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Isolation and Structural Analysis: Chromene and Flavonoid from *Orthosiphon aristatus* Aerial Part

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ABSTRACT

Three compounds were successfully isolated from the acetone extract of *Orthosiphon aristatus* leaves, methylripariochromene A **(1)**, a chromene compound; ageratoriparin **(2)**, bichromene; and 5-hydroxy-3,7,4'-trimethoxy-flavone **(3)**, a flavonoid compound. NMR spectroscopy, including both 1D and 2D methods, was used to determine the structures of the isolated compounds. In this study, it is reported for the first time that ageratoriparin **(2)** and 5-hydroxy-3,7,4'-trimethoxy-flavone **(3)** have been obtained from *Orthosiphon aristatus*. Among them, only compound **1** was assessed against caspase-3. The result revealed that compound **1** showed no activity against caspase-3.

Keywords: flavonoid, chromen, caspase-3, *Orthosiphon aristatus*.

INTRODUCTION

Orthosiphon aristatus, is a plant that commonly used in traditional medicine. It is part of the Orthosiphon genus and is classified in the Lamiaceae family. This plant is found in Southern China, South Asia, Southeast Asia, and Australia [1]. Several Orthosiphon species are used for medicinal purposes, containing chemical compounds with various pharmacological effects. In Asian, including Indonesia, Orthosiphon aristatus is used to treat a range of conditions such as fever, gout, hypertension, and diabetes. It is also used to prevent inflammation, influenza, hepatitis, cough, and arthritis, as well as to alleviate respiratory problems [2,3]. Research has shown that this plant possesses bioactive properties, including anti-inflammatory, antioxidant, and antimicrobial activities [2]. *Orthosiphon aristatus* contains several bioactive compounds, including terpenoids, polyphenols, and sterols [4]. Among these, polyphenols are the most abundant constituents in O. aristatus [5]. The polyphenols are categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins [6,7].

Neurodegenerative diseases involve progressive neuronal and nervous system damage, which is essential for bodily coordination. People suffering from these diseases typically experience speech and movement disorders along with memory and cognitive decline. Apoptosis, a programmed cell death process, is associated with both acute and chronic neurodegenerative conditions, such as Alzheimer's, Huntington's, and Parkinson's disease [8]. Caspases, a group of cysteine protease enzymes, play a role in regulating apoptosis and inflammation. Additional molecules that can inhibit caspase activity may be used to control caspase activity in neurodegenerative diseases. Caspase activity can be suppressed using peptide analogs that attach to the active site of caspases. However, peptide inhibitors have demonstrated suboptimal therapeutic effects [9]. Currently, researchers are actively seeking bioactive compounds that can inhibit caspases. There is growing interest in studies that isolate secondary metabolites from various plants, with this research focusing on *Orthosiphon aristatus*.

EXPERIMENTAL SECTION

Material and Instrumental

^{1}H and ^{13}C NMR spectra were measured using residual and deuterated solvents as reference standards with Agilent DD2 spectrometer operating at 500 MHz and 125 MHz, respectively (Agilent Technologies, Santa Clara, CA, USA). Vacuum liquid chromatography (VLC) and centrifugal planar chromatography (CPC (Chromatotron, T-Squared Technology, Inc, San Bruno, CA, USA) were conducted on Merck silica gel 60 GF254 art. 7731 and 7749, respectively. Thin layer chromatography (TLC) analysis was performed using precoated silica gel plates (Merck Kielselgel 60 GF254, 0.25 mm thickness). Solvents for extraction and chromatography were of technical grade and were distilled before use. The $CHCl_3$ used in the purification was pro analysis grade.

The Caspase Inhibitor Drug Detection Kit was purchased from Abcam (catalog number: ab102495) and stored at − 80 °C as one-time use aliquots. This kit includes reaction buffer, caspase substrate DEVD-AFC (1 mM), DTT (1 M), active caspase-3, and caspase inhibitor Z-VAD-FMK (2 mM). The luminescence in the enzyme assay was measured using a GloMax Explorer reader.

Methods

Plant material

The aerial part of *Orthosiphon aristatus* were collected in November 2021 from Solo, Central Java Indonesia.

Extraction and isolation

Dried and powdered leaves of *O. aristatus* (2.2 kg) were macerated with acetone to obtain an acetone extract (66 g). A portion of the extract (23 g) was fractionated using a vacuum liquid chromatography (VLC) method and eluted with a solvent system of *n*-hexane-EtOAc (8:2, 7:3, 6:4, 1:1, 3:7, and 2:8) to afford six major fractions A–F. Fraction B (4 g) was refractionated using the same method (*n*-hexane-diisopropyl ether 8:2) yielding ten subfractions B1–B10. Fraction B10 (175 mg) was subjected to radial chromatography (*n*–hexane:diisopropyl ether 9:1) to afford compound **1** (3 mg). Fraction D (2,93 g) was subjected to VLC to give twelve fractions D1-D12 (*n*–hexane-acetone 8:2). Fraction D11 (1.6 g) was refractionated using the same method (n-hexane-diisopropyl ether 8:2) yielding ten subfractions D111–D1110. Fraction D1110 contained compound **2** (10 mg). Fraction F (300 mg) was purified by radial chromatography (*n*–hexane-acetone 8:2) to obtain compound **3** (8 mg).

Caspase-3 Inhibition Assay

The assay for inhibitory activity against caspase-7 was adapted from Abcam [10]. All the compounds and the positive control (Z-VAD-FMK) were dissolved in 10% DMSO. Active caspase-7, buffer solution, and substrate (DEVD-AFC) were added to the mixture. The mixture was then incubated at 37ºC for 0.5-1 hour. Subsequently, the fluorescence emitted was quantified using a Glomax Exproler plate reader during the enzyme assay with a 400 nm excitation filter and a 505 nm emission filter. The inhibition activity measurement involved a comparison of the fluorescence intensity of the caspase activity positive control, which allowed for the determination of the efficiency of the tested inhibitors in inhibiting the activity.

RESULTS AND DISCUSSION

The ^{1}H–NMR spectrum of compound **(1)** (Table 1) revealed that it shared structural features with those reported for methylripariochromene A [11]. It exhibited signals characteristic of chromene moieties. Compound **1** displayed two characteristic singlet signals of methoxy groups at δ_H 3.88 and 3.97 ppm (each *s*). In the aromatic proton region, signals indicative of cis coupling was observed at δ_H 5.60 ppm (*d*, J = 9.9 Hz) and δ_H 6.30 ppm (*d*, J = 9.8 Hz). Additionally, a singlet signal was observed at δ_H 7.23 ppm (*s*), methyl at 1.49 (6H, *s*), and methyl ketone at δH 2.59 ppm (*s*). Further evidence for structure 1 was provided by HSQC and HMBC experiments. The HMBC (Table 1) exhibited correlations between δ_H 7.23 (1H, *s*) (H-5) and carbon signals at

121.7 (C-4), 198.1 (C=O), and 151.0 ppm (C-8a), suggesting the presence of an acetyl group at C-6. The remaining HMBC connectivity (Table 1) supported the structure assigned to **(1)**. Methylripariochromene A **(1)** has been isolated from various plant sources, including *Orthosiphon aristatus* [12], *Eupatorium* riparium [13], *Eupatorium glandulosum* [14] and *Stevia serrata* [11]. Several studies report on the pharmacological effects of methylripariochromene A, particularly antihypertensive, vasodilation, and diuretic action [12], and antifungal [13,15].

The ^{1}H-NMR spectrum of compound **(2)** (Table 2) revealed structural similarities to ageratoriparin, a compound previously identified in *Ageratina riparia* [16]. The spectrum exhibited signals characteristic of chromene moieties, akin to compound 1. Compound **(2)** displayed two distinct singlet signals for methoxy groups at δ_H 3.86 (*s*) and 3.90 ppm (*s*). In the aromatic region, cis coupling was evident with signals at δ_H 5.54 (*d*, 9.9) and δ_H 5.84 (*d*, 10.0). Moreover, singlet signals for methyl groups were observed at 1.40 (6H, s) (C-9;9') and 1.42 (6H, s) (C-10;10'). HSQC and HMBC experiments provided additional structural confirmation. Notably, the HMBC spectrum (Table 2) revealed correlations between δ_H 5.84 (*s*) (H-4) and carbon signals at 74.9 (C-2), 117.4 (C-4a;4a'), and importantly, the quaternary carbon at 113.5 (C-5). Unlike compound **(1)**, compound **2** has no presence of acetyl group. The remaining HMBC (Table 2) corroborated the proposed structure of **2**. This research presents the novel discovery of ageratoriparin in the Lamiaceae family, specifically extracted from *Orthosiphon aristatus*.

The ^{13}C-NMR spectrum (Table 3), displayed eighteen carbon signals, including fifteen related to flavonoid carbons and three corresponding to methoxy groups. Signals at δ_C 139.1 (C-3) and 178.9 (C-4) confirm the phenolic framework of flavonol derivatives. A methoxy carbon signal at 60.4 ppm (attached to C-3) was also observed, supporting the structure of 3-methoxyflavone derivatives. The ^{1}H-NMR spectrum showed a proton signal at 12.92 ppm, typical for a chelated hydroxyl proton. *Meta*-coupled signals at 6.69 ppm (H-6) and 6.85 ppm (H-8) were assigned to ring A, while para-substituted signals appeared at 8.02 ppm and 7.11 ppm (ring B). Combined ^{1}H-NMR, ^{13}C-NMR, HSQC, and HMBC data confirmed the structure as 5-hydroxy-3,7,4'-trimethoxyflavone, previously isolated from plants in the Lamiaceae family, such as *Vitex garderiana* and *Ballota inaequidens*[17,18] and has known antimicrobial activity [17]. This is the first report of its isolation from *Orthosiphon aristatus*.

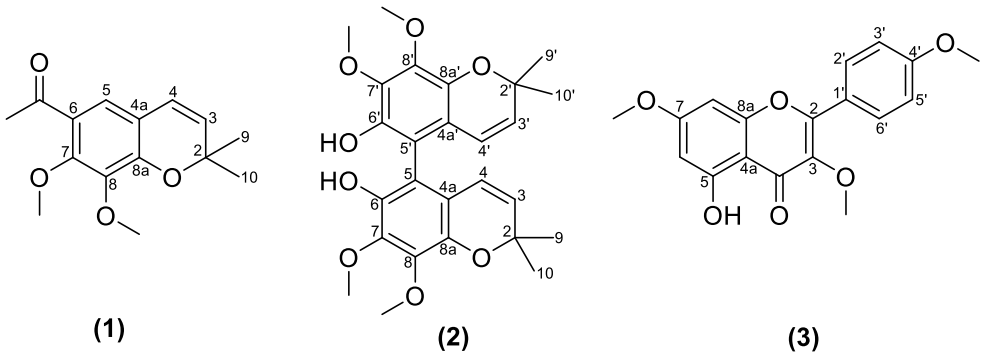

Figure 1. Structure of methylripariochromene A **(1)**, ageratoriparin **(2)**, and 5-hydroxy-3,7,4'-trimethoxy-flavone **(3)**

Table 1. Data ^{1}H and ^{13}C NMR methylripariochromene A **(1)** in $CDCl_3$.

No. C	δ_C (*mult*., *J* in Hz)	δ_C (ppm)	HMBC ($^{1}H \Leftrightarrow {}^{13}C$)
2	-	77.8	-
3	5.60 (1H, *d*, 9.9)	129.9	C-2 C-4a C-9;10
4	6.30 (2H, *d*, 9.8)	121.7	C-4a C-8a C-2
4a	-	117.9	-
5	7.23 (1H, s)	122.4	C-4 C-8a C=O
6	-	124.9	-
7	-	154.7	-
8	-	141.4	-
8a	-	151.0	-
7-OCH_3	3.88 (3H, *s*)	61.6	C-7
8-OCH_3	3.97 (3H, *s*)	61.0	C-8
9,10-CH_3	1.49 (6H, *s*)	28.4	C-2
-CO$\underline{CH_3}$	2.59 (3H, *s*)	21.3	-
-C=O	-	198.1	-

Table 2. Data ^{1}H and ^{13}C NMR ageratoriparin **(2)** in aceton-d_6.

No. C	δ_C (*mult*., *J* in Hz)	δ_C (ppm)	HMBC ($^{1}H \Leftrightarrow {}^{13}C$)
2; 2’	-	74.9	-
3; 3’	5.54 (2H *d*, 9.9)	129.6	C-2;2’ C-4a;4a’ C-9;9’ C-10;10’
4; 4’	5.84 (2H, *d*, 10.0)	120.8	C-2;2’ C-4a;4a’ C-5;5’
4a; 4a’	-	117.4	-
5; 5’	-	113.5	-
6; 6’	-	138.9	-
7; 7’	-	140.8	-
8; 8’	-	141.4	-
8a; 8a’	-	142.0	-
7; 7’-OCH_3	3.86 (6H, *s*)	60.2	C-7,7'
8; 8’-OCH_3	3.90 (6H, *s*)	60.6	C-8,8'
9; 9’	1.40 (6H, *s*)	26.5	C-2;2’ C-3;3’ C-10;10'
10;10’	1.42 (6H, *s*)	26.7	C-2;2’ C-3;3’ C-9;9'

Table 3. Data ^{1}H and ^{13}C NMR 5-hydroxy-3,7,4'-trimethoxy-flavone **(3)** in aceton-d_6.

No. C	δ_C (*mult*., *J* in Hz)	δ_C (ppm)	HMBC ($^{1}H \Leftrightarrow {}^{13}C$)
2	-	153.9	-
3	-	139.1	-
4	-	178.9	-
4a	-	106.5	-
5	-	154.1	-
6	6.69 (1H, *d*, J = 1.8)	104.3	C-4a C-8
7	-	132.9	-
8	6.85 (1H, *d*, J = 1.8)	91.9	C-6 C-4a C-7
8a	-	165.0	-
1’	-	124.7	-
2’; 6’	8.02 (2H, *brd*, J = 8.5)	129.1	C-2 C-4’
3’; 5’	7.11 (2H, *brd*, J = 8.5)	115.4	C-1’ C-4’
4’	-	163.8	-
3-OCH_3	3.98 (3H, *s*)	60.4	C-3
7-OCH_3	3.80 (3H, *s*)	56.8	C-7
4'-OCH_3	3.91 (3H, *s*)	60.5	C-4’
5-OH	12.92 (1H, *s*)	-	-

The activity of isolated compounds was tested using Abcam's Caspase Inhibitor Drug Detection Kit and measured with fluorometry. The caspase-3 substrate, DEVD–AFC, is initially non-fluorescent due to a peptide bond. Active caspase-3 cleaves this bond, releasing AFC, which fluoresces due to its amine group (N–H). If a compound inhibits caspase-3, no fluorescence is observed. Compound activity was compared to a positive control (Z–VAD–FMK, 0% activity) and a blank control (no inhibitor, 100% activity). The isolated compound tested for activity against caspase-3 was compound **(1)**. Methylripariochromene A **(1)** showed 100% caspase-3 activity, indicating that it did not inhibit caspase-3 activity.

CONCLUSION

Three compounds were isolated from *Orthosiphon aristatus* leaves, methylripariochromene A **(1)**, ageratoriparin **(2)**, and 5-hydroxy-3,7,4'-trimethoxyflavone **(3)**. The isolation used vacuum liquid chromatography (VLC) and radial chromatography, with structures identified through 1D and 2D NMR. Bioactivity tests showed that methylripariochromene A **(1)** did not inhibit caspase-3, unlike the standard inhibitor (Z-VAD-FMK), which fully inhibited it. This study is the first to report the isolation of compounds **(2)** and **(3)** from *Orthosiphon aristatus*, providing valuable insights into its secondary metabolites.

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REFERENCES

1) Febjislami, S; Kurniawati, A. N. I.; Melati, M.; Wahyu, Y. Morphological characters, flowering and seed germination of the Indonesian medicinal plant Orthosiphon aristatus. *Biodiversitas Journal of Biological Diversity*. **2019,** *20*, 328-337. DOI: https://doi.org/10.13057/biodiv/d200204.
2) Adnyana, I.K.; Setiawan, F.; Insanu, M.,Ethnopharmacology to clinical study of *Orthosiphon stamineus* benth. *Int. J. Pharm. Pharm. Sci*. **2013,** *5*, 66–73. DOI: https://doi.org/10.13040/IJPSR.0975-8232.5(10).4273-84.
3) Ameer, O.Z.; Salman, I.M.; Asmawi, M.Z.; Ibraheem, Z.O.; Yam, M.F. *Orthosiphon stamineus*: Traditional uses, phytochemistry, pharmacology, and toxicology. *J. Med. Food.* **2012**, *15*, 678–690. DOI: https://doi.org/10.1089/jmf.2011.1973.
4) Tezuka, Y.; Stampoulis, P.; Banskota, A.H.; Awale, S.; Tran, K.Q.; Saiki, I.; Kadota, S. Constituents of the Vietnamese Medicinal Plant *Orthosiphon stamineus*. *Chem. Pharm. Bull*. **2000**, *48*, 1711-1719. DOI: https://doi.org/10.1248/cpb.48.1711.
5) Hollman, P.H.; Katan, M.B. Dietary Flavonoids: Intake, Health Effects and Bioavailability. *Food Chem. Toxicol*. **1999**, *37*, 937–942. DOI: https://doi.org/10.1016/S0278-6915(99)00079-4.
6) Shahidi, F.; Yeo, J. Bioactivities of Phenolics by Focusing on Suppression of Chronic Diseases: A Review. *Int. J. Mol. Sci*. **2018**, *19*, 1573. DOI: https://doi.org/10.3390/ijms19061573.
7) Li, A.N.; Li, S.; Zhang, Y.J.; Xu, X.R.; Chen, Y.M.; Li, H.B. Resources and Biological Activities of Natural Polyphenols. *Nutrients*. **2014**, *6*, 6020–6047. DOI: https://doi.org/10.3390/nu6126020.
8) Friedlander, R. M. Apoptosis and Caspases in Neurodegenerative Diseases. *N. Engl. J. Med*. **2003**, *348*, 1365–1375. DOI: https://doi.org/10.1056/NEJMra022366.
9) White, J.B.; Beckford, J.; Yadegarynia, S.; Ngo, N.; Lialiutska, T.; d'Alarcao, M. Some natural flavonoids are competitive inhibitors of caspase–1, –3, and –7 despite their cellular toxicity. *Food Chem*. **2012,** 131, 1453–1459. DOI: https://doi.org/10.1016/j.foodchem.2011.10.026.
10) Abcam. 2023. *Caspase-7 Inhibitor Assay Kit (ab102495)*. Abcam. Retrieved August 17 from https://www.abcam.com/products/assay-kits/caspase-7-inhibitor-assay-kit-ab102495.html.
11) Kohda, H.; Yamazaki, K.; Tanaka, O.; Nishi, K. Methylripariochromene a from *Stevia serrata*. *Phytochemistry*, **1976**, *15*, 846–847.https://doi.org/10.1016/s0031-9422(00)94482-0.
12) Matsubara,T.; Bohgaki,T.; Watarai, M.; Suzuki, H.; Ohashi, K.; Shibuya, H. Antihypertensive actions of methylripariochromene methylripariochromene A from *Orthosiphon aristatus*, an Indonesian traditional medicinal plant. *Biol Pharm Bull*. **1999**, *22*, 1083-1088. DOI: https://doi.org/10.1248/bpb.22.1083.
13) Taylor, D. R.; Wright, J. A. Chromenes from Eupatorium riparium. Phytochemistry. 1971, 10, 1665-7 DOI: https://doi.org/10.1016/0031-9422(71)85045-8.
14) Thorleif, A.; New Chromenes from Eupatorium Species. *Acta Chem. Scand*. **1969**, 23, 3605-3607 DOI:10.3891/acta.chem.scand.23-3605
15) Bandara,B. M. R; Chandralal, C. M.; Karunaratne, V.; Wannanigama, G. P; Adikaraman, N.K.B. An antifungal chromene from Eupatorium riparium. *Phytochemistry*. **1992**, *31*, 1983-1985. DOI: https://doi.org/10.1016/0031-9422(92)80345-F
16) Banerjee, S.; Jakupovic, J.; Bohlmann, F.; King, R.M.; Robinson, H. Chromenes from *ageratina riparia*. *Phytochemistry*. **1985,** *24*, 2681-2683. DOI: https://doi.org/10.1016/S0031-9422(00)80693-7.
17) Çitoğlu, G.S.; Sever, B.; Antus, S.; Baitz-Gács, E.; Altanlar, N. Antifungal diterpenoids and flavonoids from Ballota inaequidens. *Pharm. Biol*. **2005**, *42*, 659–663. DOI: https://doi.org/10.1080/13880200490902626
18) Macedo, I.; da Silva, J.H.; da Silva, P.T.; Cruz, B.G.; do Vale, J.P.; Dos Santos, H.S.; Bandeira, P.N.; de Souza, E.B.; Xavier, M.R.; Coutinho, H.D; Braz-Filho, R. Structural and Microbiological Characterization of 5 Hydroxy–3,7,4–Trimethoxyflavone: A Flavonoid Isolated from *Vitex gardneriana* Schauer Leaves. Microb. *Drug Resist.* **2019**, *25*, 434–438. DOI: https://doi.org/10.1089/mdr.2018.0359.