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SHORT COMMUNICATION

CHEMICAL CONSTITUENTS AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF *Piper abbreviatum* Opiz

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(Received November 11, 2019; Revised November 30, 2020; Accepted November X, 2020)

ABSTRACT. Plants of the genus *Piper* have long been used as medicinal herbs. The chemistry of *Piper* species has been widely investigated and phytochemical investigations conducted in all parts of the World have led to the isolation of a number of physiologically active compounds. Thus, this study was carried out to investigate the phytochemicals from *Piper abbreviatum* and their acetylcholinesterase inhibitory activity, which has not been previously investigated. Fractionation and purification of the aerial parts of *P. abbreviatum* led to the isolation and identification of five methoxylated flavonoids, namely 5,7-dimethoxyflavone, 4′,5,7-trimethoxyflavone, 3',4',5,7-tetramethoxyflavone, 5-hydroxy-7-methoxyflavone, 5-hydroxy-4′,7-dimethoxyflavone, together with lupeol, lupenone, β-sitosterol, and β-sitostenone. The structures of these compounds were obtained by analysis of their spectroscopic data, as well as the comparison with that of reported data. Acetylcholinesterase inhibitory activity revealed that all isolated flavones were found to inhibit AChE with percentage inhibition values ranged from 24.2 to 58.2%. This is the first report on the isolation of methoxylated flavonoid from *P. abbreviatum.* The high variants of flavonoid compounds from this species may be used as chemotaxonomic markers for this *Piper* species.

KEY WORDS: Piperaceae, *Piper*, *Piper abbreviatum*, flavonoid, acetylcholinesterase

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease and the predominant cause of dementia among the elderly, provoking progressive cognitive decline, psycho-behavioral disturbances, memory loss, and presence of senile plaque, neurofibrillary tangles, and a decrease in cholinergic transmission. One treatment strategy to enhance the cholinergic function is to use acetylcholinesterase (AChE) inhibitors to increase the amount of acetylcholine, which is present in the synapses between cholinergic neurons [1,2]. Most of the drugs currently available for the treatment of AD are tacrine, donezepil, rivastigmine, huperzine A, and galanthamine, all of which have limited effectiveness and some kind of side effect [3]. Taking into account that the above inhibitors are related to natural products and that AChE is an important therapeutic strategy for the treatment of AD, many research groups have focused their studies on naturally-occurring compounds from plants as potential sources of either new or more effective AChE [3,4].

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Piper abbreviatum which grows in Indonesia and the Philippines is a branching climber hugging trees with pendent lateral branches. The stems are fissured longitudinally, rooting and articulated. The leaves are simple, spiral and exstipulate. In the Philippines, the leaf is used to treat splenomegaly [5]. We have recently reported the chemical compositions and biological activities of the essential oils from this species [6]. GC and GC-MS analysis of *P. abbreviatum* essential oil resulted in the identification of 33 chemical components, representing 70.5% of the total oil. The major components were spathulenol (11.2%), (*E*)-nerolidol (8.5%) and β-caryophyllene (7.8%). The essential oils also displayed weak activity towards Gram-positive bacteria with MIC values ranges between 250-500 μg/mL. In addition, the anticholinesterase activity of the leaves extract has also been reported. The hexane and EtOAc extract of *P. abbreviatum* showed moderate activity against butyrylcholinesterase enzyme with inhibition of 42.5% and 35.6%, respectively [1]. Meanwhile, phytochemical screening of this species showed the presence of alkaloids, tannins, and terpenoids [7].

In continuation of our search for bioactive compounds from *Piper* species, we have investigated a phytochemical study on the aerial parts of *P. abbreviatum.* To the best of our knowledge, this is the first report on the phytochemical study of this species and their acetylcholinesterase inhibitory activity.

EXPERIMENTAL

Plant material. Piper abbreviatum was collected from Borneo (Jan 2012) and identified by Mohizar Mohamad. The voucher specimen (UiTMKS-01/2012) was deposited at the Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

General experimental procedures. Soxhlet extraction technique was applied to extract the phytochemicals from the dried sample using different polarity solvents (*n*-hexane, ethyl acetate, and methanol). Vacuum liquid chromatography (VLC) was performed on Merck silica gel 60 (230-400 mesh) while column chromatography (CC) on Merck silica gel 60 (70-230 mesh) as the stationary phase. Thin-layer chromatography (TLC) analysis was performed on Merck pre-coated silica (SiO_2) gel F_{254} plates (0.2 mm thickness) to detect and monitor the presence of compounds in the samples. The spots were visualized under UV light at 254 and 365 nm, included with spraying reagent vanillin-sulphuric acid in MeOH followed by heating. Melting points were measured using melting point apparatus equipped with a microscope, Leica Gallen III and were uncorrected. The ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Bruker Avance 400 Spectrometer. Chemical shifts were reported in ppm and $CDCl_3$ as the solvent. The residual solvent was used as an internal standard. The IR spectra were recorded on Perkin Elmer ATR and 1600 spectrophotometer series as KBr disc. Mass spectral data were obtained from Mass Spectrometry Service, National University of Singapore (NUS), Singapore.

Extraction and isolation. The dried and powdered whole aerial parts of *P. abbreviatum* (1 kg) were extracted consecutively by Soxhlet extractor with hexane, EtOAc and MeOH. Evaporation of the respective solvents gave hexane (4.2 g), EtOAc (8.5 g) and MeOH (9.5 g) extracts.

The hexane extract was subjected to vacuum liquid chromatography (VLC) on SiO_2 60 (230–400 mesh) using hexane and $CHCl_3$ in 5% increasing polarity to give 8 fractions (PAH1–8). The combined fractions of PAH1-3 were purified by column chromatography on silica gel 70–230 mesh to afford compounds **1** (15 mg) and **2** (20 mg). The combined fractions of PAH4-6 were purified by column chromatography on silica gel 70–230 mesh to afford compounds **3** and **4**. The crude EtOAc was fractionated by VLC on SiO_2 70–230 mesh, using hexane and EtOAc in 10% increasing polarity to give 15 fractions (PAE1–15). The combined fractions PAE5-9 were purified and recrystallized from hexane–$CHCl_3$ (8:2) to yield

compounds **5** and **6**. The crude MeOH was fractionated by VLC on SiO_2 70–230 mesh, using $CHCl_3$-MeOH in 10% increasing polarity to give fractions (PAM1–5). The combined fractions PAM2-3 were purified by column chromatography to yield compounds **7** (18 mg) and **8** (20 mg), while the combined fractions PAM4-5 were purified by column chromatography to yield compound **9**.

Acetylcholinesterase inhibitory activity: AChE inhibitory activities were measured by slightly modifying the spectrophotometric method [8]. Electric eel AChE were used, while acetylthiocholine iodides were employed as substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic) acid (DTNB) was used for the measurement of the AChE activity. Briefly, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of the compound (concentration of 1 mg/mL) and 20 µL of AChE solution were added by multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 µL of acetylthiocholine iodide. Hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer). Percentage inhibition (I%) of AChE was determined by comparison of reaction rates of samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula:
$I\% = [\ E - S / E\ ] \times 100;$
where E is the activity of enzyme without test sample and S is the activity of the enzyme with test sample. Galantamine (1 mg/mL) was used as a positive control. Analyses were run in triplicate and the result was expressed as means ± SD of triplicate. Data obtained from the acetylcholinesterase activity are expressed as mean values. Statistical analyses were carried out by employing one way ANOVA (p>0.05).

Statistical analysis. Data obtained from biological activity are expressed as mean values. Statistical analyses were carried out by employing one way ANOVA (p>0.05). A statistical package (SPSS version 11.0) was used for the data analysis.

RESULTS AND DISCUSSION

Phytochemical studies on *Piper* species have resulted in the isolation and identification of various phytochemicals. In view of the attributed medicinal properties, studies were undertaken on the whole aerial parts of *P. abbreviatum* species which resulted in the isolation and structure elucidation of flavonoids and steroids. Five flavonoids have been successfully isolated which are 5,7-dimethoxyflavone (**3**), 4′,5,7-trimethoxyflavone (**4**), 3',4',5,7-tetramethoxyflavone (**5**), 5-hydroxy-7-methoxyflavone (**6**), and 5-hydroxy-4′,7-dimethoxyflavone (**9**). The chemical structures are shown in Figure 1. In addition, lupeol (**7**) [9], lupenone (**8**) [10], β-sitosterol (**1**) [11], and β-sitostenone (**2**) [12] were also identified. All secondary metabolites were identified by analyzing their spectroscopic data and comparing them with the literature data. These flavones have been previously isolated from various *Piper* genus. Flavones (**3**), (**4**), and (**6**) have been isolated previously from *P. caninum*, *P. ungaromense* [13], *P. methysticum* [14], *P. porphyrophyllum* [15], while flavone (**5**) from *P. caninum* [16] and *P. porphyrophyllum* [17]. Interestingly, flavone (**9**) was isolated for the first time from *Piper* species. All the flavonoid compounds were also reported from plants of various families such as Zingiberaceae, Winteraceae, Asteraceae, and Lamiaceae [17]. The presence of flavonoids in higher plants has been associated with various environmental conditions, such as high-light/UV stress, cold stress, nutritional deficiencies, and pathogen protection. In addition, the habitats of the samples were at about 2600 m altitude, where the plants were exposed to high UV radiation also should affect the production of different types and quantities of flavonoids in the plant [18]. The presence of those valuable flavonoids in *Piper* species definitely enriches their chemical

diversity and provides evidence for chemotaxonomic studies of *Piper* species and the family Piperaceae as well.

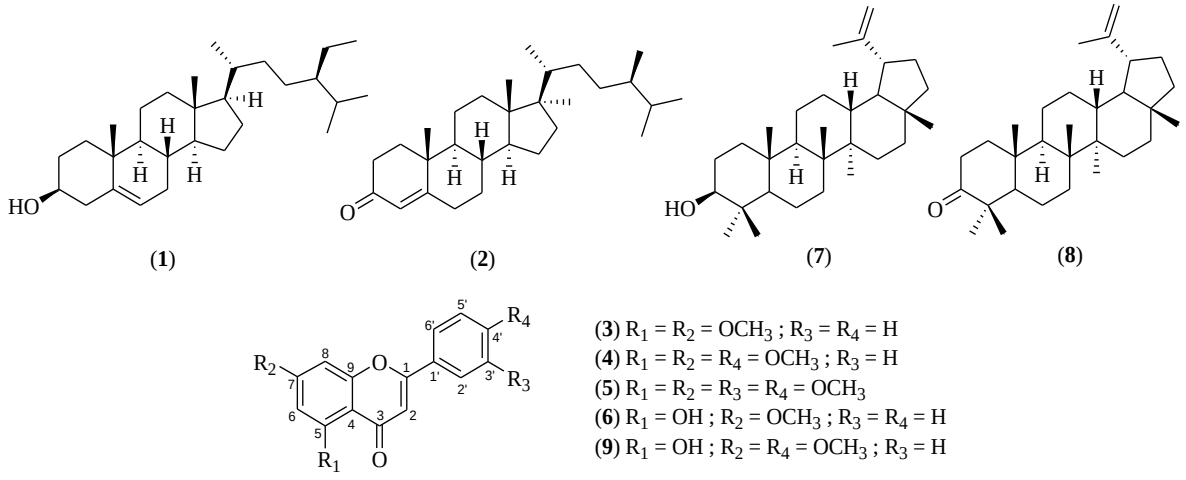

Figure 1. Chemical constituents isolated from *Piper abbreviatum*

*β-Sitosterol (**1**)* - White crystalline needles; m.p 133-134°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.70 (3H, s, H-18), 0.84 (3H, d, *J* = 6.6 Hz, H-27), 0.86 (3H, d, *J* = 6.6 Hz, H-26), 0.88 (3H, d, *J* = 3.9 Hz, H-29), 0.95 (3H, d, *J* = 6.3 Hz, H-21), 1.03 (3H, s, H-19), 1.27−2.31 (29H, m, overlapping CH and CH_2), 3.54 (1H, m, H-3), 5.37 (1H, d, J = 4.8 Hz, H-6); GC-MS *m/z* 414 [M^+, $C_{29}H_{50}O$] [11].

*β-Sitostenone (**2**)* - White solids; m.p 77-79°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.73 (3H, s, H-18), 0.82 (3H, d, *J* = 6.4 Hz, H-27), 0.84 (3H, d, *J* = 6.4 Hz, H-26), 0.86 (3H, t, *J* = 7.6 Hz, H-29), 0.93 (3H, d, *J* = 6.8 Hz, H-21), 1.18 (3H, s, H-19), 1.25−2.44 (29H, m, overlapping CH and CH_2), 5.74 (1H, s, H-4); GC-MS *m/z* 412 [M^+, $C_{29}H_{48}O$] [12].

*5,7-Dimethoxyflavone (**3**)* - White crystalline needles (50.1 mg, 1.25%); m.p 158-159°C; IR (KBr) υ_{max} cm^{-1}: 2920, 1645, 1620, 1601, 1450, 1121; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.91 (3H, s, 7-OCH_3), 3.95 (3H, s, 5-OCH_3), 6.38 (1H, d, *J* = 2.0 Hz, H8), 6.58 (1H, d, *J* = 2.0 Hz, H6), 6.68 (1H, s, H3), 7.50 (3H, m, H3′, H4′, H5′), 7.86 (2H, dd, *J* = 8.0 and 3.6 Hz, H2′, H6′); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 55.7 (5-OCH_3), 56.5 (7-OCH_3), 92.8 (C6), 96.0 (C8), 109.0 (C3), 109.3 (C4a), 125.8 (C2′, C6′), 128.8 (C3′, C5′), 131.0 (C4′), 131.5 (C1′), 159.8 (C2), 160.6 (C5), 160.9 (C7), 164.0 (C8a), 177.5 (C4); EIMS *m/z* 282 [M^+, $C_{17}H_{14}O_4$] [16].

*4,5,7-Trimethoxyflavone (**4**)* - Colourless crystalline needles (65.0 mg, 1.85%); m.p 154-155°C; IR (KBr) υ_{max} cm^{-1}: 3020, 2923, 1641, 1598, 1567, 1258; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.88 (3H, s, 4'-OCH_3), 3.92 (3H, s, 7-OCH_3), 3.95 (3H, s, 5-OCH_3), 6.38 (1H, d, *J* = 2.4 Hz, H8), 6.56 (1H, d, *J* = 2.4 Hz, H6), 6.62 (1H, s, H3), 7.02 (2H, d, *J* = 8.8 Hz, H3′ and H5′), 7.85 (2H, d, *J* = 8.8 Hz, H2′ and H6′); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 55.5 (5-OCH_3), 55.7 (7-OCH_3), 56.3 (4′-OCH_3), 92.8 (C6), 96.0 (C8), 107.6 (C3), 109.2 (C4a), 114.3 (C3′, C5′), 123.8 (C1′), 127.6 (C2′, C6′), 159.8 (C4′), 160.7 (C8a), 160.9 (C5), 162.0 (C2), 163.9 (C7), 177.6 (C4); EIMS *m/z* 312 [M^+, $C_{18}H_{16}O_5$] [16].

*3',4',5,7-Tetramethoxyflavone (**5**)* - Yellowish crystalline needles (20.0 mg, 1.80%); m.p 129-130°C; IR (KBr) υ_{max} cm^{-1}: 3012, 2925, 1648, 1605, 1515, 1253; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.94 (3H, s, 7-OCH_3), 3.97 (6H, s, 3',4'-OCH_3), 3.99 (3H, s, 5-OCH_3), 6.39 (1H, d, *J* = 2.4 Hz, H8), 6.58 (1H, d, *J* = 2.4 Hz, H6), 6.63 (1H, s, H3), 6.97 (1H, d, *J* = 8.4 Hz, H5'), 7.33 (1H, d, *J* = 2.3 Hz, H2'), 7.52 (1H, dd *J* = 8.4, 2.3 Hz, H6'); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 55.76 (3'-OCH_3), 56.06 (4'-OCH_3), 56.10 (7-OCH_3), 56.42 (5-OCH_3), 92.87 (C6), 96.10 (C8),

108.20 (C3), 108.63 (C2'), 109.20 (C4a), 119.51 (C5'), 111.10 (C6'), 124.05 (C1'), 149.25 (C3'), 151.74 (C4'), 159.87 (C8a), 160.67 (C5), 160.91 (C2), 163.90 (C7), 177.67 (C4); EIMS *m/z* 342 [M^+, $C_{19}H_{18}O_6$] [17].

*5-Hydroxy-7-methoxyflavone (**6**)* - Colourless crystalline needles (20.2 mg, 1.55%); m.p 162-163°C; IR (KBr) υ_{max} cm^{-1}: 3445, 2973, 1614, 1541, 1448, 1252; ^1H NMR (400 MHz, $CDCl_3$): δ_H 3.90 (3H, s, 7-OCH_3), 6.40 (1H, d, *J* = 2.0 Hz, H8), 6.53 (1H, d, *J* = 2.0 Hz, H6), 6.69 (1H, s, H3), 7.56 (3H, m, H3′, H4′, H5′), 7.90 (2H, dd, *J* = 2.0 and 1.6 Hz, H2′, H6′), 12.74 (1H. s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 55.8 (7-OCH_3), 92.7 (C6), 98.1 (C8), 105.7 (C3), 105.9 (C4a), 126.3 (C2′, C6′), 129.0 (C3′, C5′), 131.3 (C4′), 131.8 (C1′), 157.8 (C2), 162.2 (C5), 164.0 (C7), 165.6 (C8a), 182.5 (C4); EIMS *m/z* 268 [M^+, $C_{16}H_{12}O_4$] [16].

*Lupeol (**7**)* - White needles; m.p 214-216°C; ^1H NMR (400 MHz, $CDCl_3$): δ_H 0.71 (1H, d, *J* = 9.2 Hz, H-5), 0.77 (3H, s, H-28); 0.80 (3H, s, H-25), 0.94 (3H, s, H-27), 0.96 (3H, s, H-23), 0.98 (3H, s, H-24), 1.00 (3H, s, H-26), 1.67 (3H, s, H-30), 1.95 (2H, m, H-21), 2.36 (1H, dt, *J* = 11.2 and 5.6 Hz, H-19), 3.19 (1H, dd, *J* = 11.2 and 5.4 Hz, H-3), 4.58 (1H, s, H-29), 4.70 (1H, s, H-29); GC-MS *m/z* 426 [M^+, $C_{30}H_{50}O$] [9].

*Lupenone (**8**)* - Colourless needle; m.p 169-171°C; ^1H NMR (400 MHz, $CDCl_3$): δ_H 0.81 (3H, s, H-28), 0.95 (3H, s, H-25), 0.97 (3H, s, H-27), 1.04 (3H, s, H-24), 1.09 (3H, s, H-23), 1.22 (3H, s, H-26), 1.68 (3H, s, H-30), 1.89−1.94 (2H, m, H-21), 2.40−2.51 (1H, m, H-19), 4.59 (1H, s, H-29a), 4.71 (1H, s, H-29b); GC-MS *m/z* 424 [M^+, $C_{30}H_{48}O$] [10].

*5-Hydroxy-4',7-dimethoxyflavone (**9**)* - White crystalline needles (15.2 mg, 1.25%); m.p 158-160°C; IR (KBr) υ_{max} cm^{-1}: 3440, 2975, 1615, 1545, 1448, 1252; ^1H NMR (400 MHz, $CDCl_3$): δ_H 12.5 (1H, s,), 7.75 (2H, dd, *J* = 8.0 and 4.0 Hz, H2', H6'), 6.98 (2H, dd, *J* = 8.0 and 4.0 Hz, H3', H5'), 6.52 (1H, s, H3), 6.44 (1H, d, *J* = 4.0 Hz, H8), 6.31 (1H, d, *J* = 4.0 Hz, H6), 3.85 (3H, s), 3.85 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 55.8 (7–OCH_3), 55.9 (6'–OCH_3), 92.5 (C8), 98.0 (C6), 104.0 (C3), 105.5 (C10), 114.5 (C3'), 114.5 (C5'), 123.5 (C1'), 127.8 (C6'), 127.9 (C2'), 162.5 (C4'), 157.5 (C9), 162.0 (C5), 163.9 (C2), 165.3 (C7), 182.5 (C4); EIMS *m/z* 298 [M^+, $C_{17}H_{14}O_5$] [15].

Many natural products have been reported as acetylcholinesterase inhibitors [19]. Whereas flavonoids have long been known as excellent reactive oxygen species scavengers endowed with high metal-chelating ability. Therefore, flavonoids with AChE inhibitory activity are promising natural compounds for developing multipotent drugs against AD [20]. In the current study, the isolated flavonoids of *P. abbreviatum* were subjected to the acetylcholinesterase inhibitory activity and the results are shown in Table 1.

Table 1. Acetylcholinesterase inhibitory activity of isolated flavonoids from *P. abbreviatum*[a]

Samples	**AChE inhibition (I%)**
5,7-Dimethoxyflavone (**3**)	47.5%
4,5,7-Trimethoxyflavone (**4**)	52.8%
3',4',5,7-Tetramethoxyflavone (**5**)	58.2%
5-Hydroxy-7-methoxyflavone (**6**)	25.5%
5-Hydroxy-4′,7-dimethoxyflavone (**9**)	24.2%
Galantamine[b]	85.5%

[a]Data represent mean ± standard deviation of three replicate experiments; ($p < 0.05$); [b]positive control

The tested flavonoids displayed medium inhibitory activity, ranging from 24.2-58.2%, compared to the positive control, galantamine which inhibited the activity at 85.5%. It is worth to know that the isolated flavonoids from this species are methoxylated flavones. Previous

study reported that flavones containing highly methoxyl groups may play an important role in AChE inhibitory activity [21]. This study shows that compound (**6**) had four methoxyl groups compared to others, hence contributed to the highest activity. Nevertheless, the establishment of the structure-activity relationship for flavonoids is quite difficult due to the wide range of chemical structures of these flavonoids. Some authors suggested that not only the presence of a methoxyl group but also the presence sugar moiety may play an important role in AChE inhibitory activity, while other studies show other flavonoids with substituents in other positions with potent AChE inhibitory activity [22].

Piper species are listed together with a variety of plants that have been reported to have AChE inhibitory activity and might be relevant to the treatment of neurodegenerative disorders. At low concentrations, extracts from the stems of *P. interuptum* and seeds of *P. nigrum* exhibited 50-65% inhibitory activities on AChE [23]. The *P. sarmentosum* methanol extract was also reported to be a potential agent for the development of anti-AD [24]. AChE, is a substrate-specific enzyme, which degrades the neurotransmitter, acetylcholine, in nerve synapses. According to the cholinergic hypothesis, cholinesterase inhibitors enhance the signal transmission in nerve synapses by prolonging the effect of acetylcholine. As the meaningful results of our study, these isolated flavonoids could manifest inhibitory activity in AChE [25].

CONCLUSION

In the present study, the phytochemical investigation from the aerial parts of *P. abbreviatum* furnished five methoxylated flavones together with four triterpenoids. This study is the first report of the occurrence of methoxylated flavones from this species. Thus, high variants of flavonoid compounds from this species may be used as chemotaxonomic markers for this *Piper* species. Additionally, the isolation and identification of AChE inhibitors of these compounds may be of interest to clarify the physiological role of this enzyme.

ACKNOWLEDGMENTS

The authors would like to thank the Fundamental University Research Grant (GPUF) for financial support under vote 2019-0225-103-01 and the Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris for research facilities.

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