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Article

New Flavanol and Cycloartane Glucosides from *Landoltia punctata*

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Abstract: Chemical investigation on the constituents of *Landoltia punctata* led to the isolation and identification of 17 compounds, four of which were new and identified as (3*β*,24*S*)-9,19-cycloartane-3,22,24,25-tetraol 3-*O*-[*β*-D-glucopyranosyl-(1→2)]-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**1**), (3*β*,24*S*)-9,19-cycloartane-3,24,25-triol 3-*O*-[*β*-D-glucopyranosyl-(1→2)]-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**2**), 3,4'-dihydroxy-7,3'-dimethoxyflavan-5-*O*-*β*-D-glucopyranoside (**3**) and 3,4'-dihydroxy-4,7,3'-trimethoxyflavan-5-*O*-*β*-D-glucopyranoside (**4**). Their structures were elucidated by spectroscopic, chemical, and biochemical methods. Thus, cycloartane triterpenoids were discovered in the Lemnaceae family for the first time. Compound **3** showed antioxidant capacity in the positively charged 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical ($ABTS^{+\bullet}$) and superoxide anion radical scavenging assays.

Keywords: *Landoltia punctata*; flavanol glucoside; cycloartane glucoside; antioxidant

1. Introduction

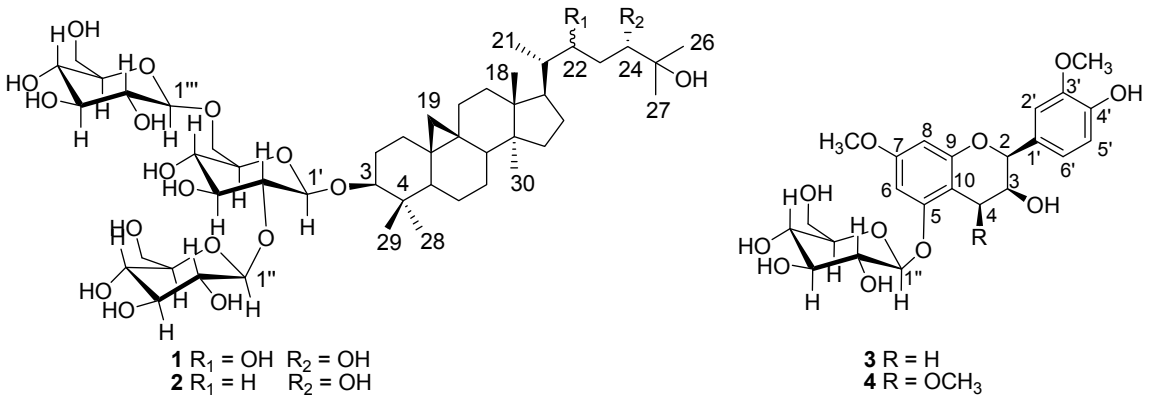
The plant of the Lemnaceae, commonly known as duckweed, is a kind of aquatic monocot angiosperm and the smallest with flowers, which is widely distributed on the surface of still, slowly flowing, and polluted waters around the World. In this family, there are 37 species, representing five genera: *Lemna*, *Landoltia*, *Spirodela*, *Wolffia* and *Wolffiella* [1,2]. Duckweeds grow very fast and proliferate quickly by budding, allowing them to colonize freshwater habitats rapidly and produce 13

to 38 metric tons/hectare/year dry weight of plant mass [3,4]. Since duckweeds can absorb pollutants (eg. N, P and heavy metals) from wastewater, they have been commonly used in the treatment of domestic and animal wastewater streams for many years [5–7]. Meanwhile, because of the relatively high starch and low lignin content in this plant, it has been proved to be an ideal bioresource for bioethanol production [4,8]. Supported by the Minister of Science and Technology and the Major Projects of Knowledge Innovation Program of Chinese Academy of Sciences, our group have been focused on the exploitation of duckweed in the fields of biofuels and natural products.

In our previous study, *Landoltia punctata* demonstrated obvious advantages in the biofuel aspect for its higher starch content and easier acquisition among the five genera in Lemnaceae [9]. Large scale cultivation of *L. punctata* has been carried out by our group, and tons of *L. punctata* biomass can be obtained in a very short time. It is no doubt that *L. punctata* has become a new resource for natural products such as proteins, polysaccharides, amino acids, and other small molecules.

There are a few reports about the chemical composition of *L. punctata*. To date, only eight compounds have been reported. One is an anthocyanin, and the other seven isolated by paper chromatography were determined by R_f values, UV spectral maxima and color reactions to be saponarin, isosaponarin, saponaretin and the glucosides of saponaretin and vitexin [10]. Detailed chemical exploration on this plant is imperative. In this study, we investigated the chemical components of *L. punctata* by chromatographic and spectroscopic methods. As a result, four new compounds: (3*β*,24*S*)-9,19-cycloartane-3,22,24,25-tetraol 3-*O*-[*β*-D-glucopyranosyl-(1→2)]-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**1**), (3*β*,24*S*)-9,19-cycloartane-3,24,25-triol 3-*O*-[*β*-D-glucopyranosyl-(1→2)]-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**2**), 3,4'-dihydroxy-7,3'-dimethoxyflavan-5-*O*-*β*-D-glucopyranoside (**3**) and 3,4'-dihydroxy-4,7,3'-trimethoxyflavan-5-*O*-*β*-D-glucopyranoside (**4**), (Figure 1), together with 13 known ones apigenin (**5**), luteolin (**6**), apigenin-7-*O*-*β*-glucoside (**7**), luteolin-7-*O*-*β*-glucoside (**8**) [11], vitexin (**9**), isovitexin (**10**), orientin (**11**), isoorientin (**12**) [12], 6,8-di-*C*-*β*-glucosylapigenin (**13**) [13], 6-*C*-*β*-glucosyl-8-*C*-*β*-galactosylapigenin (**14**) [14], *β*-sitosterol (**15**), stigmasterol (**16**) [15], 5,22-diene-3,6-dicarbonyl-stigmasterol (**17**) [16] were isolated and identified from the 95% ethanol extract of *L. punctata*.

Figure 1. Chemical structures of **1**–**4**.



This is the first report of cycloartane-type triterpenoids from the Lemnaceae family. Here the isolation and structural elucidation of these compounds as well as bioactivities of compound **3** were described.

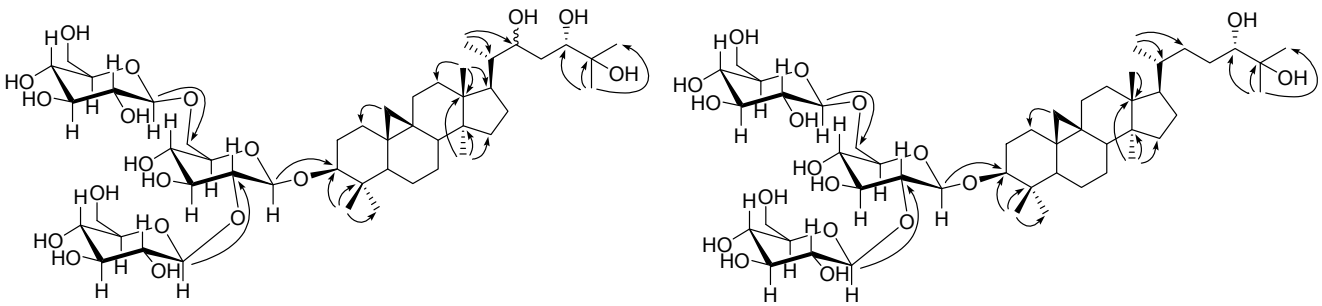
2. Results and Discussion

2.1. Structure Elucidation of Compounds **1**–**4**

Compound **1** was obtained as an amorphous powder. Its HR-ESI-MS quasi-molecular ion peak at *m/z* 985.5371 ([M+Na]$^+$) corresponded to a molecular formula of $C_{48}H_{82}O_{19}$, which was supported by the NMR signals. ^{1}H-NMR spectrum of **1** showed characteristic signals of a cyclopropane CH_2 at δ_H 0.64 (*d*, *J* = 3.72 Hz, H-19a) and 0.44 (*d*, *J* = 3.72 Hz, H-19b), six tertiary Me groups (δ_H 1.11, 1.24, 1.23, 1.13, 0.95, 1.01; respectively, H_3-18, H_3-26, H_3-27, H_3-28, H_3-29, H_3-30) and a secondary Me group at δ_H 0.95 (H_3-21) [17,18]. Additionally, three anomeric proton signals were observed at δ_H 4.74 (*d*, *J* = 7.68 Hz), 4.52 (*d*, *J* = 7.20 Hz) and 4.48 (*d*, *J* = 7.74 Hz), indicative of the presence of three *β*-linked sugar units. The ^{13}C-NMR and DEPT spectra permitted differentiation of the 48 resonances into 6 C, 22 CH, 13 CH_2, and 7 CH_3 groups, of which 30 were attributed to a triterpene skeleton and 18 to three hexose groups. Acid hydrolysis of **1** gave D-glucoses with optical rotation of $[\alpha]_D^{20}$ + 51.2, which was determined by TLC analysis and optical rotation measurement [19,20]. Therefore, **1** was considered to be a cycloartane-type triterpene glucoside.

In the HMBC spectrum (Figure 2), correlations of δ_H 1.13 (H-28) and δ_H 0.95 (H-29) with δ_C 90.9 (C-3) suggested that C-3 was substituted by the OH group; 26-CH_3, 27-CH_3 with C-24 (δ_C 76.2) and C-25 (δ_C 74.0), and 21-CH_3 with C-22 (δ_C 71.3) indicated the presence of 24-OH, 25-OH and 22-OH respectively. The glycosidic linkage was determined on the basis of following key spectral signals: HMBC correlations of H-1' with C-3, H-1'' with C-2', and H-1''' with C-6', ^{1}H,^{1}H-COSY cross peaks of H-1'/H-2', H-6'/H-5', and the NOESY cross peaks of H-1'/H-3' and H-5'. Furthermore, the stereochemistry of C-24 was assigned to be *S* by comparing its spectral data with those reported for analogs [21,22]. Additionally, NOESY correlations between δ_H 1.62 (H-8) and δ_H 0.44 (H-19a)/18-CH_3, 18-CH_3 and δ_H 1.79 (H-20), δ_H 0.44 (H-19a) and 29-CH_3, δ_H 2.04 (H-16b) and δ_H 4.01 (H-22)/30-CH_3, 21-CH_3 and δ_H 1.80 (H-17), H-17 and 30-CH_3 were observed, revealing their relative configuration (Figure 3).

Figure 2. Key HMBC correlations of **1** and **2**.

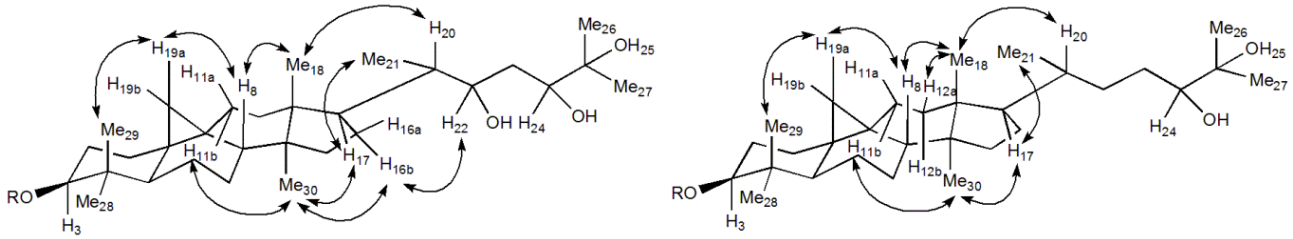


Consequently, the structure of **1** was established as (3*β*,24*S*)-9,19-cycloartane-3,22,24,25-tetraol 3-*O*-[*β*-D-glucopyranosyl-(1→2)]-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside.

The HR-ESI-MS spectrum of **2** (*m/z* 969.5406 [*M* + Na]$^+$, calc. for $C_{48}H_{82}O_{18}Na$) supported a molecular formula of $C_{48}H_{82}O_{18}$. The NMR spectra of **2** were very similar to those of **1**, except for one less oxygenated methine at δ_C 71.3. Detailed inspection of the HMBC spectrum led to a conclusion

that **2** had one less OH on C-22 than **1**, on the basis of the key HMBC correlation of 21-CH_3 (δ_H 1.01) with C-22 (δ_C 23.2). The linkage of three glucoses on the triterpenoid aglycon was determined to be the same way as **1** on the basis of HMBC and ^{1}H,^{1}H-COSY signals. The stereochemistry of **2** was determined on the basis of NOESY cross peaks of H-8 and H-19a/18-CH_3, 18-CH_3 and H-12a, H-19a and 29-CH_3, H-16b and 30-CH_3, 21-CH_3 and H-17, H-17 and 30-CH_3. Acid hydrolysis of **2** gave D-glucoses, which was identified by comparing the optical rotation value with an authentic sample. Finally the structure of **2** was determined to be (3*β*,24*S*)-9,19-cycloartane-3,24,25-triol 3-*O*-[*β*-D-glucopyranosyl-(1→2)]-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside.

Figure 3. Key NOE correlations of **1** and **2**.



Compound **3** was obtained as colorless needles. Its molecular formula was deduced to be $C_{23}H_{28}O_{11}$ from the quasi-molecular ion peak at *m/z* 479.1549 ([M–H]$^-$) in the HR-ESI-MS spectrum, indicating 10 degrees of unsaturation. The IR spectrum revealed the existence of OH (3434 cm^{-1}) and aromatic ring (1623 cm^{-1}). The ^{1}H-NMR spectrum displayed two meta-coupled protons at δ_H 6.49 (*d*, *J* = 2.16 Hz) and 6.29 (*d*, *J* = 2.16 Hz), and three ABX system aromatic protons at δ_H 7.22 (*d*, *J* = 1.06 Hz), 6.99 (*dd*, *J* = 8.16, 1.06 Hz), and 6.87 (*d*, *J* = 8.16 Hz). In addition, two methoxyl H-atoms at δ_H 3.82 (*s*, 3H) and 3.95 (*s*, 3H), two CH at δ_H 4.29 (br. *s*) and 4.98 (br. *s*), and one CH_2 at δ_H 3.09 (*d*, *J* = 17.60 Hz, 1H) and 3.02 (*dd*, *J* = 17.60, 4.38 Hz, 1H) were observed. Meanwhile an anomeric H-atom at δ_H 4.95 (*d*, *J* = 7.20 Hz) together with the signals at δ_H 3.42-3.55 indicated the presence of a *β*-linked glycosyl group. Enzymatic hydrolysis of **3** with *β*-D-glucosidase afforded D-glucose, which was identified by direct comparison with the authentic sample by TLC [19]. In view of above evidences, it was concluded that **3** was a flavanol glucoside.

In the combination of the ^{13}C-NMR and HSQC spectra of **3**, the 23 carbon resonances could be easily attributed to a flavanol moiety, a glucopyranose unit (δ_C 62.7, 71.6, 75.1, 78.2, 78.4, 102.8), and two methoxyls (δ_C 56.0, 56.6). In order to determine the location of substituent groups on the flavanol moiety, HMBC and NOESY experiments were performed. As a result, the NOESY correlations of the methoxyl H-atoms at δ_H 3.82 with H-6 and H-8, H-6 with H-1', and the other methoxyl H-atoms at δ_H 3.95 with H-2', together with the HMBC correlations of 7-OCH_3 to C-7, 3'-OCH_3 to C-3', indicated the glucose and two methoxyls were situated at C-5, C-7, and C-3' respectively (Figures 4 and 5).

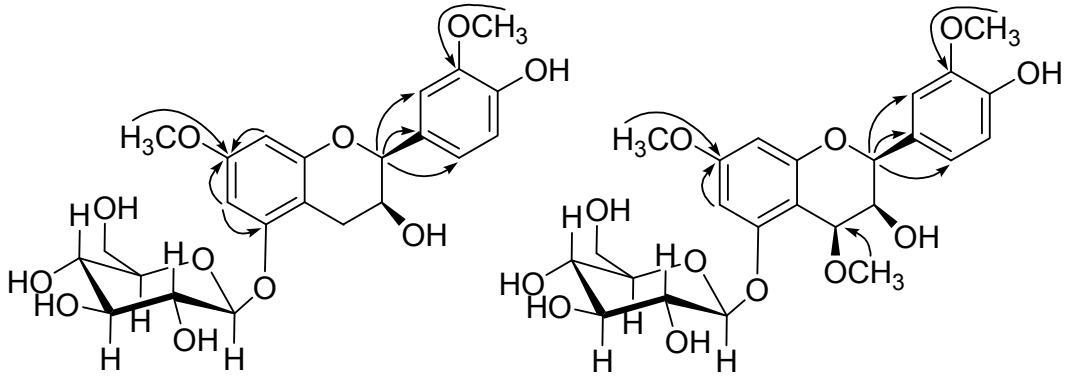
The NOESY correlation of δ_H 4.98 (H-2)/δ_H 4.29 (H-3) and resonance of H-2 as a broad singlet indicated that the relative configuration of 2,3 was cis [23–28]. Therefore, the structure of **3** was elucidated to be *cis*-3,4'-dihydroxy-7,3'-dimethoxyflavan-5-*O*-*β*-D-glucopyranoside.

Compound **4** was isolated as amorphous powder. The molecular formula was $C_{24}H_{30}O_{12}$, determined by negative-ion at *m/z* 509.1662 in the HR-ESI-MS. Enzymatic hydrolysis of compound **4**

with *β*-D-glucosidase gave D-glucose. Comparing its NMR spectra with those of **3**, it was evident that **4** contained one more methoxyl at δ_H 3.65/δ_C 56.6 and one oxygenated CH (δ_C 73.5) than **3**.

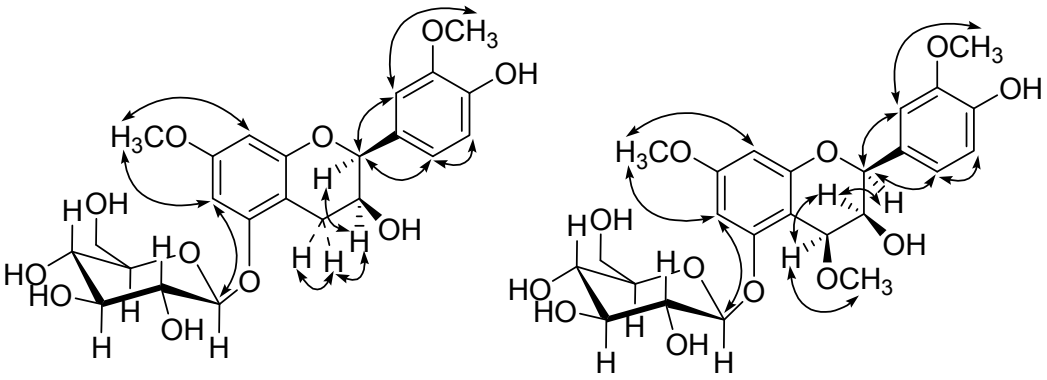
The location of one more methoxyl at C-4 was determined by the HMBC correlation of 4-OCH_3 at δ_H 3.65 with C-4 (δ_C 73.5). The other substitutions of **4** were confirmed by the same way as **3**.

Figure 4. Key HMBC correlations of **3** and **4**.



The configuration of **4** was elucidated on the basis of NOESY correlations and coupling constants. Resonances of H-2 as a broad singlet and H-4 as a small doublet (J = 2.28 Hz) were observed, which suggested that the relative 2,3/3,4-stereochemistry were both *cis* [26]. Additionally the NOESY correlation between δ_H 5.05 (H-2)/δ_H 4.62 (H-4) also supported the above assignment. The structure of **4** was identified to be *cis*-3,4'-dihydroxy-4,7,3'-trimethoxyflavan-5-*O*-*β*-D-glucopyranoside.

Figure 5. Key NOE correlations of **3** and **4**.



2.2. Biological Activity Assay

According to previous bioactivity reports on flavonoids [29–31], antimicrobial and antioxidant activities of compound **3** were evaluated. Two pathogenic bacteria (*Bacillus subtilis*, *Escherichia coli*) and two fungi (*Aspergillus niger*, *Saccharomyces cerevisiae*) were selected for antifungal and antibacterial assays, respectively. The compound **3** showed no antimicrobial activity (C > 50 μg/mL). In the ABTS cation radical scavenging assay, the inhibition rate of **3** was 82.0% at 1 mg/mL (Positive control vitamin C, 75.2%, C = 0.1 mg/mL). The superoxide anion radical scavenging assay suggested that **3** showed 53.5% superoxide anion radical scavenging capacity at 2 mg/mL. While the positive

control luteolin was 53.8% at 0.625 mg/mL. Unfortunately, the low available amount of compounds **1**, **2**, and **4** precluded the antimicrobial and antioxidant assays.

3. Experimental

3.1. General Information

Column chromatography (CC): silica gel (SiO_2, 200–300 mesh, Qingdao Marine Chemical Plant, Qingdao, P. R. China), MCI gel (Mitsubishi Chemical Corporation, Tokyo, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, USA). TLC: silica gel GF_{254} (Qingdao Marine Chemical Plant, Qingdao, P. R. China). UV absorbance of antioxide mixtures: Varioskan Flash Reader (ThermoFisher Scientific Inc., Waltham, MA). UV spectra: Perkin-Elmer S2 Lambda 35 UV/VIS spectrometer, λ in nm. IR spectra: Perkin-Elmer Spectrum One FT-IR spectrometer, as KBr tablets, ν in cm^{-1}. Optical rotations: Perkin-Elmer 341 polarimeter. NMR spectra: Bruker Avance 600 MHz instrument, δ in ppm, J in Hz, residual solvent peak as reference. HR-ESI-MS: BioTOF-Q mass spectrometer, in *m/z*.

3.2. Material

The duckweed *Landoltia punctata* (G. Meyer) Les & Crawford was grown under natural conditions and collected in Kunming, Yunnan province, P. R. China. The duckweed was collected in May, washed with water, and then dried at 60 °C. The test strains *Aspergillus niger*, *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus subtilis* were obtained from Chengdu Institute of Biology, Chinese Academy of Sciences (CAS), P. R. China.

3.3. Extraction and Isolation

The powder (7 kg) of *L. punctata* was macerated in 95% ethanol (70 L) at room temperature for 4–5 days, twice. The extract was combined and concentrated under reduced pressure. Then the concentrate was suspended in hot water, and extracted with petroleum ether (P.E.), EtOAc, and *n*-BuOH successively. The P.E. extract was separated by silica gel column chromatography (CC) using P.E./acetone (10:1–1:1, *v/v*) as eluent, affording 4 fractions (Fr. 1-4), Fr. 2 and 3 were purified by Sephadex LH-20 CC to yield compounds **15**–**17** respectively.

The EtOAc fraction (25 g) was subjected to a MCI column, and eluted with a stepwise gradient of EtOH/H_2O (0:100, 20:80, 40:60, 60:40, 100:0, *v/v*) to give three fractions (I-III), which were further purified by HPLC (ODS-C18 column, 10 × 250 mm, flow rate 2.5 mL/min). Fr. I was separated using MeOH/H_2O (40:60, containing 0.5% acetic acid, *v/v*) as the eluent to yield compounds **11** and **12**. Fr. II was purified using MeOH-H_2O (45:55, containing 0.5% acetic acid, *v/v*) as the eluent to yield compounds **7**–**10**. Fr. III was separated using MeOH-H_2O (60:40, containing 0.5% acetic acid, *v/v*) as the eluent to obtain compounds **5** and **6**.

The same CC of the n-BuOH extract (60 g) on MCI gel as that of EtOAc extract gave four fractions (I–IV). Fraction II was subjected to preparative thin layer chromatography (TLC) and Sephadex LH-20 CC to afford compounds **1** (13 mg) and **2** (10 mg). Faction III was separated over silica gel with $CHCl_3$/MeOH (7:1–0:1, *v/v*) as eluent to generate 4 fractions (IIIa-IIId). Fr. IIIb was separated by HPLC using MeOH/H_2O (45:55, *v/v*) as the eluent to yield compounds **13** and **14**. Fr. IIIc and IIId

were further purified by TLC and then Sephadex LH-20 CC to yield compounds **3** (18 mg) and **4** (8 mg) respectively.

3.4. Hydrolysis

Acid hydrolysis. Compounds **1** and **2** (10 mg) were independently dissolved in aqueous solution of 2 N HCl (6 mL) and stirred at 85 °C overnight. After cooled down, the reaction mixture was neutralized and then extracted with $CHCl_3$. The water layer were concentrated to dryness, dissolved in water to constant volume, and analyzed by comparing their TLC profile and optical rotation values with the standard sample of D-glucose [21,22], respectively.

Enzymatic hydrolysis. Compounds **3** and **4** (5.0 mg) were independently suspended in water (5 mL), and excessive *β*-D-glucosidase (Shanghai Zurui Biological Technology Co., Ltd., Shanghai, China) was added, respectively. The mixture was placed in water bath at 37 °C for 48 h. The products were analyzed by TLC [21].

3.5. Spectral Data

*(3β,24S)-9,19-Cycloartane-3,22,24,25-tetraol 3-O-[β-D-glucopyranosyl-(1→2)]-[β-*D*-glucopyranosyl(1→6)]-β-D-glucopyranoside* (**1**): Amorphous powder. $[\alpha]_D^{20}$ −3.0 (*c* 0.1, MeOH). IR (KBr) ν_{max} 3411, 2932, 1075 cm^{-1}. ^1H and ^{13}C-NMR: See Table 1. HR-ESI-MS (positive mode) *m/z* 985.5371 $[M+Na]^+$ (calc. for $C_{48}H_{82}O_{19}Na$, 985.5343).

*(3β,24S)-9,19-Cycloartane-3,24,25-triol 3-O-[β-*D*-glucopyranosyl-(1→2)]-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranoside* (**2**): Amorphous powder. $[\alpha]_D^{20}$ −4.0 (*c* 0.1, MeOH). IR (KBr) ν_{max} 3418, 2891, 1043 cm^{-1}. ^1H and ^{13}C-NMR: See Table 1. HR-ESI-MS (positive mode) *m/z* 969.5406 $[M + Na]^+$ (calc. for $C_{48}H_{82}O_{18}Na$, 969.5393).

3,4'-Dihydroxy-7,3'-dimethoxyflavan-5-O-β-D-glucopyranoside (**3**): Colorless needles. $[\alpha]_D^{20}$ −42.0 (*c* 0.1, MeOH). UV (MeOH) λ_{max} (log ε) 218 (4.60), 227 (3.96), 282 (3.52) nm. IR (KBr) ν_{max} 3434, 1623, 1080 cm^{-1}. ^1H and ^{13}C-NMR: See Table 2. HR-ESI-MS (negative mode) *m/z* 479.1549 (calc. for $C_{23}H_{27}O_{11}$, 479.1559).

3,4'-Dihydroxy-4,7,3'-trimethoxyflavan-5-O-β-D-glucopyranoside (**4**): Amorphous powder. $[\alpha]_D^{20}$ −20.0 (*c* 0.1, MeOH). UV (MeOH) λ_{max} (log ε) 226 (4.02), 280 (3.60) nm. IR (KBr) ν_{max} 3420, 2926, 1597, 1424, 1079 cm^{-1}. ^1H and ^{13}C-NMR: See Table 2. HR-ESI-MS (negative mode) *m/z* 509.1662 (calc. for $C_{24}H_{29}O_{12}$, 509.1664).

3.6. Biological Activity Assay

According to the reported [32], antifungal and antibacterial activities were determined using the Oxford cup method with Methicillin sodium as the positive control. The antioxidant activity assay was performed on the DPPH, $ABTS^{+\bullet}$, and superoxide anion radical scavenging models complying with the previously published literature [33].

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of compounds **1** (in CD_3OD, *J* in Hz) and **2** (in C_5D_5N, *J* in Hz).

No.	1		2		No.	1		2	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
1	33.3	1.65–1.67 (*m*), 1.32–1.34 (*m*) (m)	30.3	2.50–2.53 (*m*), 1.25–1.26 (*m*)	25	74.0		73.1	
2	30.7	2.08–2.11 (*m*), 1.35–1.36 (*m*)	29.3	1.81–1.82 (*m*), 1.95–1.97 (*m*)	26	26.1	1.24 (*s*, 3H)	26.5	1.56 (*s*, 3H)
3	90.9	3.34–3.36 (*m*)	89.1	3.45–3.48 (*m*)	27	25.6	1.23 (*s*, 3H)	26.2	1.54 (*s*, 3H)
4	42.4		41.7		28	26.0	1.13 (*s*, 3H)	26.0	1.29 (*s*, 3H)
5	50.4	1.68–1.70 (*m*)	47.8	1.20–1.22 (*m*)	29	15.6	0.95 (*s*, 3H)	15.7	1.19 (*s*, 3H)
6	22.3	1.67–1.68 (*m*), 1.68–1.70 (*m*)	27.2	1.87–1.88 (*m*), 1.37 (overlapped)	30	20.1	1.01 (*s*, 3H)	18.6	0.99 (*s*, 3H)
7	27.4	1.39–1.40 (*m*), 1.36–1.37 (*m*)	33.5	1.50–1.51 (*m*), 1.53 (overlapped)	1'	105.3	4.52 (*d*, *J* = 7.20, 1H)	105.2	4.90 (*d*, *J* = 7.56, 1H)
8	49.7	1.60–1.63 (*m*)	53.3	1.64–1.65 (*m*)	2'	81.4	3.62–3.63 (*m*)	83.5	4.15–4.17 (*m*)
9	21.4		20.2		3'	78.2	3.61–3.62 (*m*)	77.3	4.10–4.11 (*m*)
10	27.5		26.9		4'	71.7	3.39–3.40 (*m*)	72.0	4.20–4.21 (*m*)
11	27.7	1.70–1.72 (*m*), 2.10–2.11 (*m*)	21.5	1.45–1.46 (*m*), 0.70–0.72 (*m*)	5'	77.1	3.52–3.54 (*m*)	77.2	4.03–4.04 (*m*)
12	34.2	1.72, 1.76, overlapped	32.6	1.64–1.65 (*m*), 1.24 (overlapped)	6'	70.2	3.85–3.87 (*m*), 4.16–4.18 (br. *d*)	70.4	4.29–4.31 (*m*), 4.82 (br. *d*)
13	46.9		45.9		1"	104.8	4.74 (*d*, *J* = 7.68, 1H)	106.3	5.35 (*d*, *J* = 7.62, 1H)
14	49.8		49.3		2"	76.5	3.28–3.29 (*m*)	75.6	4.04–4.05 (*m*)
15	37.0	1.40–1.41 (*m*), 1.42–1.43 (*m*)	36.7	1.65–1.66 (*m*), 1.55 (overlapped)	3"	78.4	3.60–3.61 (*m*)	78.3	4.24–4.25 (*m*)
16	28.4	1.42–1.43 (*m*), 2.03–2.05 (*m*)	28.8	1.37–1.38 (*m*), 1.63–1.64 (*m*)	4"	72.0	3.26–3,27 (*m*)	71.8	4.28–4.29 (*m*)
17	50.0	1.79–1.81 (*m*)	48.4	1.43–1.44 (*m*)	5"	78.1	3.30–3.31 (*m*)	78.6	3.90–3.92 (*m*)
18	18.7	1.11 (*s*, 3H)	19.9	0.84 (*s*, 3H)	6"	63.0	3.71–3.72 (*m*), 3.93–3.94 (*m*)	63.0	4.45–4.46 (*m*), 4.52–4.53 (*m*)
19	30.9	0.44 (*d*, *J* = 3.72)	30.0	0.24 (*d*, *J* = 4.08)	1"'	105.0	4.48 (*d*, *J* = 7.74, 1H)	105.7	5.13 (*d*, *J* = 7.80, 1H)
		0.64 (*d*, *J* = 3.72)		0.48 (*d*, *J* = 4.08)	2"'	75.4	3.24–3.26 (*m*)	75.5	4.05–4.06 (*m*)
20	44.0	1.78–1.79 (*m*)	36.1	1.23–1.24 (*m*)	3"'	78.0	3.40–3.42 (*m*)	78.8	3.94–3.96 (*m*)
21	12.7	0.95 (*s*, 3H)	18.9	1.01 (*s*, 3H)	4"'	71.9	3.31–3.32 (*m*)	71.1	4.03–4.04 (*m*)
22	71.3	4.01 (br. *d*)	23.2	0.86–0.87 (*m*), 1.25 (overlapped)	5"'	78.1	3.29–3.30 (*m*)	78.4	4.21–4.22 (*m*)
23	32.9	1.40–1.41 (*m*), 1.53–1.57 (*m*)	34.5	1.83–1.85 (*m*), 1.68 (overlapped)	6"'	63.3	3.70–3.71 (*m*), 3.90–3.92 (*m*)	63.1	4.33–4.36 (*m*), 4.48–4.50 (*m*)
24	76.2	3.58–3.60 (*m*)	79.4	3.78–3.81 (*m*)					

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of compounds **3** and **4** (in CD_3OD, *J* in Hz).

No.	3		4	
	δ_C	δ_H	δ_C	δ_H
2	80.3	4.98 (br. *s*, 1H)	78.6	5.05 (br. *s*, 1H)
3	67.4	4.29 (br. *s*, 1H)	69.2	4.04 (br. *d*, 1H)
4	29.7	3.09 (*d*, *J* = 17.60, 1H) 3.02 (*dd*, *J* = 17.60, 4.38, 1H)	73.5	4.62 (*d*, *J* = 2.28, 1H)
5	158.6		157.9	
6	97.1	6.49(*d*, *J* = 2.10)	96.6	6.54 (*d*, *J* = 1.80)
7	160.9		162.9	
8	96.5	6.29 (*d*, *J* = 2.10)	96.3	6.30 (*d*, *J* = 1.80)
9	157.3		160.4	
10	104.0		104.4	
1'	132.3		131.6	
2'	112.1	7.22 (*d*, *J* = 1.06)	112.3	7.22 (*d*, *J* = 1.10)
3'	148.8		149.0	
4'	147.2		147.4	
5'	115.9	6.87 (*d*, *J* = 8.16)	116.0	6.90 (*d*, *J* = 8.04)
6'	120.8	6.99 (*dd*, *J* = 1.06, 8.16)	121.0	7.00 (*dd*, *J* = 1.10, 8.04)
1"	102.8	4.95 (*d*, *J* = 7.20)	102.6	4.99 (*d*, *J* = 7.50)
2"	75.1	3.50–3.55 (overlapped)	75.3	3.58–3.59 (overlapped)
3"	78.4	3.50–3.55 (overlapped)	78.5	3.53–3.56 (overlapped)
4"	71.6	3.47–3.48 (*m*)	71.6	3.46–3.48 (overlapped)
5"	78.2	3.50–3.55 (overlapped)	76.9	3.53–3.56 (overlapped)
6"	62.7	3.79 (*dd*, *J* = 5.58, 12.10) 3.98 (*d*, *J* = 12.10)	62.8	3.79 (*dd*, *J* = 5.00, 12.20) 4.00 (*d*, *J* = 12.20)
MeO-4			56.6	3.65 (*s*, 3H)
MeO-7	56.6	3.82 (*s*, 3H)	56.0	3.84 (*s*, 3H)
MeO-3'	56.0	3.95 (*s*, 3H)	57.8	3.96 (*s*, 3H)

4. Conclusions

In this study, we identified 12 flavonoids including two new flavanol glucosides from *L. punctata*. The result showed that apigenin or luteolin flavonoids are the main constituents of this species, which is in good agreement with the previous reports and our previous study results that the transcripts for key enzymes of flavonoid biosynthesis in *L. punctata* expressed in high abundance at the transcriptional level [9,10,34]. Flavonoids are generally biosynthesized to cope with environmental stressors such as ultraviolet radiation, ozone, heavy metals, nutrient limitation, herbivores, and so on. The high content of flavonoids in *L. punctata* could be related to environmental stressors. Meanwhile cycloartane triterpenoids were discovered in Lemnaceae family for the first time in this study. Many cycloartane triterpenoids possessed diverse bioactivities such as anti-inflammatory, anti-tumor, anti-viral, immuno-regulatory, hypoglycemic, cardiovascular system, nervous system and hepato-protective effects [35,36], some of which offer good prospectives in medical applications. This study demonstrates that *L. punctata* is a new source for cycloartane triterpenoids.

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Author Contributions

N.N.W performed all phytochemical parts of the work and prepared a draft manuscript. T.Y and G.B.X performed the biological activity assays. Y.F offered help on the plant material. H.Z and G.Y.L initiated the study, coordinated the project, supervised the phytochemical work and prepared the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Ge, X.M.; Zhang, N.N.; Phillips, G.C.; Xu, J.F. Growing *Lemna minor* in agricultural wastewater and converting the duckweed biomass to ethanol. *Bioresour*. *Technol*. **2012**, *124*, 485–488.
2. Zhao, H.; Appenroth, K.; Landesman, L.; Salmeán, A.A.; Lam, E. Duckweed rising at Chengdu: Summary of the 1st International Conference on Duckweed Application and Research. *Plant. Mol*. *Boil*. **2012**, *78*, 627–632.
3. Cole, C.T.; Voskuil, M.I. Population genetic structure in duckweed (*Lemna minor*, Lemnaceae). *Can*. *J*. *Bot*. **1996**, *74*, 222–230.
4. Xu, J.; Zhao, H.; Stomp, A.M.; Cheng, J.J. The production of duckweed as a source of biofuels. *Biofuels* **2012**, *3*, 589–601.
5. Ozengin, N.; Elmaci, A. Performance of duckweed (*Lemna minor* L.) on different types of wastewater treatment. *J*. *Environ*. *Biol*. **2007**, *28*, 307–314.
6. Mohedano, R.A.; Costa, R.H.; Tavares, F.A.; Filho, B.P. High nutrient removal rate from swine wastes and protein biomass production by full-scale duckweed ponds. *Bioresour. Technol.* **2012**, *112*, 98–104.
7. Cheng, J.J.; Stomp, A.M. Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *Clean* **2009**, *37*, 17–26.
8. Xu, J.; Cui, W.; Cheng, J.J.; Stomp, A.M. Production of high-starch duckweed and its conversion to bioethanol. *Biosyst*. *Eng*. **2011**, *110*, 67–72.
9. Xiao, Y.; Fang, Y.; Jin, Y.; Zhang, G.; Zhao, H. Culturing duckweed in the field for starch accumulation. *Ind*. *Crop*. *Prod*. **2013**, *48*, 183–190.
10. Jurd, L.; Geissman, T.A.; Seikel, M.K. The flavonoid constituents of *Spirodela oligorrhiza* II. The flavone constituents. *Arch. Biochem*. *Biophys*. **1957**, *67*, 284–297.
11. Lee, Y.H.; Lee, I.R.; Won, W.S.; Park, C.H. Flavonoids of *Elscholtzia cristata*. *Arch. Pharm. Res.* **1988**, *11*, 247–249.

12. Rayyan, S.; Fossen, T.; Nateland, H.S.; Andersen, Q.M. Isolation and identification of flavonoids, including flavone rotamers, from the herbal drug 'crataegi folium cum flore' (hawthorn). *Phytochem*. *Anal*. **2005**, *16*, 334–341.
13. Kawasaki, M.; Kanomata, T.; Yoshitama, K. Flavonoids in the leaves of twenty-eight polygonaceous plants. *Bot*. *Mag*. *Tokyo* **1986**, *99*, 63–74.
14. Dubois, M.A.; Zoll, A.; Markham, K.R.; Bouillant, M.L.; Dellamonica, G.; Chopin, J. 6-*C*-*β*-D-glucopyranosyl-8-*C*-*β*-D-galactopyranosylapigenin from *Cerastium arvense*. *Phytochemistry* **1984**, *23*, 706–707.
15. Kamboj, A.; Salujai, A.K. Isolation of stigmasterol and *β*-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae). *Int*. *J*. *Pharm*. *Pharm*. *Sci*. **2011**, *3*, 94–96.
16. Cui, J.; Wang, H.; Huang, Y.; Xin, Y.; Zhou, A. Synthesis and cytotoxic analysis of some disodium 3*β*, 6*β*-dihydroxysterol disulfates. *Steroids* **2009**, *74*, 1057–1060.
17. Polat, E.; Caliskan-Alankus, O.; Perrone, A.; Piacente, S.; Bedir, E. Cycloartane-type glycosides from *Astragalus amblolepis*. *Phytochemistry* **2009**, *70*, 628–634.
18. Yokosuka, A.; Sato, K.; Yamori, T.; Mimaki, Y. Triterpene glycosides from *Curculigo orchioides* and their cytotoxic activity. *J*. *Nat*. *Prod*. **2010**, *73*, 1102–1106.
19. Chaturvedula, P.V.S.; Prakash, I. A new diterpene glycoside from *Stevia rebaudiana*. *Molecules* **2011**, *16*, 2937–2943.
20. Mohan, R.; Birari, R.; Karmase, A.; Jagtap, S.; Bhutani, K.K. Antioxidant activity of a new phenolic glycoside from *Lagenaria siceraria* Stand. fruits. *Food Chem*. **2012**, *132*, 244–251.
21. Bedir, E.; Calis, I.; Khan, I.A. Macrophyllosaponin E: A novel compound from the roots of *Astragalus oleifolius*. *Chem*. *Pharm*. *Bull*. **2000**, *48*, 1081–1083.
22. Hirotani, M.; Zhou, Y.; Rut aTsutomu Furuya, H. Cycloartane triterpene glycosides from the hairy root cultures of *Astragalus. membranaceus*. *Phytochemistry* **1994**, *37*, 1403–1407.
23. Thompson, R.S.; Jacques, D.; Haslam, E.; Tanner, R.J.N. Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *J*. *Chem*. *Soc*. *Perkin Trans*. *1* **1972**, 1387–1399.
24. Fletcher, A.C.; Porter, L.J.; Haslam, E.; Gupta, R.K. Plant proanthocyanidins. Part 3. Conformational and configurational studies of natural procyanidins. *J. Chem. Soc. Perkin Trans. 1* **1977**, 1628–1637.
25. Mateus, N.; Silva, A.M.; Santos-Buelga, C.; Rivas-Gonzalo, J.C.; de Freitas, V. Identification of anthocyanin-flavanol pigments in red wines by NMR and mass spectrometry. *J*. *Agric*. *Food Chem*. **2002**, *50*, 2110–2116.
26. Otsuka, H.; Hirata, E.; Shinzato, T.; Takeda, Y. Glochiflavanosides AD: Flavanol glucosides from the leaves of *Glochidion zeylanicum* (Gaertn) A. Juss. *Chem*. *Pharm*. *Bull*. **2001**, *49*, 921–923.
27. Li, S.Y.; Fuchino, H.; Kawahara, N.; Sekita, S.; Satake, M. New phenolic constituents from *Smilax bracteata*. *J. Nat. Prod.* **2002**, *65*, 262–266.
28. Li, F.; Zhan, Z.; Liu, F.; Yang, Y.; Li, L.; Feng, Z.; Jiang, J.S.; Zhang, P. Polyflavanostilbene A, a new flavanol-fused stilbene glycoside from *Polygonum cuspidatum*. *Org*. *Lett*. **2013**, *15*, 674–677.
29. Agati, G.; Azzarello, E.; Pollastri, S.; Tattini, M. Flavonoids as antioxidants in plants: Location and functional significance. *Plant. Sci*. **2012**, *196*, 67–76.

30. Aron, P.M.; Kennedy, J.A. Flavan-3-ols: Nature, occurrence and biological activity. *Mol*. *Nutr*. *Food Res*. **2008**, *52*, 79–104.
31. Scheid, L.; Reusch, A.; Stehle, P.; Ellinger, S. Antioxidant effects of cocoa and cocoa products *ex vivo* and *in vivo*: Is there evidence from controlled intervention studies? *Curr*. *Opin*. *Clin*. *Nutr*. *Metab*. *Care* **2010**, *13*, 737–742.
32. Xu, G.B.; Yang, T.; Bao, J.K.; Fang, D.M.; Li, G.Y. Isochaetomium A2, a new bis (naphthodihydropyran-4-one) with antimicrobial and immunological activities from fungus *Chaetomium microcephalum*. *Arch*. *Pharm*. *Res*. **2013**, doi:10.1007/s12272-013-0206-3.
33. Tian, S.Z.; Pu, X.; Luo, G.Y.; Zhao, L.X.; Xu, L.H.; Li, W.J.; Luo, Y.G. Isolation and characterization of new p-terphenyls with antifungal, antibacterial, and antioxidant activities from halophilic actinomycete *Nocardiopsis gilva* YIM 90087. *J. Agric. Food Chem.* **2013**, *61*, 3006–3012.
34. Tao, X.; Fang, Y.; Xiao, Y.; Jin, Y.L.; Ma, X.R.; Zhao, Y.; He, K.Z.; Zhao, H.; Wang, H.Y. Comparative transcriptome analysis to investigate the high starch accumulation of duckweed (*Landoltia punctata*) under nutrient starvation. *Biotechnol*. *Biofuels* **2013**, *6*, 72–87.
35. Li, S.F.; Di, Y.T.; Luo, R.H.; Zheng, Y.T.; Wang, Y.H.; Fang, X.; Zhang, Y.; Li, L.; He, H.P.; Li, S.L. Cycloartane triterpenoids from *Cassia occidentalis*. *Planta. Med*. **2012**, *78*, 821–827.
36. Wang, X.Y.; Tang, G.H.; Yuan, C.M.; Zhang, Y.; Zou, T.; Yu, C.; Zhao, Q.; Hao, X.J.; He, H.P. Aphagrandinoids A–D, cycloartane triterpenoids with antibacterial activities from *Aphanamixis grandifolia*. *Fitoterapia* **2013**, *85*, 64–68.

Sample Availability: Samples of the compounds **3**, **6**, **7**–**13** are available from the authors.