Vietnam Journal of Science and Technology ***56*** *(4) (2018) 446-457*

DOI: 10.15625/2525-2518/56/4/9625

CHEMICAL CONSTITUENTS OF *HEDYOTIS PINIFOLIA* WALL. COLLECTED IN THUA THIEN HUE

Khieu Thi Tam[1, 3], Nguyen Thi Hoang Anh[1, 2, *], Nguyen Van Tuan[1, 4], Dao Duc Thien[2], Tran Duc Quan[2], Nguyen Thanh Tam[1, 2], Nguyen Chi Bao[5], Trinh Thi Thuy[1, 2], Tran Van Sung[2]

[1]*Graduate University of Science and Technology, VAST, 18 Hoang Quoc Viet, Ha Noi*

[2]*Institute of Chemistry, VAST, 18 Hoang Quoc Viet, Cau Giay, Ha Noi*

[3]*Thai Nguyen University of Sciences, Thai Nguyen University, Tan Thinh, Thai Nguyen*

[4]*Asean College, Van Lam, Hung Yen*

[5]*Department of Chemistry, Hue University, 77 Nguyen Hue, Hue City*

[*]Email: *hoanganhvhh@gmail.com*

Received: 1 February 2018; Accepted for publication: 10 May 2018

Abstract. This study reports the chemical constituents from the whole plants *Hedyotis pinifolia* Wall. Ex G. Don (now accepted as *Oldenlandia pinifolia* (Wall. Ex G. Don) Kuntze) collected in Thua Thien Hue province. Thirteen compounds were isolated by chromatography method. Their structures were elucidated using MS and NMR analysis and compared with reported data. They contain three anthraquinones, a carotenoid, two triterpenes, four iridoid glucosides and three flavonoid glycosides. Three of them were found for the first time in this genus.

Keywords: Hedyotis pinifolia (Oldenlandia pinifolia), anthraquinone, carotenoid, triterpene, iridoid glucosides and flavonoid glycosides.

Classification numbers: 1.1.1, 1.1.6, 1.2.1

1. INTRODUCTION

The *Hedyotis* genus belongs to the Rubiaceae family, which has about 180 species and is native to tropical and subtropical Asia [1]. Numerous *Hedyotis* species are used in traditional medicine for the treatment of inflammation, cancer and other diseases [2-4] such as *Hedyotis biflora, H. corymbosa, H. diffusa, H. verticillata*, etc. For example, the leaves of *H. auricularia* were used for treatment of diarrhea, dysentery in India, while using as vegetable with reducing blood pressure effect in Sri Lanka [5]. Vietnamese folk medicine used *H. herbacea* as expectorant, hypothermia and tonic; *H. tenelliflora* as detoxify, analgesic agents [4]. From this genus, many compounds with the novel structures and unique biological activities of the alkaloid, anthraquinone, iridoid, triterpenoid and lignin classes have been reported [6-9].

Hedyotis pinifolia is a small herb growing in sandy areas from Hue to the south of Viet Nam [10]. Until now, according to to our literature search, there has been only one report on the phytochemistry of this species [11]. In this paper, we isolated the chemical constituents from the *n*-hexane, ethyl acetate and *n*-butanol extracts of *H. pinifolia*. The structures of isolated compounds have been elucidated as three anthraquinones: 2-hydroxy-1-methoxy-anthraquinone (**1**); 1,6-dihydroxy-2-methylanthraquinone (**2**); digiferruginol (**3**); one carotenoid: lutein (**4**); two triterpenes: ursolic acid (**5**), oleanolic acid (**6**); four iridoid glycosides: asperuloside (**7**), deacetyl asperuloside (**8**), asperulosidic acid (**9**), scandoside methyl ester (**10**); three flavonoid glycosides: afzelin (**11**), rutin (**12**), isorhamnetin-3-*O*-β-rutinoside (**13**). Among them, compounds **1**, **3** and **11** were isolated for the first time in this genus.

2. MATERIALS AND METHODS

2.1. Equipments and methods

ESI-MS: LC-MSD-Trap-SL. NMR: Bruker Avance 500 MHz (^{1}H) and 125 MHz (^{13}C). The ^{1}H chemical shifts were referenced to the internal TMS; the ^{13}C chemical shifts to the solvent signals. The 2D experiments (HSQC and HMBC) were performed using standard Bruker pulse sequences at room temperature. Analytical TLC was performed on silica gel 60 F_{254} plates (Merck). Spots were visualized using UV light and vanillin-H_2SO_4 reagent. For preparative column chromatography silica gel 60, 60-200 μm (Merck) and sephadex LH-20 were used.

2.2. Plant material

Hedyotis pinifolia was collected on October 2014 in Phu Vang, Thua Thien Hue province of Viet Nam and determined by Dr. Do Xuan Cam, Hue University. The voucher specimen (VHH.TTH 10.2014.1) is deposited at the Institute of Chemistry, Vietnam Academy of Sciences and Technology (VAST).

2.3. Extraction and isolation

The air-dried whole of *H. pinifolia* (2.1 kg) were ground and extracted three times with 95 % MeOH at room temperature. The organic solvent was evaporated under reduced pressure and the aq. solution was partitioned with *n*-hexane, ethyl acetate and *n*-butanol, successively.

The *n*-hexane extract (36.2 g) was given on silica gel column, eluting with gradient *n*-hexane: EtOAc (from 100 % *n*-hexane to 100 % EtOAc) to yield 10 fractions (H1-H10). Fraction H7 (150 mg) was chromatographed on silica gel column, *n*-hexane: EtOAc (15:1), followed by Sephadex LH-20, CH_2Cl_2: MeOH (1:9) to furnish compound **1** (5 mg). Compound **2** (6 mg) and **4** (11 mg) were isolated when fraction H10 (410 mg) was purified over Sephadex LH-20, using CH_2Cl_2: MeOH (1:9) as eluent.

The ethyl acetate extract (34.2 g) was chromatographed over silica gel using gradient *n*-hexane:EtOAc to yield 7 fractions (E1-E7). Fraction E2 (4.42 g) was repurified over silica gel column, CH_2Cl_2: MeOH (10:1) to give 30 mg of **5**. Rechromatography of fraction E3 (1.62 g) was done on silica gel column, CH_2Cl_2: MeOH (10:1) furnished compound **6** (200 mg). Fraction E4 (1.15 g) was given on silica gel column, eluting with CH_2Cl_2: MeOH (9.5:1), followed on Sephadex LH-20 to obtain compound **3** (10 mg).

The *n*-butanol extract (32.0 g) was purified over silica gel columns to obtain 12 fractions (B1-B12). The fraction B3 (2.9 g) was rechromatographed on Sephadex LH-20 column with solvent systems of CH_2Cl_2: MeOH (1:9) to yield three subfractions (B3.1 – B3.3). Compound **7** (30 mg) was isolated by purification of subfraction B3.2 using Sephadex LH-20 column, MeOH. The purification of fraction B4 (1.35 g) on Sephadex LH-20 column, eluated with CH_2Cl_2: MeOH (1:9) to give compound **11** (10 mg). The purification of fraction B5 (2.42 g) was carried out on Sephadex LH-20 column with solvent systems of CH_2Cl_2:MeOH (1:9) to give two subfractions (B5.1-B5.2). Repeated chromatography of the subfraction B5.2 on sephadex LH-20 column, MeOH yielded compound **10** (16 mg). The fraction B6 (2.95 g) was given over Sephadex LH-20 column eluated with MeOH to furnish compound **8** (11 mg). The fraction B9 (3.9 g) was chromatographed on Sephadex LH-20 column, eluated with solvent systems of CH_2Cl_2: MeOH (1:9) to yield compound **12** (40 mg). The fraction 10 (1.5 g) was given on Sephadex LH-20 column, eluated with CH_2Cl_2: MeOH (1:9) to furnish 2 subfractions (B10.1 and B10.2). Compound **7** (10 mg) and **13** (10 mg) were obtained by repeated chromatography of subfraction B10.2. The fraction 12 (1.5 g) was chromatographed on Sephadex LH-20 column, eluated with CH_2Cl_2: MeOH (1:9) to give two subfractions (B12.1-B12.2). Rechromatography of B12.2 on sephadex LH-20 column, MeOH furnished compound **9** (10 mg).

2-hydroxy-1-methoxy-anthraquinone (1): Orange-red needles. (-)-ESI-MS *m/z*: 253 $[M-H]^-$

^{1}H NMR (500 MHz, $CDCl_3$): δ_H (ppm) 7.36 (1H, *d*, 9.0 Hz, H-3), 8.14 (1H, *d*, 9.0 Hz, H-4), 8.27 (2H, *m*, H-5, H-8), 7.74 (2H, *m*, H-6, H-7), 4.04 (3H, *s*, OMe), 6.69 (1H, *s*, OH).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C (ppm) 146.6 (C-1), 155.6 (C-2), 120.3 (C-3), 125.8 (C-4), 127.1 (C-5), 133.9 (C-6, C-7), 126.9 (C-8), 182.7 (C-9), 182.1 (C-10), 133.0 (C-11), 134.5 (C-12), 125.7 (C-13), 127.6 (C-14), 62.3 (OMe).

1,6-dihydroxy-2-methylanthraquinone (2): Orange powder. (-)-ESI-MS *m/z*: 253 $[M-H]^-$

^{1}H NMR (500 MHz, DMSO-d_6): δ_H (ppm) 7.61 (1H, *d*, 7.5 Hz, H-3), 7.55 (1H, *d*, 7.5 Hz, H-4), 7.44 (1H, *d*, 2.5 Hz, H-5), 7.21 (1H, *dd*, 2.5, 8.5 Hz, H-7), 8.08 (1H, *d*, 8.5 Hz, H-8), 13.08 (1H, *s*, 1-OH), 2.27 (3H, *s*, 2-CH_3).

^{13}C NMR (125 MHz, DMSO-d_6): δ_C (ppm) 159.9 (C-1), 114.6 (C-2), 136.8 (C-3), 118.6 (C-4), 112.5 (C-5), 163.9 (C-6), 121.4 (C-7), 129.8 (C-8), 187.6 (C-9), 181.7 (C-10), 131.1 (C-4a), 124.4 (C-8a), 134.2 (C-9a), 135.6 (C-10a), 15.7 (2-CH_3).

Digiferruginol (3): Orange-yellow needles. (-)-ESI-MS *m/z*: 253 $[M-H]^-$

^{1}H NMR (500 MHz, DMSO-d_6): δ_H (ppm) 7.77 (1H, *d*, 7.5 Hz, H-3), 7.92 (1H, *d*, 8.0 Hz, H-4), 8.20 (1H, *m*, H-5), 7.95 (2H, *m*, H-6, H-7), 8.25 (1H, *m*, H-8), 4.66 (2H, *s*, C$\underline{H}_2$OH), 5.46 (1H, *t*, 5.5 Hz, CH_2OH), 12.77 (1H, *s*, OH).

^{13}C NMR (125 MHz, DMSO-d_6): δ_C (ppm) 158.4 (C-1), 138.2 (C-2), 131.3 (C-3), 118.8 (C-4), 126.8 (C-5), 134.5 (C-6), 135.1 (C-7), 126.6 (C-8), 188.7 (C-9), 181.8 (C-10), 133.6 (C-5a), 133.2 (C-8a), 114.9 (C-9a), 132.8 (C-10a), 57.4 ($\underline{C}H_2$OH).

Lutein (4): Orange-red powder. (+)-ESI-MS *m/z*: 569 $[M+H]^+$

^{1}H-, ^{13}C-NMR (500, 125 MHz, $CDCl_3$): Table 1.

Ursolic acid (5): White amorphous powder. (-)-ESI-MS *m/z*: 455 $[M-H]^-$

^{1}H-NMR (500 MHz, $CDCl_3$): δ_H (ppm) 2.98 (*m*, H-3), 5.11 (*m*, H-12), 2.09 (*d*, 11.3 Hz, H-18), 0.88 (*s*, Me-23), 0.66 (*s*, Me-24), 0.85 (*s*, Me-25), 0.73 (*s*, Me-26), 1.02 (*s*, Me-27), 0.79 (*d*, 6.4 Hz, Me-29), 0.89 (*d*, 8.7 Hz, Me-30).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C (ppm) 38.2 (C-1), 27.0 (C-2), 76.8 (C-3), 38.4 (C-4), 54.8 (C-5), 18.0 (C-6), 30.2 (C-7), 39.1 (C-8), 47.0 (C-9), 36.5 (C-10), 23.8 (C-11), 124.6 (C-12), 138.2 (C-13), 41.6 (C-14), 32.7 (C-15), 22.8 (C-16), 46.8 (C-17), 52.4 (C-18), 38.4 (C-19), 38.5 (C-20), 27.5 (C-21), 36.3 (C-22), 28.3 (C-23); 16.9 (C-24); 16.1 (C-25), 15.2 (C-26), 23.3 (C-27), 178.3 (C-28), 17.0 (C-29), 21.1 (C-30).

Oleanolic acid (6): White amorphous powder. (-)-ESI-MS *m/z*: 455 $[M-H]^-$

^{1}H NMR (500 MHz, $CDCl_3$): δ_H (ppm) 5.27 (1H, *t*, 3.5Hz, H-12), 3.20 (1H, *dd*, 4.0, 11.0 Hz, H-3), 2.81 (1H, *dd*, 4.0, 13.5 Hz, H-18), 1.12, 0.97, 0.91, 0.90, 0.89, 0.76, 0.74 (each 3H, *s*, Me-23, 24, 25, 26, 27, 29, 30).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C (ppm) 38.4 (C-1), 27.7 (C-2), 79.1 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 32.7 (C-7), 39.3 (C-8), 47.7 (C-9), 37.1 (C-10), 23.0 (C-11), 122.7 (C-12), 143.6 (C-13), 41.7 (C-14), 27.2 (C-15), 23.4 (C-16), 46.5 (C-17), 41.1 (C-18), 45.9 (C-19), 30.7 (C-20), 33.8 (C-21), 32.5 (C-22), 28.1 (C-23), 15.6 (C-24), 15.3 (C-25), 17.1 (C-26), 25.9 (C-27), 181.6 (C-28), 33.1 (C-29), 23.6 (C-30).

Asperuloside (7): white powder. (+) ESI-MS: *m/z* = 437 $[M+Na]^+$. NMR data: Table 2.

Deacetyl asperuloside (8): white powder. (-) ESI-MS: *m/z* = 371 $[M-H]^-$. NMR data: Table 2.

Scandoside methyl ester (9): white powder. (+) ESI-MS: *m/z* = 427 $[M+Na]^+$. NMR data: Table 2.

Asperulosidic acid (10): white powder. (+) ESI-MS: *m/z* = 455 $[M+Na]^+$. NMR data: Table 2.

Afzelin (11): yellow powder. (-) ESI-MS: *m/z* = 431 $[M-H]^-$.

^{1}H-NMR (500 MHz, CD_3OD): δ_H (ppm) 6.22 (1H, *d*, 2.0 Hz, H-6), 6.40 (1H, *d*, 2.0 Hz, H-8), 7.79 (2H, *d*, 9.0 Hz, H-2', 6'), 6.96 (2H, *d*, 9.0 Hz, H-3', 5'), 5.40 (1H, *d*, 1.5 Hz, H-1"), 3.73 (1H, *dd*, 3.0, 9.0 Hz, H-2"); 3.36 (1H, *d*, 5.0 Hz, H-3"), 3.35 (1H, *d*, 5.0 Hz, H-4"), 4.24 (1H, *dd*, 2.0, 4.0 Hz, H-5"); 0.94 (3H, *d*, 6.0 Hz, H-6").

^{13}C-NMR (125 MHz, CD_3OD): δ_C (ppm) 159.3 (C-2), 136.2 (C-3), 179.6 (C-4); 163.2 (C-5), 99.9 (C-6), 166.1 (C-7), 94.8 (C-8), 158.6 (C-9), 105.9 (C-10), 122.7 (C-1'), 131.9 (C-2', C-6'), 116.5 (C3', C-5'), 161.6 (C-4'), 103.5 (C-1"), 72.0 (C-2"), 72.2 (C-3"), 73.2 (C-4"), 71.9 (C-5"), 17.6 (C-6").

Rutin (12): yellow powder. (-) ESI-MS: *m/z* = 609 $[M-H]^-$.

^{1}H-NMR (500 MHz, CD_3OD): δ_H (ppm) 6.22 (1H, br *s*, H-6), 6.42 (1H, br *s*, H-8), 7.71 (1H, br *s*, H-2'), 6.91 (1H, *d*, 8.0 Hz, H-5'), 7.64 (1H, br *d*, 8.0 Hz, H-6'), 5.07 (1H, *d*, 7.5 Hz, H-1"), 3.53 (1H, *t*, 9.0 Hz, H-2"), 3.81 (1H, br *d*, 9.5 Hz, H-6"), 4.54 (1H, br *s*, H-1"'), 3.69 (1H, br *s*, H-2"'), 3.58 (1H, *dd*, 3.5, 9.5 Hz, H-3"'), 3.31 (1H, *m*, H-4"'), 3.44 (1H, *m*, H-5"'), 1.14 (3H, *d*, 6.5 Hz, H-6"').

^{13}C-NMR (125 MHz, CD_3OD): δ_C (ppm) 158.4 (C-2), 135.6 (C-3), 179.3 (C-4), 162.7 (C-5), 100.0 (C-6), 166.0 (C-7), 95.0 (C-8), 159.4 (C-9), 105.6 (C-10), 123.1 (C-1'), 117.7 (C-2'), 145.6 (C-3'), 149.7 (C-4'), 116.1 (C-5'), 123.6 (C-6'), 104.8 (C-1"), 75.5 (C-2"), 78.0 (C-3"), 71.3 (C-4"), 77.1 (C-5"), 68.6 CH_2 (C-6"), 102.3 (C-1"'), 72.0 (C-2"'); 72.1 (C-3"'); 73.9 (C-4"'), 69.6 (C-5"'), 17.8 (C-6"').

Isorhamnetin-3-*O*-β-rutinoside (13): yellow powder. (-) ESI-MS: *m/z* = 623 $[M-H]^-$.

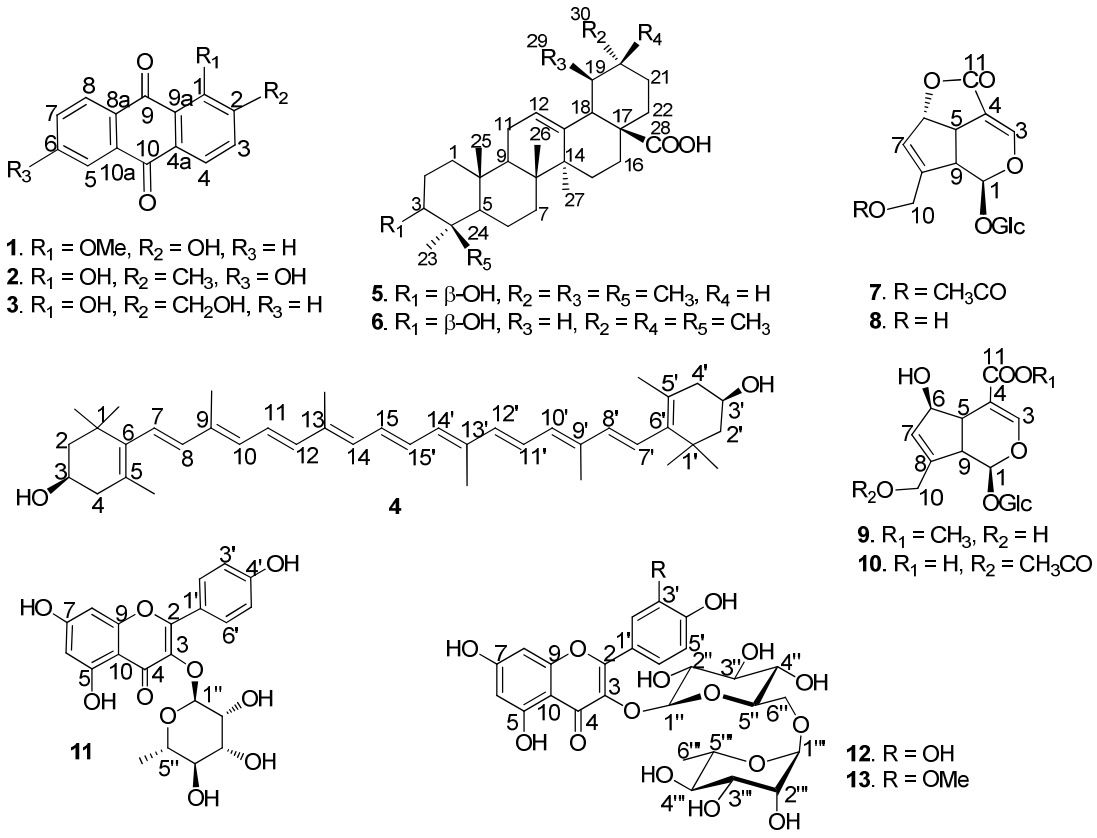
^{1}H-NMR (500 MHz, CD_3OD): δ_H (ppm) 6.22 (1H, *d*, 1.5 Hz, H-6), 6.41 (1H, *d*, 1.5 Hz, H-8), 7.95 (1H, *d*, 2.0 Hz, H-2'), 6.93 (1H, *d*, 8.5 Hz, H-5'), 7.64 (1H, *dd*, 2.0, 8.5 Hz, H-6'), 5.24

(1H, *d*, 7.5 Hz, H-1”), 4.55 (1H, *d*, 1.0 Hz, H-1”’), 1.12 (3H, *d*, 7.5 Hz, H-6”’), 3.96 (3H, *s*, OMe).

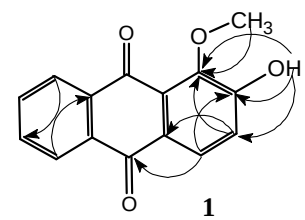
^{13}C-NMR (125 MHz, CD_3OD): δ_C (ppm) 158.5 (C-2), 135.5 (C-3), 179.3 (C-4), 162.9 (C-5), 100.0 (C-6), 166.0 (C-7), 94.9 (C-8), 158.9 (C-9), 105.7 (C-10), 123.0 (C-1’), 114.6 (C-2’), 148.3 (C-3’), 150.8 (C-4’), 116.1 (C-5’); 124.0 (C-6’), 104.4 (C-1”), 75.9 (C-2”), 78.1 (C-3”), 71.6 (C-4”), 77.3 (C-5”), 68.5 (C-6”), 102.49 (C-1”’), 72.0 (C-2”’), 72.3 (C-3”’), 73.8 (C-4”’), 69.8 (C-5”’), 17.9 (C-6”’), 56.8 (-OMe).

3. RESULTS AND DISCUSSION

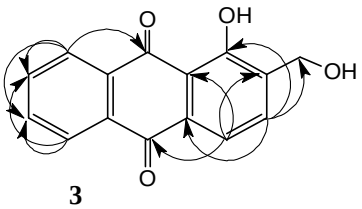
Thirteen compounds were isolated from *n*-hexane, ethyl acetate and *n*-butanol extracts of the whole plant of *H. pinifolia* by repeated column chromatography with the appropriate solvent systems. Their structures were identified as 2-hydroxy-1-methoxy-anthraquinone (**1**); 1,6-dihydroxy-2-methylanthraquinone (**2**); digiferruginol (**3**); lutein (**4**); ursolic acid (**5**) and oleanolic acid (**6**) asperuloside (**7**), deacetyl asperuloside (**8**), asperulosidic acid (**9**), scandoside methyl ester (**10**), afzelin (**11**), rutin (**12**), isorhamnetin-3-*O*-*β*-rutinoside (**13**) by the analysis of their 1D, 2D-NMR, ESI-MS spectra and compared with published data.



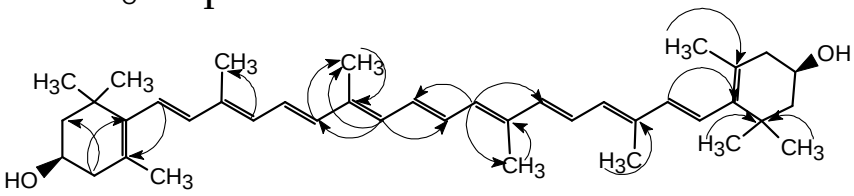
The structure of isolated compounds (1 -13)



1



3



4

HMBC correlations of compounds 1, 3, 4

Compound **1** obtained as orange-red needles, gave pseudo molecular peak at *m/z* = 253 $[M-H]^-$ in the negative ESI-MS. The ^{1}H NMR spectrum indicated the characteristic signals of the anthraquinone type, including the signals of the two aromatic rings: two *ortho*-coupled aromatic protons at δ_H 7.36 (*d*, *J* = 9.0 Hz, H-3); 8.14 (*d*, *J* = 9.0 Hz, H-4) of the first ring and typical aromatic protons of the A_2B_2 substituted ring at δ_H 8.27 (2H, *m*, H-5, H-8); 7.74 (2H, *m*, H-6, H-7) of the second together with an aromatic methoxy group at δ_H 4.04 . Beside these signals, the ^{13}C NMR spectrum gave the signals of the two carbonyl carbons at δ_C 182.7 and 182.1 and six aromatic quaternary carbons comprising one hydroxy-carbon at δ_C 155.6; a carbon connected to a methoxy group at δ_C 144.6 and four others. The position of the hydroxy group at C-2 was deduced from HMBC correlations among signals at δ_H 6.69 (2-OH), δ_C 120.3 (C-3); 155.6 (C-2) and 146.6 (C-1); among signals at δ_H 7.36 (H-3), δ_C 146.6 (C-1) and 127.6 (C-4a). The structure of **1** was thus determined to be 2-hydroxy-1-methoxy-anthraquinone or alizarin-1-methyl ether when compared to the literature [12]. This compound was isolated previously from *Hedyotis diffusa* and inhibited protein tyrosine kinases v-src and pp60src and the growth of Bcap37 cell line (IC_{50} 65 μM). Furthermore, it could induce apoptosis on SPC-1-A cell (IC_{50} 79 μM) with a close relationship to the mitochondrial apoptotic pathway [13].

Compound **2** was isolated as an orange powder, showed $[M-H]^-$ peak at *m/z* = 235 in the negative ESI-MS. Its NMR spectra showed characteristic signals of an 9,10-anthraquinone very similar to those of **1**, revealing two carbonyl carbons at δ_C 187.6 and 181.7 as well as proton signals of two aromatic rings: two *ortho*-coupled aromatic protons and typical aromatic protons of the 1,3,4-substituted ring. These signals suggested that the substituted positions of **2** are the same in compound **1** but with other groups. The connection of hydroxy group at C-1 was confirmed based on a chelated hydroxy proton in ^{1}H NMR (measured in DMSO-d_6) at δ_H 13.08, as well as the downshifted carbonyl carbon at δ_C 187.6. The position of the hydroxy group at C-6 was deduced from HMBC correlations among signals at δ_H 8.08 (H-8), δ_C 187.6 (C-9) and 163.9 (C-6); among signals at δ_H 7.44 (H-5), δ_C 181.7 (C-10) and 121.4 (C-7); and among signals at δ_H 7.21 (H-7), δ_C 112.5 (C-5) and 124.4 (C-8a). An aromatic methyl group [δ_H 2.27 (3H, *s*); δ_C 15.7] was determined to connect at C-2 by correlations among signals at δ_H 2.27 ($C\underline{H}_3$), δ_C 159.9 (C-1), 136.8 (C-3). The spectroscopic data of **2** was identical to those of 1,6-dihydroxy-2-methylanthraquinone in the literature [14]. This compound was first isolated from *Cinchona pubescens* in 1986 [14].

Table 1. ^{1}H- and ^{13}C-NMR (500 and 125 MHz) data of compound **4** and lutein ($CDCl_3$).

No	δ_H (ppm, *J*, Hz)	δ_C (ppm)	δ_H (ppm, *J*, Hz)	δ_C (ppm)
	Compound 4		**Lutein [$CDCl_3$]**	
1	-	37.1	-	37.1
2	1.48 (*t*, 12.0)	48.5	1.48 (*t*, 12.0)	48.4
3	400 (*m*)	65.1	4.0 (*m*)	65.1
4 ax 4 eq	2.04 (*dd*, 17.0; 10.0) 2.33-2.42 (*m*)	42.6	2.04 (*dd*, 17.0; 10.0) 2.33-2.45 (*m*)	42.5
5	-	126.2	-	126.2
6	-	137.6	-	137.6
7	6.12 (*s*)	125.6	6.12 (*s*)	125.6
8	6.12 (*s*)	138.5	6.12 (*s*)	138.5
9	-	135.7	-	135.6
10	6.15 (*m*)	131.3	6.15 (*m*)	131.3
11	6.58-6.67 (*m*)	124.8	6.55-6.71 (*m*)	124.9
12	6.36 (*d*, 15.0)	137.7	6.36 (*d*, 15.0)	137.6
13	-	136.5	-	136.5
14	6.25 (br *d*, 9.0)	132.6	6.26 (*m*)	132.6
15	6.58-6.67 (*m*)	130.1	6.55-6.71 (*m*)	130.0
1-Me	1.07 (*s*)	28.7	1.07 (*s*)	28.7
1-gem Me	1.07 (*s*)	30.3	1.07 (*s*)	30.2
5 -Me	1.74 (*s*)	21.6	1.74 (*s*)	21.6
9 -Me	1.97 (*s*)	12.8	1.97 (*s*)	12.7
13 -Me	1.97 (*s*)	12.8	1.97 (*s*)	12.7
1’	-	34.0	-	34.0
2’ ax 2’ eq	1.37 (*dd*, 13.0; 7.0) 1.84 (*dd*, 13.0; 6.0)	44.6	1.37 (*dd*, 13.0; 7.0) 1.84 (*dd*, 13.0; 6.0)	44.7
3’	4.25 (*m*)	65.9	4.25 (*m*)	65.9
4’	5.54 (br *s*)	125.6	5.55 (*s*)	125.6
5’	-	137.8	-	137.8
6’	2.33 – 2.42 *m*	55.0	2.33-2.45 (*m*)	55.0
7’	5.43 (*dd*, 10.0; 15.5)	128.7	5.43 (*dd*, 15.5; 10.0)	128.6
8’	6.15 (*m*)	138.0	6.15 (*m*)	137.8
9’	-	135.1	-	135.0
10’	6.15 (*m*)	130.8	6.15 (*m*)	130.8
11’	6.58-6.67 (*m*)	124.5	6.55-6.71 (*m*)	124.5
12’	6.36 (*d*, 15.0)	137.6	6.36 (*d*, 15.0)	137.6
13’	-	136.4	-	136.5
14’	6.25 (br *d*, 9.0)	132.6	6.26 (*m*)	132.6
15’	6.58-6.67 (*m*)	130.0	6.55-6.71 (*m*)	130.0
1’-Me	0.85 (*s*)	24.3	0.85 (*s*)	24.3
1’-Me	1.00 (*s*)	29.5	1.00 (*s*)	29.5
5’-Me	1.63 (*s*)	22.9	1.63 (*s*)	22.8
9’-Me	1.91 (*s*)	14.1	1.91 (*s*)	13.2
13’-Me	1.97 (*s*)	13.1	1.97 (*s*)	12.7

Compound **3** was obtained as yellow needle, showed $[M-H]^-$ peak at *m/z* = 235 in the negative ESI-MS. Its NMR spectra predicted to be an 9,10-anthraquinone from similar signals to those of **1**. Beside the same signals as in **1**, the NMR spectra of **3** appeared additional characteristic signals of the hydroxy group at δ_H 12.77 and hydroxymethylen group [δ_H 4.66 (*d*, *J* = 5.0 Hz, 2 -C<u>H</u>$_2$OH); 5.46 (*t*, *J* = 5.5 Hz, 2-CH$_2$O<u>H</u>); δ_C 57.4. The position of the hydroxyl group at C-1 and hydroxymethylen group at C-2 was deduced from HMBC correlations. Consequently, **3** was identified as digiferruginol by comparison with the published data [15]. Digiferruginol exhibited a significant cytotoxic activity against KB cancer cell line with an ED value of 0.09 µg/mL [15].

Compound **4** was isolated as an amorphous red-orange powder, indicated $[M+H]^+$ peak at *m/z* = 569 in the positive ESI-MS. The NMR spectrum of **4** appears characteristic signals of a carotenoid including conjugated olefins at δ_H 5.43-6.67; δ_C 124.5-138.5 together with 4 methylene groups. Besides, two hydroxy-methine groups [(δ_H 4.00 (1H, *m*, H-3); δ_C 65.1 (C-3) and 4.25 (1H, *m*, H-3'); 65.9 (C-3')] and 10 methyl groups were observed. The connection of two hydroxy groups at C-3 and C-3' was confirmed based on the downshifted carbons with δ_C 65.1 (C-3) and 65.9 (C-3'). The position of the allene moiety was determined to connect to alicyclic moiety at C-6 by HMBC correlations among signals at δ_H 6.12 (H-7) to δ_C 126.2 (C-5) and 137.6 (C-6). The conjugated alkadiene chain was deduced from HMBC correlations among protons H-14 with C-12 and C-15'; H-14' with C-12', C-15 and methyl group carbon connected to C-13'. The structure of **4** is concluded as lutein when compared with the spectral data in the literature [16]. Lutein plays significant roles in human health, particularly for eyes, linked to reduced risk of age-related macular degeneration and cataracts [17].

The structures of compounds **5** and **6** were elucidated as ursolic acid and oleanolic acid, respectively, by comparison with authentic sample in thin layer chromatography and ^{1}H, ^{13}C NMR spectral data [18, 19]. Oleanolic acid and ursolic acid are triterpenoid compounds that widely occur in nature in free acid form or as an aglycone of triterpenoid saponins. These compounds have shown pharmacological activities, such as hepatoprotective, anti-inflammatory, antioxidant, and anticancer effects [20].

Compound **7** showed the pseudo molecular peak at *m/z* = 437 $[M+Na]^+$ in the positive ESI-MS spectrum. The molecular formula of $C_{18}H_{22}O_{11}$ was deduced from MS and NMR data. NMR spectra showed the signals of two double bonds -CH=C< at δ_H = 7.32 (1H, *d*, 2.0 Hz), δ_C = 150.3 CH, 106.1 C and δ_H = 5.75 (1H, br *s*), δ_C = 128.9 CH, 144.2 C; two oxygenated methine at δ_H = 5.97 (1H, *d*, 1.0 Hz), δ_C = 93.3 and δ_H = 5.59 (1H, br *d*, 6.5 Hz), δ_C = 86.3 CH. Besides these, two methine and an oxy-methylene have been observed in NMR spectra. The ^{13}C NMR spectrum indicated the signals of a lactone and an acetyl groups, revealing by signals at δ_C = 172.3 C and 172.6 C, 20.6 CH$_3$, respectively. The presence of one β-D-glucopyranose unit in the molecule was confirmed by typical signals of anomer group at δ_H = 4.71 (1H, *d*, 8.0 Hz), δ_C = 100.0 and methylene group (Glc-6) at δ_H = 3.94 (*dd*, 12.0 & 2.0 Hz), 3.70 (*dd*, 12.0 & 6.0 Hz), δ_C = 62.8. The correlations in the HMBC spectrum between H-1 (δ_H 5.97) with C-3, C-5, C-8, C-9 and Glc-1; H-3 (δ_H 7.32) with C-1, C-4, C-5 and C-11; H-7 (δ_H 5.75) with C-5, C-6, C-8, C-9 and C-10 determined the substituted positions in compound **7**. The above analysis and compared with the published data [21] led to conclude that **7** is asperuloside, a glucoside of iridoid C-9, which was already isolated from *Galium verum* [22].

Compound **8** was obtained as white powder. The pseudo molecular ion peak at *m/z* = 371 $[M-H]^-$ in the negative ESI-MS and NMR spectra led to conclude that the molecular formula of **8** is $C_{16}H_{20}O_{10}$. Its NMR data were very similar to those of **7**, suggested it is an iridoid glucoside

too. The only difference between two compounds revealed by the replacement of acetyl signal group in **7** by hydroxy-methylene signals in compound **8** (δ_H = 4.21, 2H, br *s*, δ_C = 60.1). The loss of 42 mass units (CH_3CO-) of compound **2** compared with **7** confirmed the above mentioned suggestion. Therefore, the structure of **8** was determined as deacetyl asperuloside by comparison with the data in literature [21].

Compound **9** was isolated as white powder. The positive ESI-MS gave pseudo molecular ion peak at *m/z* 427 $[M+Na]^+$. Its molecular formula was determined as $C_{17}H_{24}O_{11}$ from MS and NMR data. NMR spectra of **9** showed similar signals to those of **8**. The upshifted of C-6 (δ_C 82.6) and C-11 (δ_C 170.3) together with the addition of methoxy group at δ_H = 3.77, δ_C = 52.0 suggested that lactone group in **8** was opened to form hydroxyl and methyl ester groups in **9**. Thus, the structure of **9** was elucidated as scandoside methyl ester when compared with pulished data in [21].

NMR spectra of **10** suggested that is an iridoid glucoside, very similar to those of **9** with iridoid and β-D-glucopyranose signals. But, the methyl ester and hydroxy signals in **9** were replaced by carboxylic at δ_C 170.0 (C-11) and acetyl at 172.5, 2.03/20.8. The position of substituted groups were confirmed by HMBC correlations. These analysis combined the published data in [23] led to conclude that **10** is asperulosidic acid. This compound exhibited cytotoxic activity on HL-60 and HCT15 cancer cell lines [24].

Compound **11** was obtained as yellow powder. The molecular formula was determined as $C_{21}H_{20}O_{10}$ from pseudo molecular ion peak at *m/z* = 431 $[M-H]^-$ in the negative ESI-MS and NMR spectra. The ^{1}H-NMR spectrum exhibited two meta-coupled protons at δ_H 6.22 (1H, *d*, *J* = 2.0 Hz, H-6), 6.40 (1H, *d*, *J* = 2.0 Hz, H-8), characteristic proton signals of para-substituted aromatic ring at δ_H 7.79 (2H, *d*, *J* = 9.0 Hz, H-2', 6'); 6.96 (2H, *d*, *J* = 9.0 Hz, H-3', 5'). Besides these, the presence of an α-L-rhamnopyranose have been observed, revealing by a proton anomer at δ_H 5.40 (1H, *d*, *J* = 1.5 Hz) and a methyl group at δ_H 0.94 (3H, *d*, *J* = 6.0 Hz). The ^{13}C-NMR spectrum of **11** indicated 21 carbons, among them 15 belong to flavonoid skeleton and 6 carbons of sugar unit. The above analysis combined the comparison with the published data [25] led to conclude that **11** is 3-*O*-α-L-rhamnopyranoside kaempferol, named afzelin. It was isolated for the first time from *Afzelia doussie* [26]. Afzelin inhibited the growth of prostate cancer cells *in vitro* [27].

Compound **12** was isolated as yellow powder and showed the pseudo molecular ion peak at *m/z* = 609 $[M-H]^-$ in the negative ESI-MS, corresponds to $C_{27}H_{30}O_{16}$. Its NMR spectra indicated that is a flavonoid glycoside, revealing by the signals of two meta-protons in A ring at δ_H = 6.22 br *s* (H-6), 6.42 br *s* (H-8), three protons of ABX spin system in ring B at δ_H = 7.71 br *s* (H-2'), 6.91 *d*, 8.0 Hz (H-5'), 7.64 br *d*, 8.0 Hz (H-6'), ketone group in ring C at δ_C = 179.3 (C-4). Furthermore, the characteristic signals of β-D-glucopyranose unit at δ_H = 5.07 *d*, 7.5 Hz (H-1"), δ_C = 104.8 (C-1") and α-L-rhamnopyranose at δ_H = 4.54, br *s* (H-1"'), 1.14 *d*, 6.5 (H-6"'); δ_C = 102.3 (C-1"'), 17.8 (C-6"') have been observed. The NMR data of **12** are identical with those of rutin in the literature [28]. Rutin exists widely in the nature, for example in barley, fruit types of *Citrus* genus and medicinal plant as *Sophora japonica* L. Rutin has been proven having many biological effects as inflammatory, antioxidant, etc. [29].

Compound **13**, an yellow powder, showed the pseudo molecular ion peak at *m/z* = 623 $[M-H]^-$ in the negative ESI-MS. Its molecular formula was deduced as $C_{28}H_{32}O_{16}$ (M = 624) from MS and NMR data. The NMR data of **13** were very similar to those of **12** with only one difference. The signal of hydroxy in ring B of **12** was replaced by methoxy signals (δ_H = 3.96 *s*,

3H and δ_C = 56.8) in compound **13** together with the downshifted of C-3' ($\Delta\delta_C$ = 2.67) when compared to those of compound **12**. Finally, the structure of **13** was established as isorhamnetin-3-*O*-*β*-rutinoside by comparison with the published spectroscopic data in [30]. The other name of this compound is narcissin.

Table 2. ^{1}H and ^{13}C-NMR (500 and 125 MHz) of compounds **7** – **10** (CD_3OD).

	7 δ_H (*J*, Hz)	**8** δ_H (*J*, Hz)	**9** δ_H (*J*, Hz)	**10** δ_H (*J*, Hz)	**7** δ_C	**8** δ_C	**9** δ_C	**10** δ_C
1	5.97 *d*, 1.0	5.97 *d*, 1.5	5.83 br *s*	5.07 *d*, 9.0	93.3 CH	93.3	98.3	101.2
3	7.32 *d*, 2.0	7.31 *d*, 2.0	7.53 br *s*	7.63 *s*	150.3CH	150.2	153.9	154.6
4	-	-	-	-	106.1 C	106.5	110.8	109.0
5	3.70 *m*	3.70 *m*	3.04 *m*	3.05 *m*	37.4 CH	37.5	45.6	42.7
6	5.59 br *d*, 6.5	5.58 *dd*, 1.5 & 6.5	4.57 br *s*	4.85 *m*	86.3 CH	86.6	82.6	75.5
7	5.75 br *s*	5.66 br *s*	5.21 *t*	6.04 br *s*	128.9 CH	125.7	130.1	131.9
8	-	-	-	-	144.2 C	149.8	147.5	145.9
9	3.32 *m*	3.32 *m*	3.23 br *t* 8.0	2.65 *t*, 8.0	45.2 CH	45.0	47.1	46.4
10	4.69 *dd*, 14.0 & 1.0 4,80 *dd*, 14.0 & 1.0	4.21 br *s*	4.21 br *d*, 15.0 4.36 br *d*, 15.0	4.97 br *d*, 14.5 4.83 br *d*, 14.5	61.9 CH_2	60.1	61.0	63.8
11	-	-	-	-	172.3 C	172.8	170.3	173.0
CH_3CO	-	-	-	-	172.6 C	-	-	172.5
CH_3CO	2.10 *s*	-	-	2.03 *s*	20.6 CH_3	-	-	20.8
OCH_3	-	-	3.77 *s*	-			52.0	-
Glc-1	4.71 *d*, 8.0	4.70 *d*, 8.0	4.69 *d*, 8.0	4.75 *d*, 8.0	100.0 CH	99.9	100.3	100.6
Glc-2	3.22 *dd*, 9.0 & 8.0	3.21 *dd*, 9.0 & 8.0		3.41 br *t*, 8.5	74.6 CH	74.7	74.8	74.9
Glc-3	3.31 – 3.40 *m*	3.30 – 3.60 *m*	3.20 – 3.34 *m*	3.25 – 3.33 *m*	78.3 CH	78.4	78.4	78.5
Glc-4					71.6 CH	71.6	71.5	71.6
Glc-5					77.8 CH	77.9	77.9	77.9
Glc-6	3.94 *dd*, 12.0 & 2.0 3.70 *dd*, 12.0 & 6.0	3.94 *dd*, 12.0 & 2.0 3.84 *dd*, 12.0 & 6.0	3.88 br *d*, 11.5 3.66 br *d*, 11.5	3.87 br *d*, 10.0 3.64 dd, 5.0, 12.0	62.8 CH_2	62.8	62.7	63.0

4. CONCLUSION

In summary, this report deals with the isolation and structural elucidation of thirteen compounds from the *n*-hexane, ethyl acetate and *n*-butanol extracts of *Hedyotis pinifolia*. Three of them (compounds **1**, **3** and **11**) were obtained for the first time from this genus.

REFERENCES

1. Groeninckx I., Dessein S., Ochoterena H., Persson C., Motley T. J., Kårehed J., Bremer B., Huysmans S., and Smets E. - Phylogeny of the herbaceous tribe Spermacoceae (Rubiaceae) based on plastid DNA data, Annals of the Missouri Botanical Garden **96** (2009) 109-132.
2. Kikuchi T., Matsuda S., Kadota S., Sakai Y., Nmaba T., Watanabe K., Dissanayake D. M. - Studies on the constituents of medicinal and related plants in Sri Lanka. I. New triterpenes from *Hedyotis lawsoniae,* Chem. Pharm. Bull. **32** (1984) 3906-3911.
3. Kang X. D., Li X., Zhao C. C., Mao Y. - Two new anthraquinones from *Hedyotis diffusa* W, Journal of Asian Natural Products Research **10** (2008) 193-197.
4. Vo Van Chi - Dictionary of Vietnamese medicinal plants, Medical Publishing House, 1996.
5. Kikuchi T., Matsuda S., Kadota S., Sakai Y., Nmaba T., Watanabe K., Dissanayake D. M. - Studies on the constituents of medicinal and related plants in Sri Lanka. I. New triterpenes from *Hedyotis lawsoniae,* Chem. Pharm. Bull. **32** (1984) 3906-3911.
6. Peng J. N., Feng X. Z., Liang X. T. - Iridoids from *Hedyotis hedyotidea,* Phytochemistry **47** (1998) 1657-1659.
7. Salwati O. Hamzah, Ahmad H. S., Norio A., Nordin L. M. D. - Chemical Constituents of *Hedyotis dichotoma* and their Biological Activity, Pertanika Journal of Science and Technology **4** (1996) 183-189.
8. Nguyen M. P., Porzel A., Schmidt J., Tran V. S., Adam G. - Two new β-carboline alkaloids from *Hedyotis capitellata* var. *mollis,* Planta Medica **65** (1999) 761-762.
9. Ahmad Nhlar - Phytochemical studies and pharmacological activities of plants in genus *Hedyotis*/*Oldenlandia,* Natural Products Chemistry **33** (2006) 1057-1090.
10. Pham H.H. - An Illustrated Flora of Viet Nam, Young Publisher, 1999.
11. Le H. D., Nguyen K. P. P. - Anthraquinones from *Hedyotis pinifolia,* Journal of Chemistry **47** (2009) 380-384.
12. Wu Y. B., Zheng C. J., Qin L. P., Sun L. N., Han T., Jiao L., Zhang Q. Y., Wu J. Z. - Antiosteoporotic Activity of Anthraquinones from *Morinda officinalis* on Osteoblasts and Osteoclasts, Molecules **14** (2009) 573-583.
13. Shi Y., Wang C. H., Gong X. G. - Apoptosis-inducing effects of two anthraquinones from *Hedyotis diffusa* Wild, Biol. Pharm. Bull. **31** (2008) 1075-1078.
14. Wijnsma R., Go J. T. K. A., Harkes P. A. A., Verpoorte R., Baerheim A., Svendsen B. - Anthraquinones in callus cultures of *Cinchona pubescens,* Phytochemistry **25** (1986) 1123-1126.
15. Zhang H. L., Zhang Q. W., Zhang X. Q., Ye W. C., Wang Y. T. - Chemical Constituents from the Roots of *Morinda officinalis,* Chinese Journal of Natural Medicines **8** (2010) 1-4.
16. El-Raey M. A., Ibrahim G. E., Eldahshan O. A. - Lycophene and Lutein: A review for their Chemistry and Medicinal Uses, Journal of Pharmacognosy and Phytochemistry **2** (2013) 245-254.
17. Hester P. - Egg Innovations and Strategies for Improvements, Elsevier, 2017, pp. 199-206.

18. Guvenalp Z., Ozbek H., Kuruuzum-uz A., Kazaz C., Demirezer O. - Secondary metabolites from *Nepeta heliotropifolia*, Turk J. Chem. **33** (2009) 667-675.
19. Liao C. R., Kuo Y. H., Ho Y. L., Ching-Ying Wang C. Y., Yang C. S., Lin C. W., and Chang Y. S. - Studies on Cytotoxic Constituents from the Leaves of *Elaeagnus oldhamii* Maxim. in Non-Small Cell Lung Cancer A549 Cells, Molecules **19** (2014) 9515-9534.
20. Liu J. - Pharmacology of oleanolic acid and ursolic acid, Journal of Ethnopharmacology **49** (1995) 57-68.
21. Otsuka H., Yoshimura K., Yamasaki K., Cantoria M. C. - Isolation of 10-*O*-acyl iridoid glucosides from a Philippines medicinal plant *Oldenlandia corymbosa* L. (Rubiaceae), Chem. Pharm. Bull. **39** (1991) 2049-2052.
22. Böjthe-Horváth K., Hetényi F., Kocsis Á., Szabó L., Varga-Balázs M., Máthé JrI, Tétényi P. - Iridoid glycosides from *Galium verum*, Phytochemistry **21** (1980) 2917-2919.
23. Tzakoua O., Mylonas P., Vagias C., and Petrakis P. V. - Iridoid glucosides with insecticidal activity from *Galium melanantherum*, Z Naturforsch **62C** (2007) 597-602.
24. Kozhiparambil K. P., Ayyapath S. - Structure of auricularine, a bis-indole alkaloid from *Hedyotis auricularia*, Phytochemistry **20** (1981) 351–356.
25. Trinh T. T., Tran V. S., Nguyen T. H. - Chemical constituents of *Fissistigma pallens*, Vietnam Journal of Chemistry **44** (2006) 412-417.
26. King F. E. and Acheson R. M. - Afzelin (kaempferol-3 rhamnoside), a new glycoside isolated from Doussie [Afzelia spp.], Journal of Chemistry Society No. Jan (1950) 168-170.
27. Zhu K. C., Sun J. M., Shen J. G., Jin J. Z., Liu F., Xu X. L., Chen L., Liu L. T., and Lv J. J. - Afzelin exhibits anti-cancer activity against androgen-sensitive LNCaP and androgen-independent PC-3 prostate cancer cells through the inhibition of LIM domain kinase 1, Oncology letters **10** (2015) 2359-2365.
28. Agrawal P. K. - Carbon-13 NMR of Flavonoids, Elsevier publisher, 1989.
29. Al-Dhabi N. A., Arasu M. V., Park C. H., and Park S. U. - An up-to-date review of rutin and its biological and pharmacological activities, EXCLI Journal **14** (2015) 59–63.
30. Gutzeit D., Wray V., Winterhalter P., Jerz G. - Preparative Isolation and Purification of Flavonoids and Protocatechuic Acid from Sea Buckthorn Juice Concentrate (*Hippophaë rhamnoides* L. ssp. rhamnoides) by High-Speed Counter-Current, Chromatography, Chromatographia **65** (2007) 1-7.