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UFLC-MS-IT-TOF and Bioassay Guided Isolation of Flavonoids as Xanthine Oxidase Inhibitors from *Diospyros dumetorum*

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Diospyros dumetorum is an important folk medicine for treating pulmonary abscess and inflammation. The leaves of *D. dumetorum* revealed xanthine oxidase (XOD) inhibitory activity. With the guidance of UFLC-MS-IT-TOF analyses combined with bioassay *in vitro*, 15 flavonoids were isolated from the active parts of *D. dumetorum*. Except for **11** (IC_{50} > 200μM), all compounds showed obvious XOD inhibitory activity with IC_{50} values of 32.5 ± 0.7 ~ 145.0 ± 3.3 μM. The preliminary structure-activity relationships study suggested that glycosylation on C-3 was unfavorable for XOD inhibitory activity; hydroxyl groups on ring B were essential for maintaining activity; the activity was closely related with the position of galloylation. This is the first recognition of the XOD inhibitory activity and active constituents of *D. dumetorum*, and will provide valuable information for this plant as a new resource for treating hyperuricemia and gout.

Keywords: *Diospyros dumetorum*, UFLC-MS-IT-TOF, Bioassay-guided isolation, XOD inhibitory activity, Flavonoids.

Gout is a prevalent inflammatory arthritis affecting 1~2% of adults worldwide, and is caused by the buildup of uric acid crystals in the joints. Xanthine oxidase (XOD) is a flavoprotein enzyme which plays a key role in catalyzing the reaction of purine compounds to uric acid at the end of purine nucleotide metabolism. XOD is also closely related to many pathological symptoms, *e.g.* inflammation, free radical scavenging, metabolic disorders, cellular aging, reperfusion damage, atherosclerosis, hypertension and carcinogenesis. Therefore, XOD is considered as the potent drug target for treating ischemia-reperfusion injury, hypertension, atherosclerosis, myocardial infarction, diabetes and cancer [1]. The clinically used XOD inhibitors, including allopurinol, febuxostat and oxipurinol, are unsatisfactory due to their side effects, drug resistance and rebound reaction. Traditional Chinese medicines (TCMs) and ethnic medicines used for thousands of years in China, are fascinating sources for interesting XOD inhibitors [2, 3].

Diospyros dumetorum belonging to the *Diospyros* genus of the family Ebenaceae is an ethnic medicine of the Yi nationality (Seyili) in Yunnan Province (China), which has been documented in "Yi Yao Zhi" for treating pulmonary abscess and inflammation [4,5]. However, there is no report concerning the chemical constituents and pharmacological activities of *D. dumetorum*. Therefore, a detailed investigation of the chemical constituents and their biological activity will be important for its modern application. The UFLC-MS-IT-TOF apparatus can provide chromatography and mass spectra with high efficiency and resolution, which is effective for determining the chemical constituents in medicinal herbs [6]. This study aims to characterize the active constituents of *D. dumetorum* by UFLC-MS-IT-TOF analyses and bioassay *in vitro*.

The leaves and stems of *D. dumetorum* were assayed for the XOD-inhibitory activity *in vitro*. As shown in Table 1, leaves showed better activity than stems, and thus, the leaves were selected for further investigation to yield Frs. A~C. Fr. B showed the highest XOD inhibitory activity (75.39 ± 4.33%) at the concentration of 100 μg/mL, from which three active fractions, Frs. B-2~4 with XOD-inhibitions of 63.48 ± 0.91%, 78.61 ± 2.54% and 67.33 ± 5.12% were obtained. Eventually, 15 flavonoids (**1**−**15**) were isolated from the above fractions with the guidance of LC-MS analyses and bioassay *in vitro*.

Table 1: XOD-Inhibitory activity and extraction yields of the leaves of *D. dumetorum* crude extract and fractions (100 μg/mL).

Sample	Inhibition(%)[a]	Extraction yield(% *w/w*)[c]
Leaf extract	57.08 ± 2.58	15.7
Stem extract	36.39 ± 3.93	8.1
Fr.A	8.82 ± 5.51	9.3
Fr.B	75.39 ± 4.33	3.3
Fr.C	39.86 ± 3.33	3.0
Fr.B-1	30.34 ± 3.15	0.467
Fr.B-2	63.48± 0.91	0.750
Fr.B-3	78.61 ± 2.54	1.033
Fr.B-4	67.33 ± 5.12	0.717
Allopurinol[b]	98.92 ± 0.33	-

[a]Values were expressed as mean ± SD (n=3).
[b]Allopurinol (10 μM) was used as the positive control.
[c] Extraction yield was expressed as weight percentage of original raw herb.

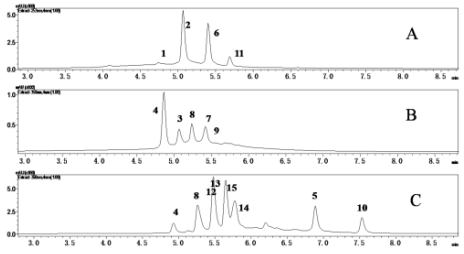

Figure 1: LC-PDA chromatogram of Frs. B-2 (A), B-3 (B) and B-4 (C).

In order to clarify the active constituents, Frs. B-2 ~ B-4 were analyzed by LC-MS (Figure.1). The MS/MS and UV data of each peak are summarized in Table S1.

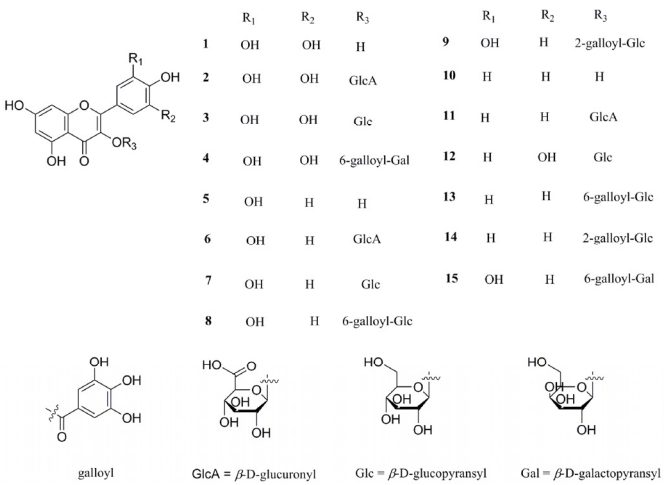

Figure 2: Structures of compounds **1**-**15**.

With the guidance of LC-MS, 15 flavonoids (Figure 2) were isolated from Frs. B-2 ~ B-4, which were determined to be myricetin (**1**), myricetin-3-*O*-*β*-D-glucuronide (**2**), myricetin-3-*O*-*β*-D-glucopyranoside (**3**), myricetin-3-*O*-(6"-galloyl)-*β*-D-galactopyranoside (**4**), quercetin (**5**), quercetin-3-*O*-*β*-D-glucuronide(**6**), quercetin-3-*O*-*β*-D-glucopyranoside (**7**), quercetin-3-*O*-(6"-galloyl)-*β*-D-glucopyranoside (**8**), quercetin-3-*O*-(2"-galloyl)-*β*-D-glucopyranoside (**9**), kaempferol (**10**), kaempferol-3-*O*-*β*-D-glucuronide (**11**), kaempferol-3-*O*-*β*-D-glucopyranoside(**12**), kaempferol-3-*O*-(6"-galloyl)-*β*-D-glucopyranoside (**13**), kaempferol-3-*O*-(2"-galloyl)-*β*-D-glucopyranoside (**14**) and kaempferol-3-*O*-(6"-galloyl)-*β*-D-galactopyranoside (**15**) by comparing the spectroscopic data with those reported in the literature [7-10].

In order to evaluate the anti-gout potency of the isolates, compounds **1**-**15** were assayed for their XOD inhibitory activity *in vitro*. As shown in Table 2, all compounds except **11** (IC_{50} > 200 μM) showed obvious XOD inhibitory activity with IC_{50} values of 32.5 ± 0.7 ~ 145.0 ± 3.3μM. Three flavonoid aglycones, myricetin (**1**), quercetin (**5**) and kaempferol (**10**) possessed the most potent XOD inhibitory activity with IC_{50} values of 32.5 ± 0.7, 36.3 ± 0.4 and 44.7 ± 1.9 μM; eight flavonoid glycosides **2-4**, **7**, **8**, **12**, **13** and **15** expressed moderate XOD inhibitory activity with IC_{50} values in the range of 50 to 100 μM; the galloylated flavonoid glycosides **6**, **9** and **14** exhibited weak activity with IC_{50} values of 145 ± 3.3, 119.0 ± 4.6 and 121.2 ± 7.3 μM, respectively.

Table 2: XOD-inhibitory activity of compounds **1-15** (100 μM)[a].

Compds	XOD Inhibition (%)	IC_{50} (μM)	Compds	XOD Inhibition (%)	IC_{50} (μM)
1	60.4 ± 2.0	36.3 ± 0.4	**9**	25.7 ± 5.7	119.0 ± 4.6
2	39.6 ± 0.6	62.7 ± 2.7	**10**	64.3 ± 3.5	44.7 ± 1.9
3	56.7 ± 0.9	89.2 ± 1.8	**11**	3.1 ± 2.1	> 200
4	77.9 ± 1.0	58.6 ± 1.5	**12**	43.7 ± 2.5	84.5 ± 3.7
5	86.3 ± 3.3	32.5 ± 0.7	**13**	55.8 ± 1.4	82.8 ± 1.4
6	22.4 ± 3.9	145.0 ± 3.3	**14**	39.5 ± 4.6	121.2 ± 7.3
7	47.9 ± 2.2	87.8 ± 5.4	**15**	58.1 ± 0.2	76.9 ± 2.5
8	40.1 ± 1.9	93.2 ± 2.1	Allop[b]	95.1 ± 0.5	0.8 ± 0.09

[a]Values were expressed as mean ± SD (n=3).
[b]Allop, allopurinol (10 μM) was used as the positive control.

From the above analyses, the flavonoid aglycones showed obviously higher activity than the glycosides, indicating that glycosylation is unfavorable for maintaining XOD inhibitory activity. For the glucuronides, the XOD inhibitory activities are increased with more hydroxyl groups on ring B (**2**>**6**>**11**). In addition, different glycosyls and the position of galloyl group are closely related to XOD inhibitory activity with the fact that the galactosides (**4**, **15**) show better activity than the glucosides (**8**, **14**), and a galloyl group at C-6" (**8**, **13**) is preferable than at C-2" (**9**, **14**).

D. dumetorum is an important ethnic medicine of the Yi minority in Yunnan Province of China; however, its XOD inhibitory activity is still unclear. In order to clarify its active constituents, 15 flavonoids were isolated under the guidance of LC-MS analyses and bioassay *in vitro*. Most of the isolates showed obvious XOD inhibitory activity suggesting that flavonoids should be responsible for the XOD inhibitory activity of *D. dumetorum*. This is the first time to reveal the XOD inhibitory activity and active constituents of *D. dumetorum*, which will provide valuable information for this plant as a new resource for treating hyperuricemia and gout.

Experimental

General experimental procedures: LC-MS analyses were performed on a UFLC-MS-IT-TOF apparatus (Shimadzu, Kyoto, Japan) equipped with a diode array detector (DAD) and an electrospray ionization (ESI) source coupled to an ion trap (IT) and time of flight (TOF) mass analyzers. Chromatographic separation was achieved on a Hypersil GOLD C_{18} column (1.8 μm, 2.1 × 100 mm, *i.d.*). The DAD profiles were recorded from 190 to 400 nm, and monitored at 254 nm. Accurate masses were calibrated using CF_3CO_2Na clusters. MS/MS experiments in positive and negative ion modes were performed in an automatic pattern. Molecular formulae were speculated by the Shimadzu Composition Formula Predictor. The analytical conditions were set as previously reported [6].

Silica gel (200-300 mesh) for column chromatography (CC) and TLC plates (GF_{254}) was purchased from Linyi Haixiang Chemical Co. Ltd. (Linyi, China) and Merck Chemical (Shanghai) Co. Ltd., respectively. Sephadex LH-20 for chromatography was obtained from Pharmacia Fine Chemical Co., Ltd. (Uppsala, Sweden). CHP20P MCI gel (Mitsubishi Chemical Corporation, Tokyo, Japan) was applied for MPLC separation on a Dr-Flash-II MPLC system (Lisui, Suzhou, China). Chuang Xin Tong Heng LC3000 apparatus (Beijing Chuang Xin Tong Heng Science and Technology Co. Ltd., Beijing, China) was used for HPLC purification on an Agilent SB-PHENYL column (5 μm, 9.4 × 250 mm). ^{1}H and ^{13}C NMR data were recorded on Bruker DRX-500 or AVANCE III-600 spectrometers (Bremerhaven, Germany).

Plant materials: The aerial parts of *Diospyros dumetoru* W. W. Smith were collected in Luquan, Yunnan Province, China, in August 2015, which were authenticated by Prof. Hua Peng (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (No.20150801) was deposited in the Laboratory of Anti-virus and Natural Medicinal Chemistry, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and bioassay-guided fractionation: The dried leaves of *D. dumetorum* (600g) were extracted twice with 50% EtOH under reflux (1:8, *w/v*), 3h each time. The combined EtOH extract was concentrated in *vacuo* and subjected to macroporous resin D101 column chromatography (CC) with EtOH-H_2O gradient (0:10, 5:5, 9:1, *v/v*) to yield a water fraction (Fr. A, 55.8 g), a 50% EtOH fraction (Fr. B, 19.8 g) and a 90% EtOH fraction (Fr. C, 18 g). Fr. B showed the most potent XOD inhibitory activity, and was further separated by MPLC on a CHP20P MCI column (100 g, 2.5 × 30 cm) eluted with MeOH-H_2O gradient (from 1:9 to 9:1, *v/v*) to generate four sub-fractions, Fr.B-1(2.8 g), Fr.B-2 (4.5 g), Fr.B-3 (6.2 g) and Fr.B-4 (4.3 g). Subsequent bioassay revealed that Frs.B-2 ~ B-4 had XOD inhibitory activity, from which compounds **1**−**15** were isolated under the guidance of LC-MS analyses. Fr.B-2 was purified

by HPLC on an Agilent SB-PHENYL column (5 μm, 9.4 × 250 mm) with MeCN-H_2O (23:77, *v/v*) to yield compounds **1** (24 mg, t_R = 13.5 min), **2** (142 mg, t_R = 14.8 min), **6** (83 mg, t_R = 17.2 min) and **11** (47 mg, t_R = 26.0 min). Fr. B-3 was purified by HPLC on an Agilent SB-PHENYL column (5 μm, 9.4 × 250 mm) using MeCN-H_2O (19:81, *v/v*) system to yield compounds **3** (17 mg, t_R = 25.5 min), **4** (72 mg, t_R = 25.0 min), **7** (19 mg, t_R = 28.2 min), **8** (44 mg, t_R = 29.9 min) and **9** (13mg, t_R = 31.5 min). Fr. B-4 was separated by silica gel CC (80 g, 3.0 × 40 cm) eluted with MeOH-$CHCl_3$ gradient (10:90, 20:80, 50:50, *v/v*, each 500 mL) to afford Frs.B-4-1~5. Fr. B-4-1 was purified by Sephadex LH-20 CC to obtain compounds **5** (116 mg) and **10** (78 mg); Fr. B-4-2 was purified by semi-preparative HPLC on a Welch C-4 column (5 μm, 7.8 × 250 mm) using MeCN-H_2O (20:80, *v/v*) system to obtain compound **12** (14 mg, t_R = 21.1 min); Fr. B-4-4 was separated by Sephadex LH-20 CC and further purified by semi-preparative HPLC on an Agilent SB-PHENYL column (5 μm, 9.4 × 250 mm) using MeOH-H_2O (35:65, *v/v*) to yield compounds **13** (39 mg, t_R = 33.1 min), **14** (16 mg, t_R = 35.0 min) and **15** (19 mg, t_R = 41.8 min).

Xanthine oxidase inhibitory assay in vitro: The xanthine oxidase (XOD) inhibitory activity was evaluated by measuring the uric acid production from xanthine or hypoxanthine substrate at 295 nm [2]. The negative group consisted of 60 μL of 100 μmol/L phosphate buffer (pH 7.5) and 40 μL of 400 μmol/L xanthine (pH 7.5). The enzyme group consisted of 20 μL of 100 μmol/L phosphate buffer (pH 7.5), 40 μL of 400 μmol/L xanthine (pH 7.5) and 40 μL of enzyme solution (2.5 U/L). The sample group consisted of 40 μL of 400 μmol/L xanthine (pH 7.5), 20 μL of tested samples and 40 μL of enzyme solution (2.5 U/L). Allopurinol was used as the positive control at the concentration of 10 μM. The absorption at 295nm was measured at 25 ^oC after the addition of enzyme for 3 min. All experiments were performed in triplicate. Inhibition of xanthine oxidase was calculated as $(\Delta_{Enzyme}-\Delta_{Sample}/\Delta_{Enzyme}-\Delta_{Negative})\times100\%$, and IC_{50} values of compounds **1**−**15** were obtained by Graphpad Prism 5.0 (GraphPad Software, Inc.).

All experimental results were presented as mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett's test.

Supplementary data: Table S1 is included in the Supporting information

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