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CHARACTERIZATION OF TEAGHRELIN-LIKE COMPOUNDS FROM TEA CULTIVARS IN THAILAND AND *IN SILICO* STUDY OF THEIR BIOACTIVITY

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Abstract – In the present research, four tea cultivars in Thailand were screening to search for the teaghrelin-like compounds and totally six components were identified. Among these, one new constituent isolated form Assam tea varieties was assigned as quercetin 3-*O*-[2-*O*-(*E*)-*p*-coumaroyl][*α*-L-rhamnopyranosyl(1→6)]-*β*-D-glucoside 4'-*α*-L-rhamnoside (**1**) through the comprehensive 1D- and 2D-NMR and mass spectrometric analysis. The isolated compounds were examined for their ghrelin receptor binding affinity *in silico* and antioxidant bioactivity by free radical scavenging model. However, no significant bioactivity was observed according to the experimental results.

Tea (*Camellia sinensis*) is considered as the major drink consumption by most Asian people distributed in India, China, Myanmar, Laos, Vietnam, and Thailand.1 In Thailand, tea has been cultivated mainly in the

northern area such as Chiang Rai, Chiang Mai, and Nan provinces, and is usually employed for green, oolong, and black tea production.1 Various chemical compounds in teas such as catechins, alkaloids, polyphenols, flavonoids, amino acids, polysaccharides, and volatile components had been reported.1 Among these reports, polyphenols and their derivatives are evaluated as the major compounds to possess antioxidant, anti-inflammatory, antibacterial, anti-allergic activities,[2] and reducing the risk of cancer,[3] Parkinson's disease,[4,5] and Alzheimer's disease.6 In addition, flavonoids in tea have also been reported to provide similar health benefits to those observed by polyphenols.[7] For example, teaghrelins, unique acylated flavonoid tetraglycosides, along with their derivatives, have been detected in oolong tea and are responsible for the hunger induction as the endogenous hunger hormone.[8,9] They have been demonstrated to induce hunger sensation and stimulate growth hormone secretion of primary anterior pituitary cells in rats.9 Moreover, several teaghrelin-like compounds, acylated flavonoid tetraglycosides with different attachments of glycosides, have been reported in various oolong tea cultivars[10-12] and Assam teas[13] that also provided significant biological activity.[14,15] Therefore, teaghrelins and teaghrelin-like compounds were evidenced to displayed promising potential as oral drug candidates with medicinal effects similar to ghrelin. Although teaghrelins and teaghrelin-like compounds have been reported in several teas, there were fewer literature studies of these compounds and their biological activities of tea cultivars grown in Thailand. Thus, the aim of this study was to characterize the teaghrelin-like compounds of different tea cultivars in Thailand and examine their antioxidant bioactivity. Moreover, molecular docking was used to evaluate the receptor binding affinities of ghrelin and purified compounds. Hopefully, these results may improve the economic values of tea industry and tea consumption population on the basis of additional scientific data regarding its teaghrelin analogues and biological activities.

The HPLC chromatograms of the extracts of four tea cultivars, including Ju Hua Xiang (No. 311), Assam in Chiang Rai (No. 317), Nan (No. 318), and Chiang Mai (No. 319) were obtained and shown in Figure 1. There were various polyphenols and alkaloids were characterized in tea cultivars, comprising 5-galloylquinic acid (5-GA), gallic acid (GA), theobromine (TB), crypto-chlorogenic acid (crypto-CGA), epicatechin (EC), epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), and caffeine (CAF). The identification of those compounds was furnished by comparison of their relative retention times with the standards and previously published reports, and the results were labeled on the chromatogram (Figure 1A). In addition, based on our previous reports,[13] teaghrelin-like compounds were separated in the regions of retention time at 100-120 min. As shown in Figure 1B, there were two observed peaks (**1** and **2**) at the low polarity region of HPLC chromatograms of the samples No. 317, 318, and 319. Among these three samples, No. 318 (Assam in Nan) exhibited the highest contents of **1** and **2** and hence, this sample was further applied for isolation of compounds **1** and **2**. It is the first report of **1** from natural sources, and the

structural elucidation was described in the next paragraph. Compound **2** was identified as kaempferol 3-*O*-[2-*O*-(*E*)-*p*-coumaroyl][*α*-L-rhamnopyranosyl(1→6)]-*β*-D-glucoside 4'-*α*-L-rhamnoside (Figure 2) by comparison of their physical and spectroscopic data with those previously reported.[16] In addition, the HPLC signals of Ju Hua Xiang tea (No. 311) were totally different from those of other three Assam teas, and ingredients **3-5** (Figure 2) were identified, among which **3** and **4** were already reported from Chin-shin oolong tea,9 and **5** was characterized from Shy-jih-chuen tea.[11] Moreover, compound **6** was purified from Ju Hua Xiang tea and its structure was known[17] (Figure 2).

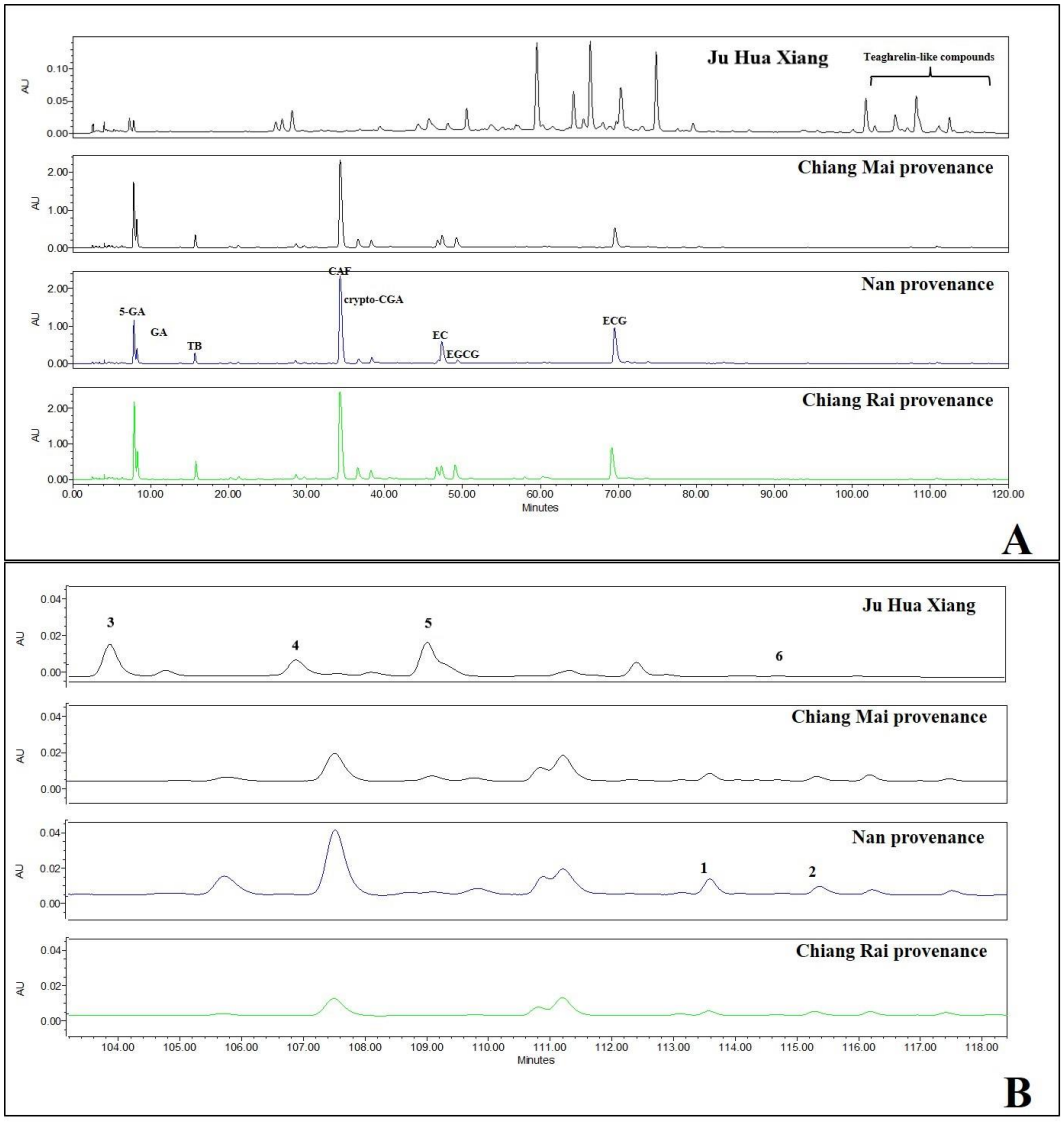
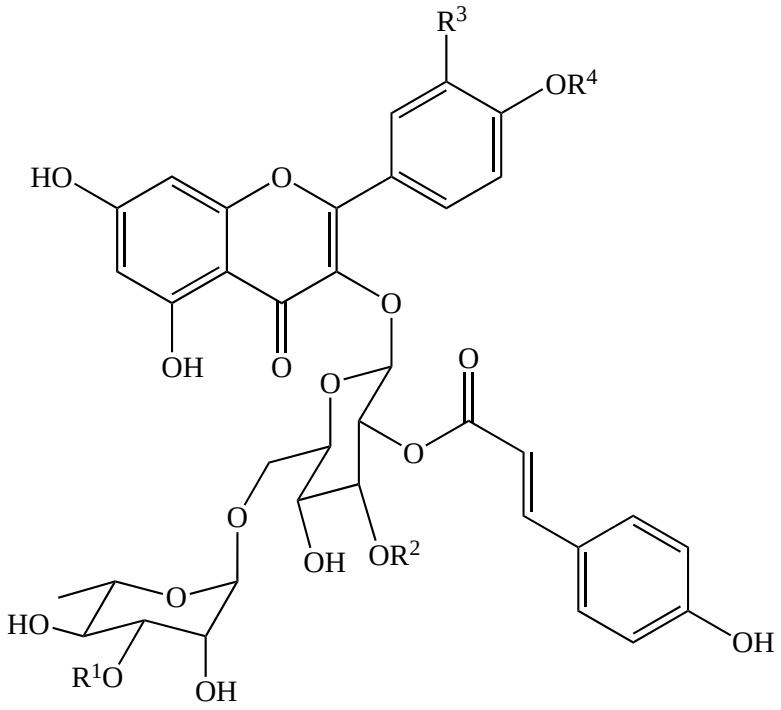


Figure 1. HPLC chromatograms of tea infusions obtained from different samples. (A) Total chromatograms at 280 nm. (B) Expanded chromatograms of Teaghrelin-like compounds.



	R^1	R^2	R^3	R^4
1	H	H	OH	Rha
2	H	H	H	Rha
3	Glc	Glc	OH	H
4	Glc	Glc	H	H
5	Glc	Ara	OH	H
6	H	Glc	H	H

Figure 2. Chemical structures of **1-6**

Compound **1** was isolated as optically active yellow solid with negative optical rotation ($[\alpha]_D^{25}$ –128) and mp 185-186 °C. The HR-ESI-MS analytical data demonstrated the molecular formula of **1** as $C_{42}H_{46}O_{22}$ according to a sodium adduct ion signal (*m/z* 925.2378) (Figure S1). A flavone skeleton was determined by the UV absorption maxima at 314 and 268 nm.[18] In its IR spectrum, the significant absorption bands at 3379 and 1602 cm^{-1} indicated the presences of hydroxy and carbonyl functional groups, respectively. In ^{1}H-NMR spectrum of **1** (Figure S2), there were a set of ABX-coupled proton signals at δ 7.65 (1H, d, *J* = 2.0 Hz, H-2'), 7.25 (1H, d, *J* = 9.0 Hz, H-5'), and 7.61 (1H, dd, *J* = 9.0, 2.0 Hz, H-6') presented the trisubstituted B-ring. The two *meta*-coupled protons at δ 6.17 (1H, d, *J* = 2.0 Hz, H-6) and 6.34 (1H, d, *J* = 2.0 Hz, H-8) constructed the A-ring of flavone, and these supported the basic skeleton of **1** as quercetin. In addition, a *trans-p*-coumaric acid moiety was determined by the proton signals at δ 6.38 (1H, d, *J* = 16.0 Hz, H-α), 7.69 (1H, d, *J* = 16.0 Hz, H-β), 7.46 (2H, d, *J* = 8.5 Hz, H-2", -6"), and 6.81 (2H, d, *J* = 8.5 Hz, H-3", -5"). Combination of the ^{13}C-NMR and DEPT analytical data (Figure S3), D-glucose, L-rhamnose, and L-rhamnose were identified. The configurations of these sugar units were assigned as β, α, and α according to the corresponding *J* values of anomeric protons at δ 5.52 (1H, d, *J* = 8.0 Hz), 4.55 (1H, d, *J* =

1.5 Hz), and 5.56 (1H, d, J = 1.5 Hz), respectively.[19] There are significant ^{2}J- and ^{3}J-correlations observed in its HMBC spectrum (Figure S4), from Glc H-1 to C-3, from Glc H-2 to carbonyl group of *p*-coumaroyl, from Rha H-1 to Glc C-6, and from Rha' H-1 to C-4' established the linkage of sugar units, and the complete chemical structure of **1** was determined by full HMBC and NOESY analysis (Figure 3). In our previous report, two new teaghrelin-like compounds were characterized from another Assam tea varieties.[13] Comparison of their NMR spectral characteristics, **1** displayed high similarity to those compounds, with the significant difference that **1** lose an arabinose unit. Conclusively, **1** was assigned as quercetin 3-*O*-[2-*O*-(*E*)-*p*-coumaroyl][*α*-L-rhamnopyranosyl(1→6)]-*β*-D-glucoside 4'-*α*-L-rhamnoside and its ^{1}H- and ^{13}C-NMR spectroscopic data were listed in Table 1.

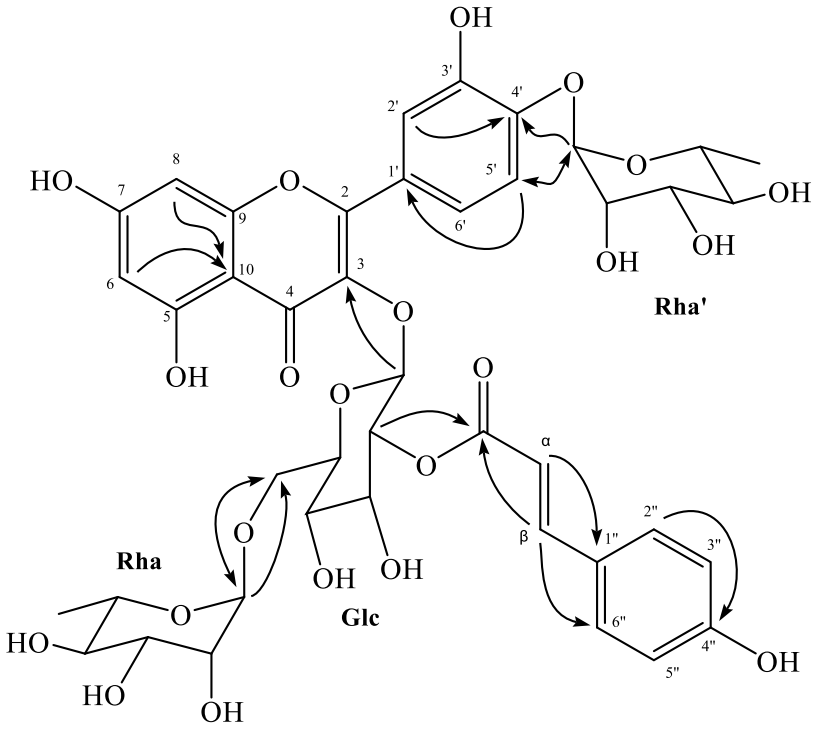


Figure 3. Significant HMBC (→) and NOESY (↔) correlations of **1**

The isolated teaghrelin-like compounds **1**, **2**, and **6** were performed molecular docking to evaluate their binding affinity with ghrelin receptor (Figure S8). As expected, the extra glycoside substitution on the flavonoid backbone resulted in lower docking scores compared with a positive control GHRP-6 (Table 2). This prediction has been published by our research team.[10] Anti-inflammation examination experiments demonstrated the low inhibition for this type of compounds.[13] In addition, antioxidant potential of **1**, **2**, and **6** was also examined by free radical scavenging assay, however, no notable significant bioactivity was observed (data not shown).

Table 1. ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR spectroscopic data of **1** in MeOH-d_4

Position	1	
	δ_H	δ_C
2	-	158.1
3	-	135.5
4	-	179.1
5	-	163.1
6	6.17 (1H, d, *J* = 2.0 Hz)	100.0
7	-	166.3
8	6.34 (1H, d, *J* = 2.0 Hz)	94.9
9	-	158.5
10	-	105.9
1'	-	126.2
2'	7.65 (1H, d, *J* = 2.0 Hz)	118.2
3'	-	148.1
4'	-	148.0
5'	7.25 (1H, d, *J* = 9.0 Hz)	117.3
6'	7.61 (1H, dd, *J* = 9.0, 2.0 Hz)	122.9
p-coumaroyl		
1"	-	127.3
2", 6"	7.46 (2H, d, *J* = 8.5 Hz)	131.2
3", 5"	6.81 (2H, d, *J* = 8.5 Hz)	116.8
4"	-	161.3
α	6.38 (1H, d, *J* = 16.0 Hz)	115.2
β	7.69 (1H, d, *J* = 16.0 Hz)	147.0
C=O	-	168.6
Glc		
1	5.52 (1H, d, *J* = 8.0 Hz)	101.0
2	5.04 (1H, dd, *J* = 10.0, 8.0 Hz)	75.7
3	3.61 (1H, dd, *J* = 10.0, 9.0 Hz)	76.3
4	3.36 (1H, dd, *J* = 9.0, 9.0 Hz)	71.8
5	3.43 (1H, m)	77.4
6	3.44 (1H, m) 3.89 (1H, dd, *J* = 9.5, 5.0 Hz)	68.4
Rha		
1	4.55 (1H, d, *J* = 1.5 Hz)	102.3
2	3.66 (1H, dd, *J* = 3.5, 1.5 Hz)	72.1
3	3.55 (1H, dd, *J* = 9.5, 3.5 Hz)	72.3
4	3.28 (1H, dd, *J* = 9.5, 9.5 Hz)	73.9
5	3.48 (1H, m)	69.8
6	1.13 (3H, d, *J* = 6.5 Hz)	17.9
Rha'		
1	5.56 (1H, d, *J* = 1.5 Hz)	100.7
2	4.16 (1H, dd, *J* = 3.5, 1.5 Hz)	71.9
3	4.00 (1H, dd, *J* = 9.5, 3.5 Hz)	72.1
4	3.50 (1H, m)	73.9
5	3.77 (1H, m)	71.0
6	1.28 (3H, d, *J* = 6.5 Hz)	18.1

Table 2. *In silico* computing binding energies of compounds **1**, **2**, **6**, and GHRP-6

Compound	Affinity (kcal/mol)
1	−7.9
2	−7.6
6	−8.9
GHRP-6	−10.3

EXPERIMENTAL

General. The melting points was recorded on an WRX-4 melting-point apparatus without correction. Optical rotations were recorded on a JASCO P-2000 digital polarimeter. The UV spectra were obtained by a Hitachi U-2001 UV/Vis spectrometer. The IR spectra were examined with a JASCO FT/IR-4100 spectrophotometer. ^{1}H-, ^{13}C-, and 2D NMR spectra were recorded on the Bruker AV-500 spectrometers. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal standard. The ESI-MS data were obtained on Shimadzu LC-8040 systems, and HR-ESI-MS were taken on a JEOL JMS-700 spectrometer (operated in the positive-ion mode). Column chromatography (CC) was performed on Sephadex LH-20 (Sigma-Aldrich) and LiChroprep® RP-18 gel (Sigma-Aldrich). High performance liquid chromatographic (HPLC) analysis of samples was conducted on Water high performance liquid chromatography Model G10460F 421M comprising the vacuum degasser, quaternary pump, auto-sampler, thermostatted column compartment and photo diode array detector. The column used was a Syncronis C_{18} column (250 × 4.6 mm, 5 μm).

Tea samples. Fresh tea leaves of four samples, including Ju Hua Xiang tea cultivated at Boon Rawd tea plantation in Chiang Rai province with age 15 years, and three Assam teas cultivated in Chiang Rai, Nan, and Chiang Mai provinces, were collected in October 2014. All the tea samples were authenticated by Mr. Thawipich Aryanan, an agricultural expert of Tea and Coffee Institute of Mae Fah Luang University, and voucher specimens (No. 311, 317, 318, and 319 for Ju Hua Xiang, Chiang Rai, Nan, and Chiang Mai provenance) were deposited in the Mae Fah Luang Botanical Garden, Mae Fah Luang University, Chiang Rai, Thailand. Harvested plant materials were indoor withered at room temperature and turned 2-3 times prior roasting by pan-firing machine for 5 min at 250-300 °C. They were further rolled by rolling machine for 5 min. The rolled tea leaves were finally dried using a hot-air oven at 70 °C for 10 h. The obtained tea samples were individually milled using a miller to produce a tea powder. All samples were further stored in plastic bags at room temperature until extraction.

Chemicals. All chemicals were purchased from E. Merck Co. (Merck KGaA, Darmstadt, Germany). High-performance liquid chromatography (HPLC)-grade acetonitrile (MeCN) was bought from Fisher Scientific (Fair Lawn, NJ). Methanol (MeOH) was purchased from Aencore Chemical PTY, LTD (Surrey Hills,

Australia). Deuterated solvent methanol-d_4 was purchased from Sigma-Aldrich Co. (St. Louis, MO). Purified water was afforded by a Millipore clear water purification system (Direct-Q, Millipore, Billerica, MA).

Liquid chromatography analysis of polyphenols, alkaloids, and teaghrelin-like compounds. Two grams of each tea sample was sonicated with 25 mL of distilled water at 70 °C for 30 min. The tea solutions were then centrifuged at 8,000 rpm for 5 min. All solutions were filtered through a 0.45 μm syringe polytetrafluoroethylene (PTFE) filter (Millipore Ltd., Bedford, USA) before analysis by high performance liquid chromatographic (HPLC). A gradient solution of water and acetonitrile was used as mobile phase for this experiment by starting with a 5% MeCN at 0 min, then increased to 25% MeCN until 100 min, 30% MeCN until 110 min, and decreased to 5% MeCN until 120 min. The flow rate was 1.0 mL/min and injection volume was 20 μL at ambient temperature. The absorption wavelength was monitored by the UV absorbances at 280 and 320 nm for analysis of polyphenols, alkaloids, and teaghrelin-like compounds.

Isolation of teaghrelin-like compounds. Two tea samples (each 100 g), including Assam tea cultivated in Nan province (No. 318) and Ju Hua Xiang tea (No. 311) were sonicated with water (1 L x 3) at 70 °C for 1 h. Each afforded tea infusion was subjected to a Sephadex LH-20 column and eluted with a gradient of water and MeOH (0, 10, 20, 30, 40, 50, 60, 70, 80, and 100%) to achieve ten fractions. The teaghrelin-like compounds retained in the sixth subfraction (50%) was further purified by reversed-phase HPLC system with a Syncronis C_{18} column (250 × 4.6 mm, 5 μm). The mobile phase consisted of MeCN and water using a gradient elution of 5% MeCN at 0 min, 20% MeCN at 0-10 min, 30% MeCN at 10-50 min, 5% MeCN at 50-60 min. The flow rate was 1.0 mL/min and injection volume was 20 μL at ambient temperature. Compounds **1** (7.2 mg) and **2** (4.3 mg) were purified from the subfraction of Assam tea (No. 318). Compounds **3-5** were identified in Ju Hua Xiang tea (No. 311) by comparison of their UV absorbance and retention times with those of standards purified in our group. **6** (4.1 mg) was isolated from Ju Hua Xiang tea (No. 311) and characterized by the comparison of their physical and spectral data reported in the literature.

Quercetin 3-*O*-[2-*O*-(*E*)-*p*-coumaroyl][*α*-L-rhamnopyranosyl(1→6)]-*β*-D-glucoside 4'-*α*-L-rhamnoside (1): Yellow solids; mp: 185-186 °C (MeOH); $[\alpha]_D^{25}$ –128 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε): 314 (4.22), 268 (4.15) nm; IR (neat) ν_{max} 3379, 1648, 1602, 1508, 1363, 1254, 1165, 1070 cm^{-1}; ^{1}H- and ^{13}C-NMR, see Table 1; ESI-MS (*rel. int. %*) *m/z* 925 ($[M+Na]^+$, 67), 903 ($[M+H]^+$, 100); HR-ESI-MS *m/z* 925.2379 $[M+Na]^+$ (calcd for $C_{42}H_{46}NaO_{22}$, 925.2378).

Molecular dockings. *In silico* calculation was based on AutoDock Vina program (v.1.1.2).[20] The crystal structure of ghrelin receptor with a resolution of 3.30 Å has been identified,[21] and the .PDB file was retrieved from the Research Collaboratory for Structural Bioinformatics-Protein Data Bank (PDB ID: 6KO5). The Chem3D program was used for constructing the three-dimension structure of ligands.

Hydrogen supplement, Gasteiger charge measurement, and flexible torsions were completed by AutoDockTools (ADT v.1.5.6). The grid box with size (18.5 Å × 18.5 Å × 18.5 Å) was set center at 9.7, –19.2, 14.6 (x, y, z). Binding affinity energy represents docking score with unit of kcal/mol. The top-scoring configuration was considered the most suitable interaction between protein and ligand. The visualization of stable complex was executed by Biovia Discovery Studio client 2020 (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

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SUPPORTING INFORMATION

Supplementary (synthesis of the starting azides, HPLC chromatograms, IR, 1H and 13C NMR, MS spectra, etc.) data associated with this article can be found, in the online version, at URL: https://www.heterocycles.jp/newlibrary/downloads/PDFsi/27596/104/6

REFERENCES

1. T. Theppakorn, A. Luthfiyyah, and K. Ploysri, *J. Microbiol. Biotechnol. Food Sci.*, 2014, **3**, 364.
2. C. Xu, L. Liang, T. Yang, L. Feng, X. Mao, and Y. Wang, *LWT Food Sci. Technol.*, 2021, **152**, 112234.
3. E. J. O'Neill, D. Termini, A. Albano, and E. Tsiani, *Molecules*, 2021, **26**, 987.
4. M. Barooah and D. J. Hazarika, 'Roles of functional foods in neuroprotection, in antioxidants and functional foods for neurodegenerative disorders,' CRC Press, Boca Raton, pp. 365–374, 2020.
5. C. F. Jhuo, S. K. Hsieh, C. J. Chen, W. Y. Chen, and J. T. C. Tzen, *Nutrients*, 2020, **12**, 3665.
6. C. A. Polito, Z. Y. Cai, Y. L. Shi, X. M. Li, R. Yang, M. Shi, Q. S. Li, S. C. Ma, L. P. Xiang, K. R. Wang, J. H. Ye, J. L. Lu, X. Q. Zheng, and Y. R. Liang, *Nutrients*, 2018, **10**, 655.
7. Y. Wang, X. Cheng, T. Yang, Y. Su, S. Lin, S. Zhang, and Z. Zhang, *J. Agric. Food Chem.*, 2021, **69**, 10002.
8. V. S. Lee, C. R. Chen, Y. W. Liao, J. T. C. Tzen, and C. I. Chang, *Chem. Pharm. Bull.*, 2008, **56**, 851.
9. Y. H. Lo, Y. J. Chen, C. I. Chang, Y. W. Lin, C. Y. Chen, M. R. Lee, V. S. Lee, and J. T. C. Tzen, *J. Agric. Food Chem.*, 2014, **62**, 5085.
10. S. K. Hsieh, Y. H. Lo, C. C. Wu, T. Y. Chung, and J. T. C. Tzen, *J. Food Drug Anal.*, 2015, **23**, 660.
11. Y. C. Li, C. J. Wu, Y. C. Lin, R. H. Wu, W. Y. Chen, P. C. Kuo, and J. T. C. Tzen, *J. Food Biochem.*,

2019, **43**, e12810.

12. P. C. Kuo, Y. C. Li, R. H. Wu, and J. T. C. Tzen, *Nat. Prod. Res.*, 2021, **35**, 57.
13. Y. C. Li, C. Tanapichatsakul, P. Pripdeevech, T. L. Hwang, P. C. Kuo, and J. T. C. Tzen, *Nat. Prod. Res.*, 2022, **36**, 305.
14. M. K. Tao, M. Xu, H. Zhang, H. Chen, C. Liu, H. T. Zhu, D. Wang, C. R. Yang, and Y. J. Zhang, *Nat. Prod. Res.*, 2016, **30**, 776.
15. Y. Z. Tian, X. Liu, W. Liu, W. Y. Wang, Y. H. Long, L. Zhang, Y. Xu, G. H. Bao, X. C. Wan, and T. J. Ling, *Nat. Prod. Res.*, 2016, **30**, 2637.
16. S. Yang, W. Liu, S. Lu, Y. Z. Tian, W. Y. Wang, T. J. Ling, and R. T. Liu, *ACS Chem. Neurosci.*, 2016, **7**, 505.
17. L. Shi and D. Yu, China Patent: CN103951723A, 2014.
18. T. J. Mabry, K. R. Markham, and M. B. Thomas, 'The Ultraviolet spectra of flavones and flavonols,' In 'The systematic identification of flavonoids,' Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 41–164, 1970.
19. M. M. Manir, J. K. Kim, B. G. Lee, and S. S. Moon, *Bioorg. Med. Chem.*, 2012, **20**, 2376.
20. O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455.
21. Y. Shiimura, S. Horita, A. Hamamoto, H. Asada, K. Hirata, M. Tanaka, K. Mori, T. Uemura, T. Kobayashi, S. Iwata, and M. Kojima, *Nat. Commun.*, 2020, **11**, 4160.