ฤทธิ์การยับยั้งเอนไซม์ฮิสโทนดีอะเซทิลเลสของไบฟลาโวน อะกาทิสฟลาโวนจากดอกรักใหญ่

Histone Deacetylase Inhibitory Activity of a Biflavone, Agathisflavone from *Gluta Usitata* Flowers

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บทคัดย่อ

จากการสกัดและแยกสารจากดอกรักใหญ่ พบว่าได้อะกาทิสฟลาโวน (**1**) ซึ่งเป็นสารกลุ่มไบฟลาโวนเป็นสารหลัก โดยใช้ข้อมูลทางสเปกโทรสโกปี IR, 1D และ 2D NMR ในการพิสูจน์โครงสร้างของสาร ผลการทดสอบฤทธิ์การต้านเอนไซม์ฮีสโทนดีอะเซทิลเลสและการยับยั้งอนุมูลอิสระ พบว่ามีฤทธิ์ต้านเอนไซม์ฮีสโทนดีอะเซทิลเลสที่ดีโดยมีค่าการยับยั้งเท่ากับ 67 μM แต่มีฤทธิ์การยับยั้งอนุมูลอิสระที่อ่อนโดยมีค่าการยับยั้งเท่ากับ 182 μM การทดลองโมเลคิวลาร์ด็อกกิ้งของอะกาทิสฟลาโวนกับเอนไซม์ฮีสโทนดีอะเซทิลเลสกลุ่ม 1 (HDAC2 และ HDAC8) และกลุ่ม 2 (HDAC4 และ HDAC7) พบว่าอะกาทิสฟลาโวนแสดงคุณสมบัติเป็นสารยับยั้งเอนไซม์ฮีสโทนดีอะเซทิลเลสที่มีความจำเพาะเจาะจง ผลการทดลองโมเลคิวลาร์ด็อกกิ้งสอดคล้องกับผลในหลอดทดลอง โดยอะกาทิสฟลาโวนมีความจำเพาะเจาะจงกับเอนไซม์ฮีสโทนดีอะเซทิลเลส 7 (HDAC7) ที่สูง และนี่เป็นรายงานการพบอะกาทิสฟลาโวนครั้งแรกในดอกรักใหญ่

ABSTRACT

A biflavone, agathisflavone (**1**) was extracted and purified as a major product from the flower of *Gluta usitata*. The structure was determined by IR, 1D and 2D NMR spectroscopic data. The histone deacetylase inhibitory activity and antioxidant activity of the compound were evaluated. Agathisflavone (**1**) acted as good HDAC inhibitor with IC_{50} values of 67 μM but weak antioxidant activity with IC_{50} values of 182 μM. Molecular docking experiments of the compound with representatives of class I (HDAC2 and HDAC8) and class II (HDAC4 and HDAC7) HDAC isoforms displayed potential isoform-selective HDAC inhibitor. Molecular docking data showed consistent results to the *in vitro* experiments with the high selectivity towards HDAC7. The occurrence of this compound in *G. usitata* is reported here for the first time.

คำสำคัญ: ฮีสโทนดีอะเซทิลเลส รักใหญ่ สารต้านมะเร็ง สารต้านอนุมูลอิสระ โมเลคิวลาร์ด๊อกกิ้ง
Keywords: Histone deacetylase, *Gluta usitata*, Anticancer, Antioxidant, Molecular docking

INTRODUCTION

Reversible chemical modification of DNA and histones are important cellular events that promote activation or repression of DNA transcription (Konstantinopoulos et al., 2007; Weichert, 2009). Histone deacetylases (HDACs) have been discovered as a class of enzymes which regulate the removal of acetyl group from a lysine residue of histone. HDACs lead to a transcriptionally inactive and condensed form of chromatin. On the contrary, histone acetyl transferases (HATs) transfer an acetyl group to the histone, are associated to active chromatin (Bolden et al., 2006; Roth, et al., 2001). Overexpression of HDACs has been reported in many disorders including cancers. Therefore, HDAC inhibitors have been validated as a promising anti-cancer treatment (Rasheed et al., 2007; Dokmanovic et al., 2007). In total, HDACs are grouped into four classes based on sequence similarity. HDAC classes I (HDAC1, 2, 3 and 8), IIa (HDAC4, 5, 7 and 9), IIb (HDAC6 and 10), and IV (HDAC11) employ Zn^{2+} as an essential cofactor while HDAC Class III, also known as the Sirtuin class, need NAD^{+} to exert activity (Micelli and Rastelli, 2015). The different isoforms are mainly found in diverse diseases or cancer cells. For example, Class I, HDAC1, HDAC2 and HDAC3 are founded in ovarian and lung cancers (Bartling et al., 2005; Khabele et al., 2007), whereas HDAC2 is detected in neurodegenerative disease (Guan et al., 2009), including ovarian and gastric cancers (Song et al., 2005). Moreover, class IIa, HDAC4 is found in colon cancer (Wilson et al., 2008), ovarian cancer (Shen et al., 2016), and gastric cancer (Kang et al., 2014). While, HDAC7 plays an important role in cardiovascular development and disease (Chang et al., 2006).

Plants of the cashew family (Anacardiaceae) are a family of flowering plants; comprising about 83 genera and 860 species distributed throughout the tropical forests of Southeast Asia, especially in Burma and Thailand (Christenhusz and Byng, 2016). *Gluta usitata* (Wall.) Ding Hou (synonym *Melanorrhoea usitata*), locally known as Rakyai or Black lacquer tree, is a medium to large tree with dark green leaves and straight clean cylindrical trunks (Smitinand, 2014). Phytochemical studies on this plant have led to the identification of various structures of urushiol, laccol, thitsiol, resorsinol and phenol derivatives (Du et al., 1986; Lu et al., 2006; Lu et al., 2007). In addition, we have reported the bioactive compounds from the twigs of this plant. Many compounds acted as good HDAC inhibitors such as depsides, chalcone, flavonoids including biflavonoid (Kumboonma et al., 2018).

In the present work, the isolation, antioxidant activity as well as histone deacetylase (HDAC) inhibitory activity of the isolated compound is discussed. Furthermore, the molecular docking studies of the compound with HDAC2, HDAC8, HDAC4 and HDAC7 isoforms are described for the putative discovery of isoform-selective inhibitors.

RESEARCH METHODOLOGY

1. General experimental procedures

The compounds 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) was purchased from Sigma-Aldrich (USA). Ascorbic acid and gallic acid were purchased from Qrec (New Zealand). Column chromatography (CC) was carried out on a silica gel P60 (Silicycle, Canada). TLC and precoated TLC were performed on silica gel 60 F_{254} (Merk, Germany). NMR spectra were recorded in the indicated solvents ($CDCl_3$ and CD_3OD) on a Varian

Mercury Plus spectrometer operated at 400 MHz (^{1}H) or 100 MHz (^{13}C). The fluorescence was measured using spectra Max Gemini XPS microplate spectrofluorometer. UV spectra were obtained using a model Agilent 8453 UV-Vis Spectrometer.

2. Plant material

The flowers of *Gluta usitata* were collected in November 2014, at Ubolratana-phitthayakhom School, Khon Kaen and were identified by Dr. Boonchuang Boonsuk. A voucher specimen (P. Kumboonma 01) was deposited at the Herbarium of Khon Kaen University (KKU), Khon Kaen, Thailand.

3. Preparation

Upon arrival at the laboratory, the flowers were cleaned with running water, dried in an oven at 40 °C for 48 hours, and then cut into small sizes. The sample was ground with grinder.

4. Extraction

The flower powder (1.8 kg) was extracted with methanol 5L for 15 days at room temperature three times. The mixture was filtrated through the Whatman-1 filters, and then evaporated to dryness under vacuum (35-40 °C) to give a dark brown gum (65.5 g).

5. Isolation

The methanol extract (65.5 g) was subjected to a normal phase column using a gradient of dichloromethane-methanol (10:0 to 5:5), yielding five fractions (M_1-M_5) based on TLC profiles. Fraction M_2 (5.5 g) was chromatographed on silica gel column, eluting with an isocratic of dichloromethane-methanol (9:1) to yield agathisflavone **1** (105 mg).

6. DPPH free radical-scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical assay (Parejo et al. 2002), was used to determine the free radical activity of the biflavone. Briefly, a 1 mL of various concentrations (120, 140, 160, 180 and 200 µM) of agathisflavone (in methanol) was added to 3 mL of a solution of 0.1 mM DPPH solution (in methanol). The mixture was shaken vigorously and left to stand for 30 min in the dark condition. Absorbance at 517 nm was determined and the percent inhibition was calculated using Eq (1) (A_c = absorbance without the sample, A_s = absorbance with the sample).

$$\text{Inhibition (\%)} = [(A_c - A_s)/A_c] \times 100 \quad (1)$$

IC_{50}, the amount of agathisflavone decreasing by 50% the initial DPPH concentration, was derived from the % inhibition vs concentration plot. All experiments were carried out in triplicate.

7. Histone deacetylase activity assay

Agathisflavone was evaluated for their ability to inhibit HDAC enzymes. Inhibition of HDAC activity *in vitro* was assessed using the Fluor-de-Lys HDAC activity assay kit (Biomol, Enzo Life Sciences International, Inc., USA). HeLa nuclear extract provided with the kit was used as a source of HDAC enzymes for *in vitro* study. A 5 µL of various concentrations of agathisflavone (10, 20, 60, 80, 100 and 120 µM), 1 µL of the Hela nuclear extract and 19 µL of buffer were added into the 96-well plate and incubated at 37 °C for 5 minutes. After that, 25 µL of the substrate was added and incubated again at 37 °C for 15 minutes and then 50 µL of the developer was added to generate a fluorophore. Finally, the fluorophore was measured fluorescence signal with excitation at 360 nm and emission at 460 nm. A decrease in fluorescence signal indicated an inhibition of HDAC activity. All experiments were carried out in triplicate.

8. Molecular docking studies

The crystal structures of HDAC2, HDAC4, HDAC7 and HDAC8 [PDB entry code: 3MAX, 2VQW, 3C0Z and 1T64, respectively] were obtained from the Protein Data Bank. All water and non-interacting ions as well as ligands were removed. Then all missing hydrogens and sidechain atoms were added using the ADT program.

Gasteiger charges were calculated for the system. For ligand setup, the molecular modeling program Hyperchem 8.0 was used to build the ligand. Energy of the molecule was minimized with the AM1 level. Molecular docking studies were performed for 50 runs using AutoDockTools 1.5.4 (ADT) and AutoDock 4.2 programs. Docking software AutoDock 4.2 Program supplied with AutoGrid 4.0 was used to produce grid maps (60x60x60). The spacing between grid points was 0.375 angstroms. The Lamarckian Genetic Algorithm (LGA) was chosen to search for the best conformers.

RESULTS

1. Isolation and structure elucidation

The methanol extract of *G. usitata* flowers was purified separately by column chromatography (CC) using normal phase silica gel to obtain agathisflavone (**1**) as a major product: Yellow powder; IR (KBr) υ_{max} (cm^{-1}): 3424, 1647, 1409, 1245, 836; ^{1}H-NMR (400 Hz, CD_3OD): δ (ppm) = 7.86 (d, *J*=8.4 Hz, 2H, H-2'), 7.51 (d, *J*=8.4 Hz, 2H, H-2''), 6.93 (d, *J*=8.4 Hz, 2H, H-3''), 6.74 (d, *J*=8.4 Hz, 2H, H-3'), 6.69 (s, 1H, H-8''), 6.62 (s, 1H, H-3''), 6.54 (s, 1H, H-3), 6.39 (s, 1H, H-6). ^{13}C-NMR (100 Hz, CD_3OD): δ (ppm) = 182.9 (C-4), 182.6 (C-4''), 164.9 (C-2''), 164.8 (C-2), 162.9 (C-7), 162.6 (C-5), 161.3 (C-4'''), 161.2 (C-4'), 161.0 (C-7''), 159.9 (C-5''), 157.7 (C-9''), 155.5 (C-9), 128.1 (C-2'''), 127.9 (C-2'), 122.1 (C-1'), 121.9 (C-1'''), 115.7 (C-3'''), 115.6 (C-3'), 104.3 (C-10), 104.0 (C-10''), 103.4 (C-6''), 102.6 (C-3''), 102.2 (C-3), 98.9 (C-8), 98.7 (C-6), 93.4 (C-8''). The data were consistent with previous report (Meselhy, 2003). Agathisflavone is reported from *G. usitata* for the first time.

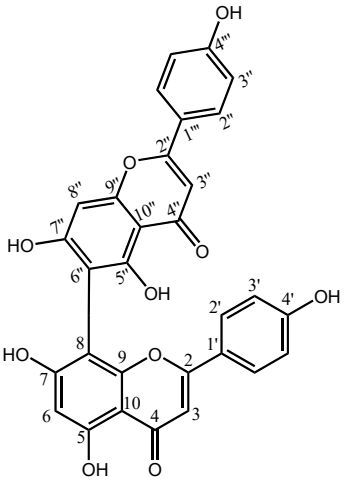

Figure 1. Structure of agathisflavone (**1**).

2. DPPH scavenging activity

The DPPH radical is a free radical compound which has been widely used to test free radical scavenging ability. Antioxidants, on interaction with the DPPH radical, transfer either an electron or hydrogen atom to DPPH, thus neutralizing its free-radical character. The reagent color changes from purple to yellow and its absorbance at wavelength 517 nm decreases. Agathisflavone (**1**) showed weak DPPH radical-scavenging activity with IC_{50} value of 182 µM.

3. Histone deacetylase inhibitory activity

Histone deacetylase inhibitory activitiy of the agathisflavone (**1**) was evaluated as IC_{50} value with various concentrations (10, 20, 60, 80, 100 and 120 µM) by a fluorimetric assay (Fluor de LyseTM). The results are showed in Figure 2. Agathisflavone (**1**) displayed good HDAC inhibitory activity with IC_{50} value of 67 µM.

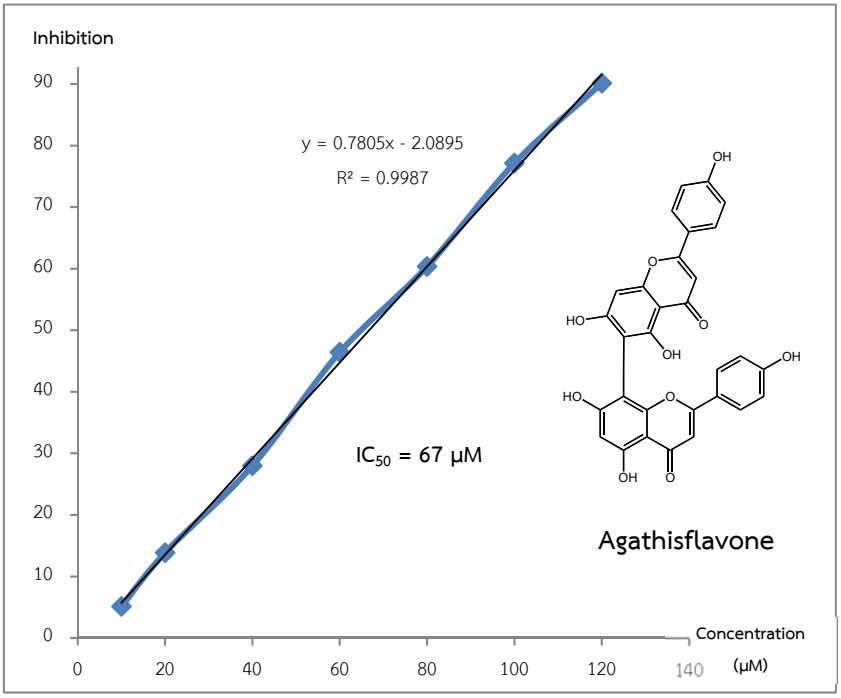

Figure 2. HDAC inhibitory activity of agathisflavone (**1**).

Table 1. *In silico* histone deacetylase inhibitory activity.

Compound	Class I HDACs				Class II HDACs			
	HDAC2		HDAC8		HDAC4		HDAC7	
	ΔG^*	K_i^{**}	ΔG^*	K_i^{**}	ΔG^*	K_i^{**}	ΔG^*	K_i^{**}
TSA	-8.67	0.44	-8.85	0.33	-8.39	0.70	-7.97	1.44
SAHA	-9.38	0.13	-7.74	1.78	-7.51	3.14	-6.70	12.36
1	-5.85	50.65	-6.95	8.01	-6.25	26.32	-8.26	0.90

* kcal/mol; ** µM

4. Molecular docking study

To explore a possibility of being HDAC isoform selective inhibitor, agathisflavone (**1**) was docked into the catalytic pocket of representative isoforms of class I (HDAC2 and HDAC8) and class II (HDAC4 and HDAC7). Trichostatin A (TSA), which is the most potent HDAC inhibitor with IC_{50} value of 12 nM (Yosida et al., 1990) and SAHA, were used as positive controls for molecular docking experiment.

The free energy of binding (ΔG) and the calculated inhibition constant (K_i) for each enzyme-inhibitor complex are demonstrated in Table 1. The interaction mode of agathisflavone (**1**) in the active sites of HDAC2, HDAC4, HDAC7 and HDAC8 are shown in Figure 3.

Agathisflavone (**1**) showed moderate HDAC inhibitions against HDAC2 (ΔG = -5.85, K_i = 50.65 µM) and HDAC4 (ΔG = -6.25, K_i = 26.32 µM). Interestingly, agathisflavone (**1**) showed good HDAC inhibitors against HDAC7 (ΔG = -8.26, K_i = 0.90 µM) and HDAC8 (ΔG = -6.95, K_i = 8.01 µM). TSA was selective towards HDAC8 (ΔG = -8.85, K_i = 0.33 µM). Whereas, SAHA showed selectively to HDAC2 (ΔG = -9.38, K_i = 0.13 µM).

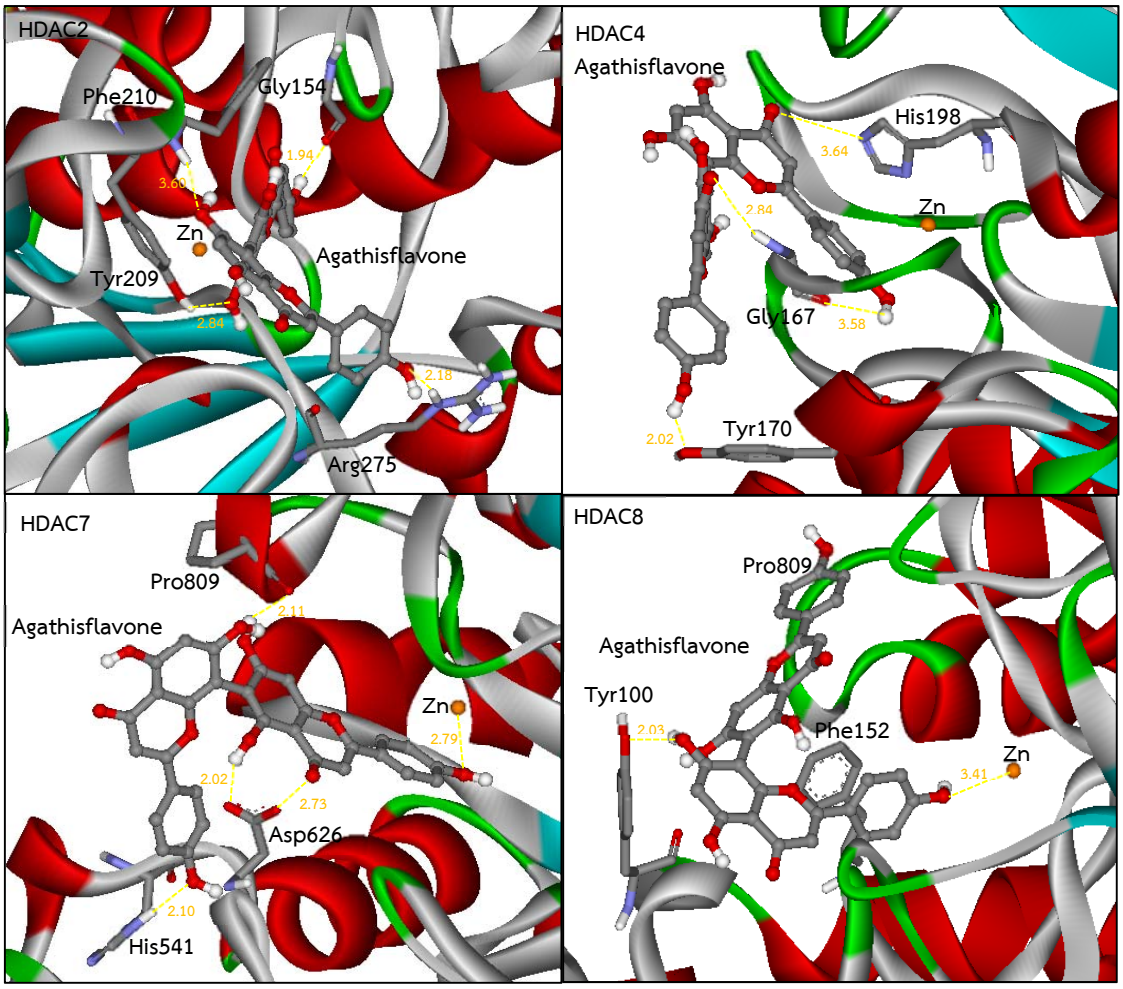

Figure 3. The interaction mode of agathisflavone (**1**) in the active site of HDAC2, HDAC4, HDAC7 and HDAC8.

DISCUSSION

Molecular docking studies were conducted to gain more details on the binding mode and the selectivity of agathisflavone (**1**) with HDAC2, HDAC8, HDAC4 and HDAC7. Visual inspection of the binding mode for **1** at HDAC2 binding site revealed that there are four important interactions considered as hydrogen bonds. The hydrogen bond between the hydroxyl group of Tyr209 and the phenol group was calculated as 2.84 Å. Other hydrogen bonds are observed between the hydroxyl groups of **1** and Phe210, Gly154 and Arg275 with a distance of 3.60 Å, 1.94 Å and 2.18 Å, respectively.

The agathisflavone-HDAC8 complex presented a dentate zinc chelation via the phenolic group and a hydrogen bond interaction with Tyr100 (2.03 Å). Visual inspection of the position of **1** in the HDAC4 binding shows that Gly167, Tyr170 and His198 are residues near the phenolic and the carbonyl regions of **1**. Four important interactions are considered to be hydrogen bonds. One hydrogen bond occurs between the backbone imidazole group of His198 and the oxygen atom of the carbonyl group that belongs to **1** (3.64 Å). Two hydrogen bonds are observed from coordinating between carboxylic group of Gly167 and the phenolic group of **1** (3.58 Å), amino group of gly167 and the carbonyl group of **1** (2.84 Å). The other hydrogen bond is between the phenolic group of **1** and the phenolic group of Tyr170 (2.02 Å).

Surprisingly, agathisflavone (**1**) acted as good HDAC inhibitor and selective to HDAC7 more than TSA

and SAHA. The major interaction between HDAC7 and **1** are the coordination to zinc ion and four hydrogen bonds. One hydrogen bond between the carboxylic group of Pro809 and the phenolic group of **1** was calculated as 2.11 Å. Two hydrogen bonds are observed from coordinating between carboxylic group of Asp626 with the carbonyl group (2.73 Å) and the phenolic group (2.02 Å) of **1**. Finally, a hydrogen bond is observed between the phenolic group of **1** and the imidazole moiety of His541 (2.10 Å). Analysis of the molecular docking results of **1** also revealed that the phenolic groups and the carbonyl group played a critical role for zinc ion binding and hydrogen bonding.

HDAC7 play a significant role in breast cancer stem cells (CSCs) and necessary to maintain CSCs (Ouaissi et al., 2008). High HDAC7 protein level was observed in 9 out of 11 human pancreatic cancers (Ouaissi et al., 2008). Moreover, HDAC7 promotes lung tumorigenesis by inhibiting Stat3 activation (Lei et al., 2017). Therefore, this compound could serve as promising lead for safe and selective anticancer agent with HDAC inhibitory activity.

CONCLUSION

The isolation of chemical constituent from the flowers of Rakyai or *G. usitata*, is reported for the first time. Agathisflavone was isolated. The compound showed high HDAC inhibitory activity but weak antioxidant activity. Although the HDAC inhibitory activity was not comparable to that of TSA, agathisflavone was already proved to be a potential and safe anticancer agent. Molecular docking studies provided important data regarding the potential HDAC isoform-selectivity. These results also confirmed the significance of phenolic compound as a putative HDAC inhibitor.

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