxy-2-(2-phenylethyl)chromon **2**. They were isolated by other research groups using *A. sinensis* and *A. malaccensis*, which are different species of *Aquilaria*.

Acknowledgements

This work was financially supported by 2016 grant from the Ministry of Research and Higher Education. The authors are grateful to Bukit Bangkirai Forest Conservation, Samboja, Samarinda, Kalimantan Timur for the sample material provision.

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