Original article

Triterpenoids from the stem of *Diospyros glandulosa*

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Abstract:

Six pentacyclic triterpenoids, including friedelin, β-amyrin, oleanolic acid, lupeol, betulin and ursolic acid, have been isolated from the stem of *Diospyros glandulosa* Lace. Identification of the isolated compounds was accomplished by analysis of their IR, MS, 1-D and 2-D NMR spectral data, as well as comparison with reported data.

Keywords: *Diospyros glandulosa;* Ebenaceae; Triterpenoid

Introduction

Diospyros, the largest genus of the family Ebenaceae, comprises about 400 species widespread mainly in the tropics [1]. Plants in this genus have long been known for their medicinal uses and it is so interesting that almost all parts of these plants were found to possess therapeutic properties [2]. Phytochemical investigation of more than 130 *Diospyros* species led to the isolation of a variety of compounds, the majority of which is triterpenoids and naphthoquinones [2]. Several compounds of these two groups have been found to exhibit interesting bioactivities [2]. In Thailand, sixty species of *Diospyros* have been found, excluding two exotic species [1, 3]. About ten of them have been recorded of their uses in Thai traditional medicine [4-6] and the most well-known is *D. mollis* (Ma-kluea), the fruit of which is popularly employed as anthelmintics. Twelve species of Thai *Diospyros* plants have been reported for their chemical constituents [2, 4-6]. Some of them have been biologically investigated and several biological activities have been demonstrated [2, 5-6].

Diospyros glandulosa Lace, known in Thai name as "Kluai-ruesi", is an evergreen or partly deciduous tree that can grow up to 15 meters and commonly found in the hill forests [7]. The plant is one of the *Diospyros* species with no previous report on phytochemical investigation. We herein report the isolation and the identification of six pentacyclic triterpenoids, friedelin, β-amyrin, lupeol, betulin, ursolic acid and oleanolic acid, from the stem of this plant.

Material and Methods

General experimental procedures

Melting points were determined on the Fisher-Johns melting point apparatus (U.S.A.) and were uncorrected. Infrared (IR) spectra (KBr disc and thin film) were recorded on a Perkin Elmer infrared spectrophotometer model 283. Electron impact-mass spectra (EI-MS) were obtained with a Finnigan Polaris Q gas chromatography-mass spectrometer (Direct Probe Controller) operating at 50 eV. Nuclear magnetic resonance (NMR) spectra in $CDCl_3$ and DMSO-d_6 were recorded on a JEOL JNM-A500 (Alpha series) 500 MHz NMR spectrometer or a Bruker Avance DPX-300 300 MHz FT-NMR spectrometer. Thin layer chromatography (TLC) was performed with precoated silica gel 60 F254 plates (0.25 mm), and detection was done by spraying with 10% sulfuric acid in EtOH and heating at 110°C.

Plant material

The stem of *Diospyros glandulosa* Lace was collected from Doi Phukha National Park, Nan province, Thailand, in May, 2000. The plant was identified by comparison with a herbarium specimen at the Royal Forest Department, Bangkok, Thailand. Its herbarium specimen was deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and isolation

The dried, powdered stem of *Diospyros glandulosa* Lace (750 g) was extracted with 95% EtOH (8 × 3 L) at room temperature to yield 54.7 g of dried crude extract. The crude EtOH extract was then suspended in aq. MeOH and partitioned successively with *n*-hexane and $CHCl_3$ to give 16.1 g of hexane extract and 14.7 g of $CHCl_3$ extract. The hexane extract was subjected to a silica gel column, eluted with solvent mixtures of increasing polarity (*n*-hexane-$CHCl_3$ to $CHCl_3$-MeOH), to give six fractions (A-F). Fraction B (hexane-$CHCl_3$ = 2:3 eluate) yielded friedelin (**1**, 50.3 mg) upon recrystallization from MeOH. Fraction D (hexane-$CHCl_3$ = 1:4 eluate) was rechromatographed on a silica gel column with $CHCl_3$-MeOH (99:1) to afford four subfractions (D1-D4). Subfraction D2 was further purified by recrystallization from MeOH to yield β-amyrin (**2**, 20.3 mg). Fraction E ($CHCl_3$ eluate), upon recrystallization from MeOH, afforded lupeol (**3**, 33.1 mg).

The $CHCl_3$ extract was divided into two parts. One portion (8.6 g) was subjected to a silica gel column, eluted with increasing gradient of MeOH in $CHCl_3$ to give five fractions (G-K). Fraction H ($CHCl_3$-MeOH = 99:1 eluate) was further separated on a silica gel column,

eluted with $CHCl_3$, to give five subfractions (H1-H5). Subfraction H3 yielded betulin (**4**, 41.1 mg) upon recrystallization from MeOH. Fraction I ($CHCl_3$-MeOH = 98:2 to 95:5 eluate) was rechromatographed on a silica gel column with $CHCl_3$-MeOH (99:1) to afford five subfractions (I1-I5). Further separation of subfraction I5 on a silica gel column, using $CHCl_3$-MeOH (99:1) as eluent, afforded ursolic acid (**5**, 108.2 mg).

The other portion of $CHCl_3$ extract (5.3 g) was subjected to a silica gel column, eluted with increasing gradient of MeOH in $CHCl_3$, to give five fractions (L-P). Fraction N ($CHCl_3$-MeOH = 97:3 eluate) was further separated on a silica gel column, eluted with increasing gradient of acetone in $CHCl_3$, to afford oleanolic acid (**6,** 50.4 mg). Fractions G and L, each of which was the first fraction obtained from each portion of $CHCl_3$ extract, were combined and subjected to a silica gel column, eluted with $CHCl_3$, to give lupeol (**3**, 25.4 mg).

Friedelin (1) : colorless needles (MeOH), mp 262-263 ˚C; IR ν_{max} (KBr) cm^{-1} : 3405, 2927, 2869, 1715, 1463, 1389, 1050; EI-MS *m/z* (% rel. int.): 426 $[M]^+$ (26), 341 (24), 302 (22), 273 (62), 246 (66), 231 (84), 123 (80), 109 (100), 95 (98), 81 (79); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.70 (3H, *s*, Me-24), 0.85 (3H, *s*, Me-25), 0.86 (3H, *d*, *J* = 6.0 Hz, Me-23), 0.93 (3H, *s*, Me-29), 0.94 (1H, *m*, H-22a), 0.98 (6H, *s*, Me-26, Me-30), 1.03 (3H, *s*, Me-27), 1.16 (3H, *s*, Me-28), 1.27 (1H, *m*, H-6a), 1.33 (1H, *m*, H-7a), 1.37 (1H, *m*, H-8), 1.45 (1H, *m*, H-7b), 1.48 (1H, *m*, H-22b), 1.50 (2H, *m*, H-10), 1.53 (1H, *m*, H-18), 1.67 (1H, *m*, H-1a), 1.74 (1H, *m*, H-6b), 1.96 (1H, *m*, H-1b), 2.23 (2H, *m*, H-2a, H-4), 2.39 (1H, *m*, H-2b); ^{13}C-NMR ($CDCl_3$, 75 MHz): see Table 1.

β-amyrin (2) : colorless needles (MeOH), mp 195-196 ˚C; IR ν_{max} (KBr) cm^{-1} : 3282, 2948, 1464, 1386, 1360, 1036, 996; EI-MS *m/z* (% rel. int.): 426 $[M]^+$ (9), 218 (100), 203 (35), 189 (12); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.77 (3H, *s*, Me-25), 0.81 (3H, *s*, Me-28), 0.85 (6H, *s*, Me-29, Me-30), 0.92 (3H, *s*, Me-24), 0.95 (3H, *s*, Me-26), 0.98 (3H, *s*, Me-23), 1.12 (3H, *s*, Me-27), 3.20 (1H, *dd*, *J* = 10.7, 5.0 Hz, H-3), 5.17 (1H, *t*, *J* = 3.0 Hz, H-12); ^{13}C-NMR ($CDCl_3$, 75 MHz): see Table 1.

Lupeol (3) : white amorphous powder (MeOH), mp 214-215˚C; IR ν_{max} (KBr) cm^{-1} : 3486, 2934, 1474, 1443, 1384, 1037, 815; EI-MS *m/z* (% rel. int.): 426 $[M]^+$ (27), 411 (25), 393 (10), 218 (93), 207 (66), 204 (82), 189 (100); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.66 (1H, *d*, *J* = 8.7 Hz, H-5), 0.74 (3H, *s*, Me-24), 0.76 (3H, *s*, Me-28), 0.81 (3H, *s*, Me-25), 0.92 (3H, *s*, Me-27), 0.94 (3H, *s*, Me-23), 1.01 (3H, *s*, Me-26), 1.66 (3H, *s*, Me-30), 2.36 (1H, *m*, H-19), 3.17 (1H, *dd*, *J* = 10.2, 5.1 Hz, H-3), 4.55 (1H, *br s*, H-29 a), 4.67 (1H, *br s*, H-29b); ^{13}C-NMR ($CDCl_3$, 75 MHz): see Table 1.

Betulin (4) : white amorphous powder (MeOH), mp 251-252˚C; IR ν_{max} (KBr) cm^{-1} : 3443, 2934, 2867, 1641, 1459, 1373, 1011, 879; EI-MS *m/z* (% rel. int.): 442 $[M]^+$ (12), 411 (33), 393 (17), 234 (27), 207 (48), 203 (100), 189 (99), 175 (41), 147 (42), 133 (45), 119 (46), 95 (487), 81 (31); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.66 (1H, *d*, *J* = 9.3 Hz, H-5), 0.74 (3H, *s*, Me-24), 0.80 (3H, *s*, Me-25), 0.95 (3H, *s*, Me-23), 0.96 (3H, *s*, Me-27), 1.00 (3H, *s*, Me-26), 1.66 (3H, *s*, Me-30), 2.36 (1H, *m*, H-19), 3.16 (1H, *dd*, *J* = 10.8, 5.1 Hz, H-3), 3.31 (1H, *d*, *J* = 10.8 Hz, H-28a), 3.78 (1H, *d*, *J* = 10.8 Hz, H-28b), 4.56 (1H, *br s*, H-29a), 4.66 (1H, *br s*, H-29b); ^{13}C-NMR ($CDCl_3$, 75 MHz): see Table 1.

Ursolic acid (5) : colorless prisms (hexane), mp 282-283˚C; IR ν_{max} (KBr) cm^{-1}: 3417, 2927, 2871, 1694, 1456, 1386, 1030, 997; EI-MS *m/z* (% rel. int.): 248 (100), 219 (26), 203 (68), 189 (22), 133 (61); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 0.67 (3H, *s*, Me-24), 0.74 (3H, *s*, Me-26), 0.80 (3H, *d*, *J* = 6.4 Hz, Me-29), 0.86 (3H, *s*, Me-25), 0.88 (3H, s, Me-23), 0.89 (3H, *d*, *J* = 8.9 Hz, Me-30), 1.03 (3H, *s*, Me-27), 2.10 (1H, *d*, *J* = 11.6 Hz, H-18), 2.99 (1H, *dd*, *J* = 10.1, 5.2 Hz, H-3), 5.12 (1H, *br t*, *J* = 3.4 Hz, H-12); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 1.

Oleanolic acid (6) : colorless needles (MeOH), mp 297-298˚C; IR ν_{max} (KBr) cm^{-1}: 3432, 2948, 1695, 1463, 1387, 1182, 1029; EI-MS *m/z* (% rel. int.): 248 (100), 207 (26), 203 (75), 189 (12); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.73 (3H, *s*, Me-26), 0.75 (3H, *s*, Me-24), 0.88 (3H, *s*, Me-29), 0.89 (3H, *s*, Me-25), 0.91 (3H, *s*, Me-30), 0.96 (3H, *s*, Me-23), 1.11 (3H, *s*, Me-27), 2.80 (1H, *d*, *J* = 10.2 Hz, H-18), 3.20 (1H, *dd*, *J* = 10.1, 4.7 Hz, H-3), 5.26 (1H, *br s*, H-12); ^{13}C-NMR ($CDCl_3$, 75 MHz): see Table 1.

Table 1 ^{13}C-NMR spectral data of compounds **1-6.** (**1-4** and **6**, in $CDCl_3$; **5**, in DMSO-d_6)

C	1	2	3	4	5	6
1	22.3	38.6	38.7	38.7	38.2	38.4
2	41.5	27.2	27.4	27.0	27.0	27.2
3	213.2	79.0	79.0	79.0	76.8	79.0
4	58.2	38.8	38.9	38.8	38.5	38.7
5	42.1	55.2	55.3	55.3	54.8	55.2
6	41.3	18.4	18.3	18.3	18.0	18.3
7	18.2	32.5	34.3	34.2	32.7	32.6
8	53.1	39.8	40.8	40.9	39.1	39.2
9	37.4	47.6	50.4	50.4	47.0	47.6
10	59.5	36.9	37.2	37.1	36.5	37.1
11	35.6	23.6	20.9	20.8	22.8	23.4
12	30.5	121.7	25.1	25.2	124.6	122.6
13	39.7	145.2	38.0	37.3	138.2	143.6
14	38.3	41.7	42.8	42.7	41.6	41.6
15	32.4	26.1	27.4	27.3	27.5	27.7
16	36.0	26.9	35.6	29.2	23.8	22.9
17	30.0	32.6	43.0	47.8	46.8	46.5
18	42.8	47.2	48.3	48.7	52.4	40.9
19	35.3	46.8	48.0	47.8	38.5	45.8
20	28.2	31.1	151.0	150.5	38.4	30.6
21	32.8	34.7	30.0	29.7	30.2	33.8
22	39.2	37.1	40.0	33.9	36.3	32.4
23	6.8	28.1	28.0	28.0	28.2	28.1
24	14.6	15.5	15.4	15.3	16.1	15.5
25	17.9	15.6	16.1	16.1	15.2	15.3
26	20.2	16.8	16.0	16.0	16.9	17.1
27	18.6	26.0	14.5	14.7	23.3	25.9
28	32.1	28.4	18.0	60.5	178.3	183.6
29	35.0	33.3	109.3	109.7	17.0	33.0
30	31.8	23.7	19.3	19.1	21.0	23.6

Results and Discussion

Chromatographic separation of the hexane and $CHCl_3$ extracts of *Diospyros glandulosa* stem led to the isolation of six compounds. Compounds **1** and **2** were obtained from the hexane extract whereas compounds **4-6** from the $CHCl_3$ extract. Both of the extracts yielded compound **3**. All of these compounds appeared to be triterpenoids, based on the positive Liebermann-Burchard test and the number of ^{13}C-NMR signals. Their structures are shown in Figure 1.

Compound **1** was identified as friedelin [8]. Its EI-MS molecular ion peak at *m/z* 426 corresponded to the molecular formula of $C_{30}H_{50}O$. The fragment peak at *m/z* 273, together with those at *m/z* 302 and 341, was suggestive of a friedelane derivative with 3-keto substituent [9]. Its IR spectrum displayed the carbonyl absorption at 1715 cm^{-1}. One secondary and seven tertiary methyls of the friedelane skeleton were observed in the ^{1}H-NMR spectrum as a doublet at δ 0.86 (J = 6.0 Hz, Me-23) and singlets at δ 0.70 (Me-24), 0.85 (Me-25), 0.93 (Me-29), 0.98 (Me-26, Me-30), 1.03 (Me-27), 1.16 (Me-28), respectively. The most downfield carbon signal at δ 213.2 represented the 3-keto group of friedelin.

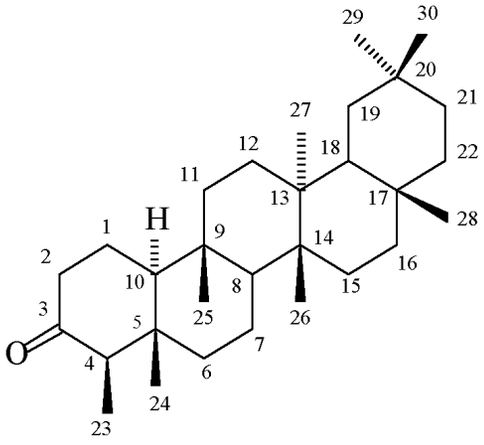
Compound **2** was found to be β-amyrin [10], a C-12 unsaturated triterpenoid of the oleanane type. Its EI-MS showed molecular ion peak at *m/z* 426, corresponding to the molecular formula of $C_{30}H_{50}O$. The base peak at *m/z* 218, produced through retro-Diels-Alder fragmentation, was the characteristic feature of a C-12 unsaturated oleanane containing no substiuents in both ring D and E [11]. The further loss of one methyl group produced the mass fragment peak at *m/z* 203, and the loss of water from the other retro-Diels-Alder fragment led to the peak at *m/z* 189. The ^{1}H-NMR spectrum of **2** displayed singlets of eight tertiary methyls, indicative of the oleanane skeleton, at δ 0.77 (Me-25), 0.81 (Me-28), 0.85 (Me-29, Me-30), 0.92 (Me-24), 0.95 (Me-26), 0.98 (Me-23) and 1.12 (Me-27). A double doublet (J = 10.7, 5.0 Hz) at δ 3.20 was assignable to the carbinylic proton (H-3) whereas the most downfield signal, a triplet (J = 3.0 Hz) at δ 5.17, to the olefinic proton (H-12). A pair of downfield carbon signals at δ 121.7 and 145.2 represented the C-12 unsaturation of the compound.

Compounds **3** and **4** appeared to be lupane-type triterpenoids, as suggested by the very intense fragment peak at *m/z* 189 in their EI-MS [11]. The molecular ion peak of **3** at *m/z* 426 corresponded to the molecular formula of $C_{30}H_{50}O$, whereas that of **4** appeared at 16 mass units higher, suggesting an additional hydroxy group. The ^{1}H-NMR spectrum of **3** displayed characteristic proton signals of the isopropenyl group, a downfield singlet of vinylic methyl (Me-30) at δ 1.66 and a pair of broad singlets due to exomethylene protons (H-29) at δ 4.55 and 4.67. In that of **4**, the former appeared at δ 1.66 and the latter at δ 4.56 and 4.66. The double doublet at δ 3.17 (J = 10.2, 5.1 Hz) in the spectrum of **3** or at δ 3.16 (J = 10.8, 5.1 Hz) in that of **4** was typical for a triterpenoid with 3-hydroxy substituent. The NMR data of **4** were similar to those of **3** except for the absence of one methyl singlet and the addition of a pair of doublets (J = 10.8 Hz) at δ 3.31 and 3.78 in the ^{1}H-NMR spectrum, as well as the absence of one methyl signal and the addition of hydroxymethylene signal at δ 60.5 in the ^{13}C-NMR spectrum. This information suggested that **4** was a derivative of **3**, of which one methyl was replaced by the primary alcoholic group. All spectral data of **3** were found to be in full agreement with those of lupeol [12], and in case of **4**, with those of betulin [13]. Therefore, **3** and **4** were identified as lupeol and betulin, respectively.

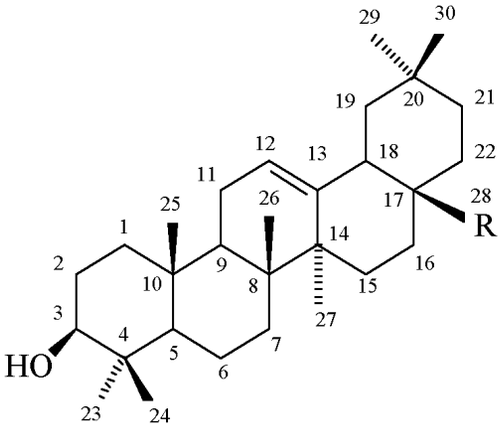
Compounds **5** and **6** are isomeric triterpenoid acids, identified as ursolic acid [14] and oleanolic acid [15], respectively. The base peak at *m/z* 248 of each compound was characteristic of a C-12 unsaturated triterpenoid with ursane or oleanane skeleton containing carboxylic group in ring D or E, resulting from cleavage through retro-Diels-Alder reaction [11]. The further loss of the carboxylic group led to the peak at *m/z* 203, whereas the loss of water from the other retro-Diels-Alder fragment produced the peak at *m/z* 189. The carbonyl band in the IR spectrum was observed at 1694 cm^{-1} (compound **5**) or 1695 cm^{-1} (compound **6**).

The ^{1}H-NMR spectrum of **5** exhibited two doublets of secondary methyls and five singlets of tertiary methyls, signifying its ursane nature, while seven methyls of **6**, an oleanane, were all observed as singlets. The olefinic proton (H-12) of **5** resonated as a triplet at δ 5.12 (*J* = 3.4 Hz) and that of **6** as a broad singlet at δ 5.26. The carbinylic protons (H-3) of **5** and **6** were observed as a double doublet at δ 2.99 (*J* = 10.1, 5.2 Hz) and at δ 3.20 (*J* = 10.1, 4.7 Hz), respectively. The most downfield signals at δ 178.3 in ^{13}C-NMR spectra of **5** and δ 183.6 in that of **6** represented their carboxylic groups.

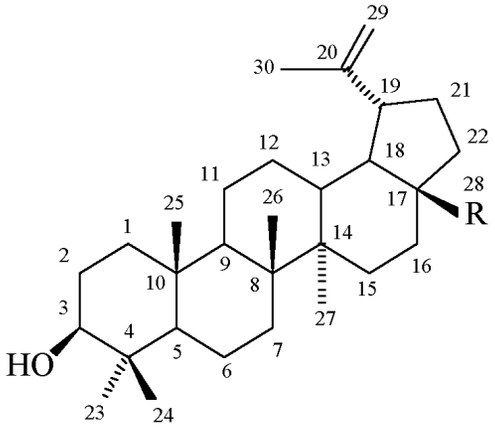
It is noted here that the ^{13}C-NMR assignment of **5** as shown in Table 1 is slightly different from that previously reported [14]. The difference is reversed chemical shift assignments for C-11 (**5**, δ 22.8; reported value, δ 17.1) and C-29 (**5**, δ 17.0; reported value, δ 23.2). The signal assignments for C-11 and C-29 of **5** were based on data obtained from the DEPT (Distortionless Enhancement by Polarization Transfer) experiments which indicated that those signals belonged to a methylene and a methyl, respectively. The assignment for C-11 was also confirmed by the



1



2: R = CH_3
6: R = COOH



3: R = CH_3
4: R = CH_2OH

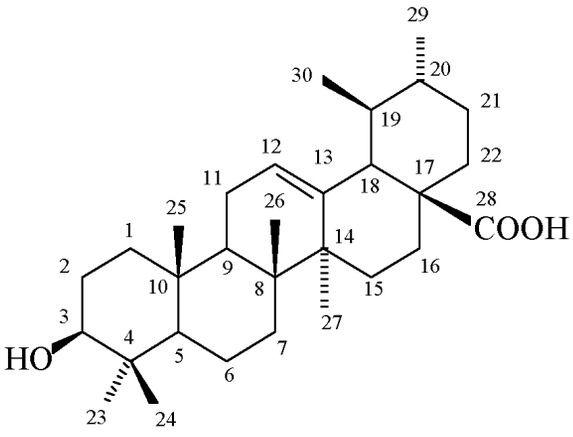


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Figure 1 Structures of the isolated compounds [**1-6**] from the stem of *Diospyros glandulosa*.

HMBC (^{1}H-detected Heteronuclear Multiple-Quantum Coherence via Multiple-Bond Coupling) spectrum in which the correlation between the proton signal at δ 5.12 (*t*, *J* = 3.4 Hz, H-12) and the carbon signal at δ 22.8 (C-11) was observed.

The six triterpenoids isolated from *D. glandulosa* are all biologically active. All of them have been reported to exhibit anti-inflammatory activity [16-18], and, except for friedelin, the cytotoxic activity of the compounds has been demonstrated [16, 19]. Lupeol, betulin, ursolic acid and oleanolic acid have been found to be antimycobacterial [20]. Other bioactivities of these compounds have also been documented e.g. antitumor activity of lupeol and betulin, antihepatotoxic and antifertility activities of oleanolic acid, and antileukemic and hepatoprotective activities of ursolic acid [16].

Of more than 130 *Diospyros* species subjected to phytochemical investigation, about forty species, including some Thai species (*D. castanea*, *D. cauliflora*, *D. curranii* and *D. rhodocalyx*), have never been reported as sources of naphthoquinones. In this study, the detection for the presence of quinone derivatives in the stem of *D. glandulosa* by modified Borntrager's test has also been performed and the result was found to be negative. The phytochemical data of the genus *Diospyros* pointed out that the stem was one of the plant parts in which naphthoquinones have been frequently found. The absence of naphthoquinones in the stem of *D. glandulosa* may imply the limited ability of the plant in producing compounds of this group.

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