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Original Article

Further Results about Chemical Constituents of *Heterostemma grandiflorum* Cost.

Nguyen Thi Thuy Linh[1,2], Khieu Thi Tam[3], Pham Duc Thang[4], Ba Thi Cham[1,2], Nguyen Thanh Tam[2], Nguyen Thi Hoang Anh[1,2,*]

[1]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*
[2]*Institute of Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*
[3]*University of Sciences, Thai Nguyen University, Tan Thinh, Thai Nguyen, Vietnam*
[4]*Danang University of Medical Technology and Pharmacy, Vietnam*

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Abstract: *Heterostemma grandiflorum* Cost. is a flowering plant of Asclepiadaceae family, distributed in Hoa Binh, Hanoi, Ha Nam,... provinces. We have reported first results about chemical constituents and cytotoxicity of *H. grandiflorum* collected in Me Linh, Hanoi in the previous paper. Further study on dichloromethane and butanol extracts of *H. grandiflorum*'s twigs and leaves, 8 compounds have been isolated. Their structures were elucidated by the 1D and 2D NMR spectroscopy and comparison with published data. They include two flavonoids: apigenin and thalassiolin C; a phenolic: vanillic acid; two triterpenes: 3β-stearyloxy-urs-12-ene, and 3β-acetoxy-22,23,24,25,26,27-hexanordammaran-20-one; and three nitrogen-containing compounds: heteromin A, uracil, and uridine. With exceptions to apigenin and heteromin A, the rest six compounds were found for the first time from *Heterostemma* genus.

Keywords: Heterostemma grandiflorum, flavonoid, triterpene, nitrogen-containing compounds.

1. Introduction

Heterostemma genus belongs to Asclepiadaceae family, consisting of 7 species in Vietnam: *H. grandiflorum*, *H. balansae*, *H. acuminatum*, *H. lutea*, *H. oblongifolium*, *H. suberosum*, and *H. villosum*. They are distributed in many regions such as Hoabinh, Hanoi, Dongnai, Ho Chi Minh City, etc [1]. The traditional medicine of Taiwan used *H. brownii* for cancer treatment and *H. alatum* for sputum consumption and detoxification [2, 3]. According to our literature search, *H. brownii* and *H. alatum* are two species, which were mostly studied for chemical compositions in *Heterostemma* genus [4]. Many compounds of steroids, fatty acids, flavonoids, flavonoid glycosides, adenine, uridine, puriniums and

* Corresponding author.
E-mail address: hoanganhvhh@gmail.com

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pyrimidines have been isolated from the studied species [2-4]. A series of new compounds: heteromines A - E, I and heteromines F - H have been detected. In particular, heteromines A and B have been reported to show toxic activities against esophageal, liver, lymphadenopathy and leukemia cell lines, while heteromine D has strongly inhibited HL-60 cell lines with the IC_{50} value of 4.04 nmol/mL [2, 3]. The above mentioned results prompt us to study chemical compositions and cytotoxic activities of *H. grandiflorum*, collected in Me Linh, Hanoi. The first results about chemical constituents and cytotoxic activities of this species have been published by our group in 2020 [5]. In continuation of our studies on chemical constituents of dichloromethane and *n*-butanol extracts of *H. grandiflorum*'s leaves and twigs, this paper deals with the isolation and structural elucidation of 8 compounds. Chemical structures were determined by spectroscopic analyses and comparisons with previously published data. They are apigenin (1), thalassiolin C (2), vanillic acid (3), 3*β*-stearyloxy-urs-12-ene (4), 3*β*-acetoxy-22,23,24,25,26,27-hexanordammaran-20-one (5), heteromine A (6), uracil (7) and uridine (8). With exception to apigenin and heteromine A, the rest six compounds (2-5, and 7-8) were isolated as the first presentatives of *Heterostemma* genus.

2. Experimental

2.1. Plant Materials

Twigs and leaves of *H. grandiflorum* were collected in Me Linh, Ha Noi in October 2019. A voucher specimen (VHH.ML.10.2019.1) is deposited in Institute of Chemistry, VAST, Hanoi, Vietnam. The scientific name was identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST, Hanoi, Vietnam.

2.2. General Experimental Procedures

NMR: Bruker Avance 500, Germany with TMS as internal reference (for ^{1}H) and solvent signal (for ^{13}C). HR-ESIMS: Agilent 6530 Accurate-Mass Q-TOF LC/MS system. ESI-MS: 5989B-MS. CC used silica gel 60 G, size 0,043-0,063 mm (Merck), TLC: precoated silica gel $G60F_{254}$ plates (Merck), spots were detected by spraying with vanillin 1% in conc. H_2SO_4 and heating at 110 ^{o}C.wigs and leaves of *H. grandiflorum* were collected in Me Linh, Ha Noi in October 2019. A voucher specimen (VHH.ML.10.2019.1) is deposited in Institute of Chemistry, VAST, Hanoi, Vietnam. The scientific name was identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST, Hanoi, Vietnam.

2.3. Extraction and Isolation

The dried leaves and twigs of *H. grandiflorum* (5,0 kg) were extracted with MeOH:H_2O (95:5) (four times) at room temperature. The methanol extract was concentrated under *vacuum* and then the aqueous solution was partitioned with *n*-hexane, EtOAc and *n*-BuOH, successively. After solvent evaporation in *vacuum*, *n*-hexane (34,0 g), CH_2Cl_2 (23,0 g) và *n*-BuOH (27,0 g) extracts were obtained.

The dichloromethane extract (23,0 g) was chromatographed on a silica gel column, eluting with gradient *n*-hexane:EtOAc to furnish 7 fractions (D1 - D7). The first one (2,4 g) was rechromatographed on silica gel, eluted with *n*-hexane:CH_2Cl_2 (95:5 - 80:20) to yield 12 subfractions (D1.1 - D1.12). Compound 1 (10 mg), 5 (5 mg) were obtained by purification of subfraction D1.5 (150 mg) using silica gel column, *n*-hexane:CH_2Cl_2 = 90:10. The separation of fraction D3 (2.8 g) was carried out on repeat silica gel columns with suitable eluting solvent systems to furnish 5 mg of compound 4.

The *n*-butanol extract (27,0 g) was given on a Sephadex LH-20 column with MeOH as eluate giving 6 fractions (B1 - B6). Fraction B1 (2.2 g) was reseparated on silica gel column, EtOAc:MeOH (95:5 - 70:30) and then on Sephadex LH-20, MeOH to yield compound **7** (5 mg). Fraction B3 (3,4 g) was purified on RP-18 column, MeOH:H_2O (50:50) to furnish 6 subfractions (B1.1 - B1.6). Repeat chromatography of subfraction B1.3 (350 mg)

on Sephadex LH-20 and then on silica gel columns to yield compounds 2 (5 mg) and 3 (4 mg). The separation of fraction B5 (2,9 g) was carried out at first with silica gel column, *n*-hexane:acetone (95:5 - 80:20), and then Sephadex LH-20 column, MeOH to yield compounds 6 (7 mg) and 8 (5 mg).

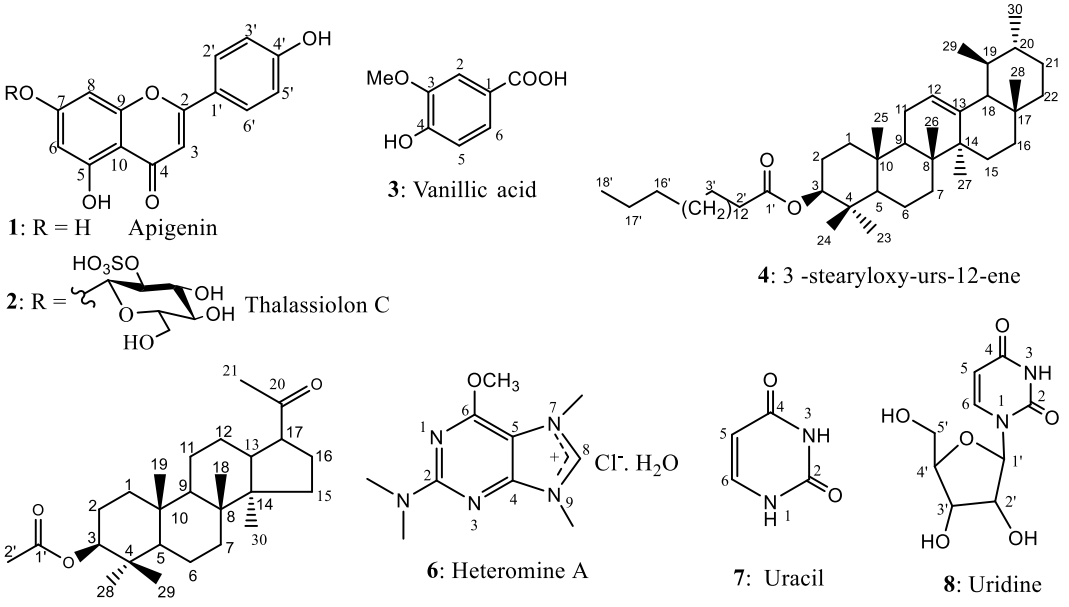

Figure 1. Chemical structures of compounds 1-8.

Apigenin (1): (+) ESI-MS: *m/z* = 271 $[M + H]^+$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.57 (s, H-3), 6.18 (d, J = 2.0 Hz, H-6), 6.41 (d, J = 2.0 Hz, H-8), 7.86 (d, J = 9.0 Hz, H-2′, 6′), 6.94 (d, J = 9.0 Hz, H-3′, 5′).

Thalassiolin C (2): HR-ESIMS: *m/z* = 513.0686 $[M + H]^+$ (calcd. for $C_{21}H_{21}O_{13}S$ 513.0702). ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 6.868 s (H-3), 6.41 d, 2.5 (H-6), 6.81 d 2.5 (H-8), 7.97 d, 9.0 (H-2′, 6′), 6.94 (d 9.0, 1H) (H-3′, 5′), 5.28 d, 8.0 (H-1″).

Vanilic acid (3): (-) ESI-MS: *m/z* = 167 $[M - H]^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H 7.57 d, 2.0 (H-2), 6.76 d, 8.0 (H-5), 7.48 dd, 2.0 & 8.0 (H-6). ^{13}C NMR (125 MHz, CD_3OD): δ_C 129.5 (C-1), 114.1 (C-2), 148.1 (C-3), 150.3 (C-4), 115.2 (C-5), 124.3 (C-6), 173.5 (C-7), 55.7 (3-OCH_3).

3β-stearyloxy-urs-12-ene (4): (+) ESI-MS: *m/z* = 693 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 4.51 (dd, 6.5, 9.0, H-3), 5.13 (t, 3.5, H-12), 1.30 (m, H-18), 1.32 (m, H-20), 0.87 (s, H_3-23), 1.01 (s, H_3-24), 0.98 (s, H_3-25), 0.88 (s, H_3-26), 1.07 (s, H_3-27), 0.80 (s, H_3-28), 0.80 (d, 4.0, H_3-29), 0.89 (d, 4.0, H_3-30), 2.30 (t, 7.5, 14.5, H_2-2’), 0.87 (t, 7.0, H_3-18’).

3β-acetoxy-22,23,24,25,26,27 hexanor-dammaran-20-one (5): (+) ESI-MS: *m/z* = 403 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 4.48 (dd, 5.0, 11.0, H-3), 2.60 (td, 6.0, 11.0, H-17), 0.98 (s, H_3-18), 0.85 (s, H_3-19), 2.13 (s, H_3-21), 0.85 (s, H_3-28), 0.85 (s, H_3-29), 0.87 (s, H_3-30).

Heteromine A (6): HR-ESIMS: *m/z* = 222.1357 $[M]^+$ (calcd. for $C_{10}H_{16}N_5O$, 222.1354). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 4.20 (s, OMe-6), 4.11 (s, H_3-7), 3.89 (s, H_3-9), 3.29 (s, N-CH_3)$_2$. ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 162.0 (C-2), 153.5 (C-4), 106.0 (C-5), 159.6 (C-6), 141.0 (C-8), 55.2 (6-OCH_3), 37.7 (N-(CH_3)$_2$), 36.6 (CH_3-7), 31.4 (CH_3-9).

Uracil (7): HR-ESIMS: *m/z* = 113.0349 $[M + H]^+$ (calcd. for $C_4H_5N_2O_2$ 113.0351). ^{1}H

NMR (500 MHz, CD_3OD): δ_H 5.62 (d, 7.5, H-5), 7.41 (d, 7.5, H-6). ^{13}C NMR (125 MHz, CD_3OD): δ_C 153.7 (C-2), 167.4 (C-4), 101.7 (C-5), 143.8 (C-6).

Uridine (8): ^1H-NMR (500 MHz, CD_3OD): δ_H 5.71 (d, 8.0, H-5), 8.01 (d, 8.5, H-6), 5.91 (d, 5.0, H-1′), 4.20 (t, 5.0, H-2′), 4.17 (t, 5.0, H-3′), 4.02 (m, H-4′), 3.85 (dd, 2.5, 12.0, H-5′a), 3.75 (dd, 2.5, 12.0, H-5'b). ^{13}C-NMR (125 MHz CD_3OD): 166.3 (C-2), 152.1 (C-4), 90.8 (C-5), 142.7 (C-6), 102.6 (C-1'), 75.7 (C-2'), 71.3 (C-3') 86.4 (C-4'), 62.3 (C-5').

^{13}C NMR data of compounds 2, 4 and 5 were given in the Table 1.

Table 1. ^{13}C-NMR data (125 MHz) of compounds **2** (DMSO-d_6), 4 ($CDCl_3$) and 5 ($CDCl_3$)

Position	δ_C	Position	δ_C	δ_C	Position	δ_C	δ_C
	2		4	5		4	5
2	164.3	1	38.5 CH_2	38.8 CH_2	21	31.3 CH_2	30.1 CH_3
3	102.6	2	23.7 CH_2	23.7 CH_2	22	41.6 CH_2	
4	182.0	3	80.6 CH	80.9 CH	23	28.1 CH_3	
5	161.1	4	37.8 C	37.9 C	24	16.9 CH_3	
6	99.9	5	55.3 CH	56.0 CH	25	15.7 CH_3	
7	*163.0*	6	18.3 CH_2	18.2 CH_2	26	16.8 CH_3	
8	95.1	7	32.9 CH_2	35.5 CH_2	27	23.3 CH_3	
9	157.0	8	40.1 C	40.5 C	28	28.8 CH_3	28.0 CH_3
10	105.4	9	47.7 CH	50.7 CH	29	17.5 CH_3	16.5 CH_3
1′	120.0	10	36.8 C	37.1 C	30	21.4 CH_3	15.9 CH_3
2′	127.7	11	23.4 CH_2	21.3 CH_3	1′	173.7 C	171.0 C
3′	116.0	12	124.4 CH	26.0 CH_2	2′	34.9 CH_2	21.3 CH_3
4′	163.0	13	139.7 C	45.2 CH	3′	25.2 CH_2	
5′	116.0	14	42.1 C	50.1 C	4′-15′	29.7-29.2 CH_2	
6′	127.7	15	26.7 CH_2	31.6 CH_2	16′	31.9 CH_2	
1″	97.3	16	28.1 CH_2	25.6 CH_2	17′	22.7 CH_2	
2″	78.4	17	33.8 C	54.3 CH	18′	14.1 CH_3	
3″	75.9	18	59.1 CH	15.6 CH_3			
4″	69.4	19	39.7 CH	16.3 CH_3			
5″	76.4	20	39.7 CH	222.4 C			
6″	60.4						

3. Results and Discussion

From dichloromethane and butanol extracts of the twigs and leaves of *H. grandiflorum* eight compounds were isolated by chromatographic methods. The isolates are two flavonoides (1, 2), a phenolic (3), two triterpenes (4, 5) and three nitrogen compounds (6 - 8). Compound 1 was isolated as light yellow powder. It's ^1H-NMR spectrum indicated typical signals of flavonoid skeleton, including two doublet signals at δ_H 7.86 (d, J = 9.0 Hz, H-2′, 6′), 6.94 (d, J = 9.0 Hz, H-3′, 5′) of ring B and two meta-aromatic protons at δ_H 6.18 (d, J = 2.0 Hz, H-6), 6.41 (d, J = 2.0 Hz, H-8) of ring A and a singlet

signal at δ_H 6.57 (s, H-3) of ring C. These data are total identical with those of apigenin [6]. Many studies have revealed that apigenin has cytotoxic effects on the various cancer cells, prevents the atherogenesis, hypertension, inhibits the asthma, abnormal behavior,... [7]. Compound 2 (yellow powder) was obtained in the mixture with apigenin-7-O-*β*-D-glucopyranoside (2A), which was reported as compound 3 in our previous study of this plant [5]. Its HR-ESIMS indicated the *pseudo-molecular* peaks at *m/z* = 433.1134 ($C_{21}H_{21}O_{10}$) and 513.0686 ($C_{21}H_{21}O_{13}S$). NMR spectral data of 2 were almost identical with those of apigenin-7-O-*β*-D-glucopyranoside except only difference on the downfield shift of C-2" (**(**$\Delta\delta_C$ = 5.3 ppm). This difference together with 80 mass units more of 2 compared to 2A suggested that this compound has a sulfate group connected at position C-2″ of *β*-D-glucopyranose. Finally, 2 was determined as apigenin-7-O-*β*-D-glucopyranosyl-2"-sulfate, named thalassiolin C when compared with published literature [7]. Thalassiolins A–C are first flavones having a sulfated *β*-D-glucose at C-7 position isolated from the Caribbean sea grass *Thalassia testudinum* [8]. Compound 3 was obtained as a white powder. Its NMR spectra showed signals of a tri-substituted benzene ring revealing by three aromatic protons at δ_H 7.57 d, 2.0 (H-2), 6.76 d, 8.0 (H-5), 7.48 dd, 2.0 & 8.0 (H-6). The substitutes were determined as a methoxy (δ_H 3.88 s, δ_C 55.7), a carboxyl (δ_C 173.5) and a hydroxyl (downfield shift of C-4 at δ_C 150.3) groups. These data led to conclusion that 3 is vanillic acid by comparison with reported data [9].

NMR spectroscopic data of compound 4 (white powder) indicated the typical signals of a derivative of urs-12-ene triterpene, revealed by 6 singlet methyls at δ_H 0.87, 1.01, 0.98, 0.88, 1.07, 0.80 and two doublet methyls at δ_H 0.80 (d, 4.0, H_3-29), 0.89 (d, 4.0, H_3-30); a double bond (δ_H 5.13 t, 3.5, H-12; δ_C 124.4 CH, 139.7 C), an oxy-methine: δ_H 4.51 (dd, 6.5, 9.0, H-3), δ_C 80.6 CH. Beside these signals, the presence of long chain ester group was also observed, including a triplet methyl signal at δ_H 0.87 (t, 7.0, H_3-18′), a methylene connected to carbonyl carbon at δ_H 2.30 (t, 7.0, H_2-2′) and a carbonyl ester carbon (δ_C 173.7 C). The connecting position of long chain ester was established at C-3 due to the correlations between C-1′ (173.7 ppm) and H-3 (4.51 ppm), H-2′ (2.30 ppm) in the HMBC spectrum. ESI-MS of 4 showed a *pseudo-molecular* peak at *m/z* = 693 $[M + H]^+$, corresponding to $C_{48}H_{85}O_2$. The above-mentioned data were identical with those of 3*β*-stearyloxy-urs-12-ene [9]. This compound was isolated previously from the *n*-hexane extract of *Maytenus salicifolia* Reissek (Celastraceae) leaves [10].

^1H and ^{13}C-NMR spectra of compound 5 (white powder) indicated the presence of five singlet methyl signals, eight methylenes, five methines with one is oxygenated: δ_H 4.48 dd, 5.0, 11.0; δ_C 80.9 (CH-3), four quaternary carbons, an acetoxyl: δ_H 2.04 s, δ_C 21.3 CH_3, 171.0 C and a methyl-ketone group: δ_H 2.13 s, δ_C 30.1 CH_3, 222.4 C. Comparison of the ^{13}C data with those of dammarane-type triterpenes led to conclude that compound 5 is 3*β*-acetoxy-22,23,24,25,26,27 hexanor-dammaran-20-one and the assignment of ^{13}C was given in the table 1 [11, 12]. This compound is one of dammarane-type acetylated triterpenes isolated from methanol extract of *Ficus pumila* fruit [12].

Compound 6 was separated as white crystals. Its molecular formula $C_{10}H_{16}N_5O$ was deduced from the molecular ion peak at *m/z* = 222.1357 $[M]^+$ in the HR-ESIMS spectrum. ^1H and ^{13}C NMR data exhibited signals for a dimethylamino group at δ_H 3.29 (s, 6H), δ_C 37.7; two methyl groups connected on two quaternary amines at δ_H 4.11, 3.89; δ_C 36.6, 31.4; one methoxy (δ_H 4.20, δ_C 55.2) and a typical purinium methine CH-8 at δ_H 9.25, δ_C 141.0. Furthermore, the signals of four olefinic quaternary carbons at δ_C 106.0, 153.5, 159.6, 162.0 have been seen. HMBC correlations (3.89/141.0, 153.5; 4.11/141.0, 106.0; 3.29/ 162.0) suggested the structure of 6-methoxy-7,9-dimethyl-2-(N,N dimethylamino) purinium chloride, named heteromine A for 6. This suggestion was confirmed by comparison

with those of reported data [13]. Heteromine A together with heteromines B and C were detected as new purinium natural compounds from the aerial parts of *Heterospemma brownii* Hay (Asclepiadaceae), a species used in Taiwan traditional medicine for the treatment of tumors [14]. It was proved to inhibit K562 and HL-60 cell lines [13].

Both compounds 7 and 8 were isolated as yellow powder. Their structures were elucidated as uracil and uridine by comparison with those of published spectral data [15, 16]. Both of these compounds were found in many plant species such as *Colysis hemionitedea, Phlomis samia, Phlomis carica* [4, 16].

4. Conclusion

In this paper we report the further results on chemical constituents of *H. grandiflorum*'s leaves and twigs. In detail, 8 compounds were furnished from dichloromethane and *n*-butanol extracts. Their structures were established as apigenin, thalassiolin C, vanillic acid, 3β-stearyloxy-urs-12-ene, 3β-acetoxy-22,23,24,25, 26,27-hexanordammaran-20-one, heteromine A, uracil and uridine.

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