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Chemical Constituents of the Fruits of *Crataegus dahurica* and the Antihyperlipidemic Activity in HepG2 Cells

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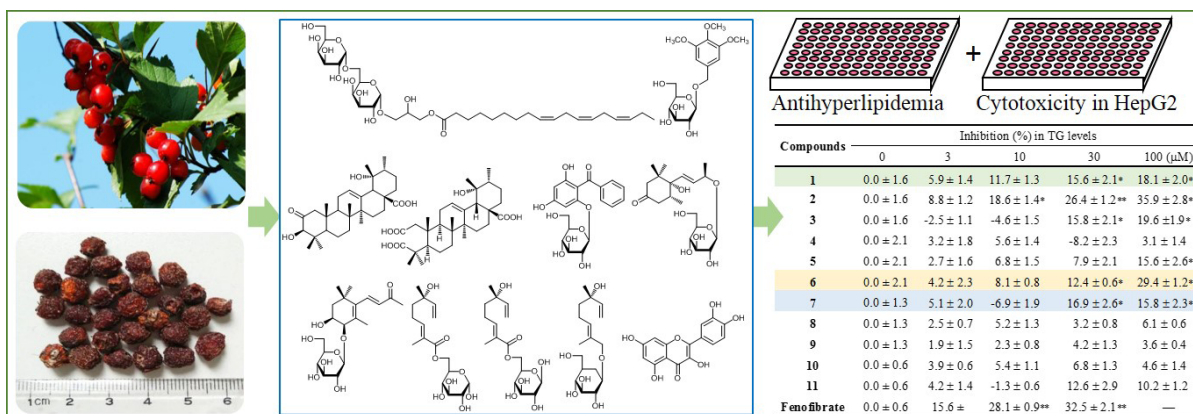
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Abstract

The fruit of *Crataegus dahurica* Koehne ex C.K. Schneid is an edible wild fruit and herb for the treatment of various diseases. However, few studies were conducted to search the chemical constituents of the edible herb. In the present study, a new glycoglycerolipid (1), together with ten known compounds, 2-oxo pomolic acid (2), cecropiacic acid (3), nikoenoside (4), garcimangosone D (5), ampelopsisionoside (6), saussureosides B (7), 6-O-[(*2E*, 6S)-2, 6-dimethyl-6-hydroxy-2, 7-octadienoyl]-α-D-glucopyranoside (8), 6-O-[(*2E*, 6S)-2, 6-dimethyl-6-hydroxy-2, 7-octadienoyl]-β-D-glucopyranoside (9), betulalbuside A (10), quercetin (11), were isolated from the fruits of *C. dahurica* with various chromatographic techniques. Their structures were determined by extensive spectroscopic data analysis including 1D and 2D NMR and HR-ESI MS and comparison with previous literature. The inhibitory activity on TG accumulation in HepG2 cells was assayed to evaluate the antihyperlipidemic activity. The results showed that compound 6 significantly inhibited the accumulation of TG in cells without inducing cytotoxic activity. In addition, compounds 1 and 7 also exhibited the moderately inhibitory activity on TG in cells. The results in this present study would be helpful to further exploration and utilization of the edible herb.

Compounds	Inhibition (%) in TG levels				
	0	3	10	30	100 (µM)
1	0.0 ± 1.6	5.9 ± 1.4	11.7 ± 1.3	15.6 ± 2.1*	18.1 ± 2.0*
2	0.0 ± 1.6	8.8 ± 1.2	18.6 ± 1.4*	26.4 ± 1.2**	35.9 ± 2.8**
3	0.0 ± 1.6	-2.5 ± 1.1	-4.6 ± 1.5	15.8 ± 2.1*	19.6 ± 1.9*
4	0.0 ± 2.1	3.2 ± 1.8	5.6 ± 1.4	-8.2 ± 2.3	3.1 ± 1.4
5	0.0 ± 2.1	2.7 ± 1.6	6.8 ± 1.5	7.9 ± 2.1	15.6 ± 2.6*
6	0.0 ± 2.1	4.2 ± 2.3	8.1 ± 0.8	12.4 ± 0.6*	29.4 ± 1.2**
7	0.0 ± 1.3	5.1 ± 2.0	-6.9 ± 1.9	16.9 ± 2.6*	15.8 ± 2.3*
8	0.0 ± 1.3	2.5 ± 0.7	5.2 ± 1.3	3.2 ± 0.8	6.1 ± 0.6
9	0.0 ± 1.3	1.9 ± 1.5	2.3 ± 0.8	4.2 ± 1.3	3.6 ± 0.4
10	0.0 ± 0.6	3.9 ± 0.6	5.4 ± 1.1	6.8 ± 1.3	4.6 ± 1.4
11	0.0 ± 0.6	4.2 ± 1.4	-1.3 ± 0.6	12.6 ± 2.9	10.2 ± 1.2
Fenofibrate	0.0 ± 0.6	15.6 ±	28.1 ± 0.9**	32.5 ± 2.1**	—

Graphical Abstract

Keywords: Crataegus dahurica Koehne ex C.K. Schneid; Edible Herbs; Glycoglycerolipid; Chemical Constituents; Antihyperlipidemic Activity

Introduction

Crataegus dahurica Koehne ex C.K. Schneid, belonging to Rosaceae family, mainly distributed in northeastern provinces and Great Xing'an Mountains areas in China, as well as in the Far East region of Russia. The fruits of *C. dahurica* is not only as a wild food eaten by Oroqen people, a nomadic minority in Northeast China, but also used as an herb for the treatment of dyspepsia, hepatitis, and hyperlipidemia, etc [1,2]. However, no systematic chemical constituents and pharmacological activities of the wild edible and medicinal fruit has been reported except our previous study [3]. In the continuous research on the chemical constituents and biological activities of this edible herb, a new glycoglycerolipid together with ten known compounds were isolated and characterized, and the antihyperlipidemic activity in HepG2 cells was also evaluated. In this paper, we report the isolation

and the structure elucidation of these compounds and their antihyperlipidemic activity *in vitro*. The findings in this study will be helpful to further utilize and develop this wild fruit as a dietary supplement or potential medicine.

Experimental

General

^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance III-400 spectrometer (Bruker, Germany), with tetramethylsilane (TMS) for the internal standard. Chemical shifts were reported in units of δ (ppm) and coupling constants (J) were expressed in Hz. IR spectra were recorded on a Bruker Tensor II spectrometer. The purification was conducted on a medium pressure system (PrepChromaster 6000plus, Kezhe, China) and a semi-preparative HPLC system (PuriMaster 5000, Kezhe, China). The silica gel (Qingdao Haiyang Chemical Co., Shandong, China), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden) and ODS (Merck Co., Germany) were used in the isolation and purification of the compounds. A triglyceride detection kit was obtained from Nanjing jiancheng bioengineering institute (Nanjing, China) and the absorbance measurements of triglycerides were conducted on a multiplate reader (SuPerMax 3100, Kezhe, China). The other chemicals and reagents were bought from Sigma-Aldrich (Sigma, St. Louis, MO, USA).

Plant Material

The fruits of *C. dahurica* were collected in August 2015, Tongbei, Heilongjiang province and identified as the previously reported [3]. A voucher specimen (MLN150816) has been deposited in the herbarium of the School of Pharmacy, Health Science Center, Xi'an Jiaotong University. And the CdME and the fractions were prepared according to the method described by Wang *et al.* [3].

Extraction and Isolation

The *n*-BuOH fraction (115.7 g) of *C. dahurica* was fractionated to thirty-six fractions (Fr.Bu1~Fr.Bu36) by silica gel (200-300 mesh, 1000 g) CC eluting with $CHCl_3/CH_3OH$ (1:0, 80:1, 50:1, 20:1, 10:1, 5:1, 2:1, 0:1, v/v). Fr.Bu3 was further separated by silica gel CC eluting with PE/acetone (10:1, v/v), the sub-fractions were purified by semi-preparative HPLC with 80% MeOH in 0.5% acetic acid as the mobile phase to afford the compound 1 (10.3 mg), compound 2 (16.9 mg) and 3 (5.2 mg). Compound 4 (11.2 mg) was obtained from Fr.Bu13 which was separated by Sephadex LH-20 CC (methanol) and semi-preparative HPLC (30% methanol in 0.5% acetic acid). Fr.Bu14 was further separated to fourteen subfractions (Fr. Bu14-1 ~ Fr. Bu14-14) by ODS (212 g) CC eluting with CH_3OH/H_2O (15:85, 30:70, 45:55, 60:40, 80:20, 100:0, v/v). Fr. Bu14-6 to Fr. Bu14-8 were purified by semi-preparative HPLC with 35% methanol in 0.5% acetic acid to get compound 5 (4.7 mg), compound 6 (12.4 mg) and 7 (10.3 mg), respectively. Fr. Bu16 was divided into sixteen subfractions (Fr. Bu16-1 ~ Fr. Bu16-16) by ODS CC eluting with CH_3OH/H_2O (25:75, 34:66, 44:56, 57:43, 74:26, 100:0, v/v). Compound 8 (6.8 mg), 9 (4.1 mg) and 10 (5.1 mg) were purified from Fr. Bu16-8 with 40% methanol in 0.5% acetic acid through semi-preparative HPLC. The EtOAc fraction (36.2 g) was separated by silica gel (200-300 mesh, 500 g) CC gradient eluting with $CHCl_3/CH_3OH$ (1:0 ~ 1:5, v/v) to yield twenty-three fractions (Fr.EA1 ~ Fr.EA23). Compound 11 (9.2 mg) was purified from Fr.EA16 by Sephadex LH-20 CC eluting with CH_3OH/H_2O (1:1, v/v).

1-*O*-(9Z, 12Z, 15Z-octadecatrienoyl)-3-*O*-[α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl] glycerol

White powder. HR-ESI MS m/z 699.35740 [M+Na]$^+$ (calcd for $C_{33}H_{56}O_{14}Na$, 699.35678); IR (KBr) n$_{max}$ 3401, 1736, 1645 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-*d6*): 3.96 (1H, dd, 11.2, 6.5, H-1), 4.04 (1H, dd, 11.2, 4.0, H-1), 3.80 (1H, m, H-2), 3.60 (1H, m, H-3), 3.55 (1H, m, H-3), 2.28 (2H, m, H-2′), 1.50 (2H, m, H-3′), 1.26 (2H, m, H-4′), 1.25 (2H, m, H-5), 1.26 (2H, m, H-6′), 1.30 (2H, m, H-7′), 2.02 (2H, m, H-8′), 5.34 (1H, m, H-9′), 5.29 (1H, m, H-10′), 2.77 (2H, m, H-11′), 5.32 (1H, m, H-12′), 5.31 (1H, m, H-13′), 2.77 (2H, m, H-14′), 5.28 (1H, m, H-15′), 5.35 (1H, m, H-16′), 2.03 (2H, m, H-17′), 0.92 (3H, t, 7.5, H-18′), 4.67 (1H, d, 3.4, H-1′′), 3.55 (1H, m, H-2′′), 3.28 (1H, m, H-3′′), 3.67 (1H, m, H-4′′), 3.54 (1H, m, H-5′′), 3.66 (1H, m, H-6′′), 3.43 (1H, m, H-6′′), 4.09 (1H, d, 3.6, H-1′′′), 3.28 (1H, m, H-2′′′), 3.60 (1H, m, H-3′′′), 3.68 (1H, m, H-4′′′), 3.61 (1H, m, H-5′′′), 3.44 (1H, m, H-6′′′), 3.50 (1H, m, H-6′′′) ; 13C NMR (100 MHz, DMSO-d6): 65.5 (C-1), 67.4 (C-2), 66.5 (C-3), 173.0 (C-1′), 33.5 (C-2′), 24.5 (C-3′), 28.64 (C-4′), 28.56 (C-5′), 29.0 (C-6′), 28.5 (C-7′), 26.7 (C-8′), 129.9 (C-9′), 127.6 (C-10′), 25.2 (C-11′), 127.9 (C-12′), 128.0 (C-13′), 25.1 (C-14′), 127.0 (C-15′), 131.5 (C-16′), 20.1 (C-17′), 14.2 (C-18′), 99.5 (C-1′′), 72.9 (C-2′′), 70.5 (C-3′′), 68.1 (C-4′′), 68.9 (C-5′′), 70.4 (C-6′′), 104.0 (C-1′′′), 73.1 (C-2′′′), 71.3 (C-3′′′), 68.4 (C-4′′′), 69.6 (C-5′′′), 60.6 (C-6′′′).

Antihyperlipidemic and Cytotoxic Activities in HepG2 Cells

The assay was performed according to the previous method with slight modification [4-14]. Briefly, HepG2 cells were seeded in 48-well plates (1×10^4 cells/well) in DMEM (high glucose). After incubation for 24 h, the medium was changed and the samples dissolved in DMSO were added into the wells. The cells with or without the samples were cultured for another 4 days (change the medium every two days). At the end of the incubation, the medium was aspirated, and the cells were sonicated in distilled water (150 μL/well). The concentration of TG in every well was measured by a TG kit. Fenofibrate as a reference compound was tested in this assay. After 5 days' culture, cytotoxic activity was also assayed by adding 10 μL of MTT to each well and incubating for another 4 h. Then 200 μL of DMSO was added to each well to dissolve the formazan after the removal of the supernatant. The absorbance was measured at 570 nm on a multiplate reader (SuPerMax 3100, Kezhe, China).

Hydrolysis of Compound 1

The hydrolysis of compound 1 was conducted as the previous literature [15,16].
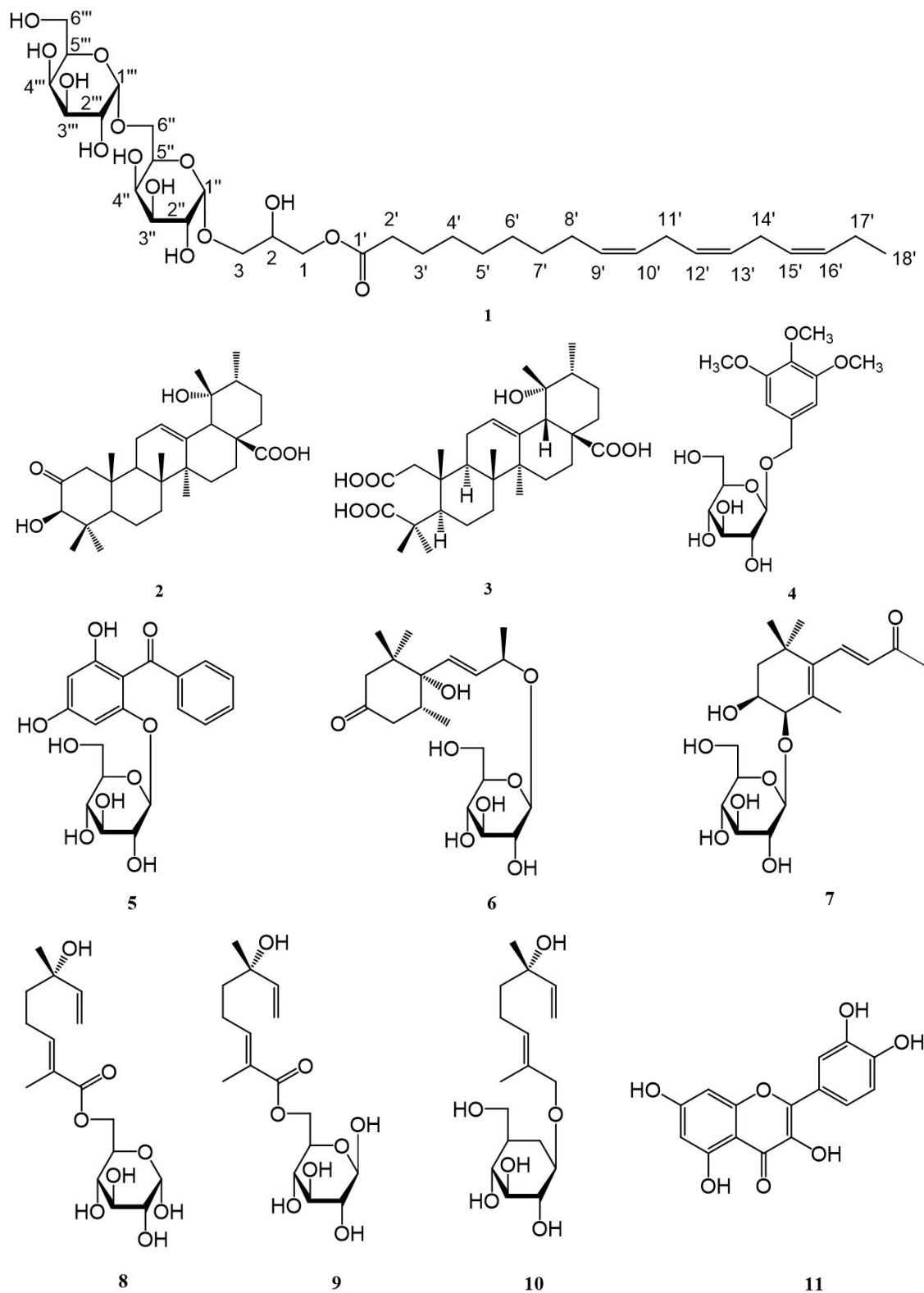
Results and Discussion

Figure 1: Chemical structures of compounds 1-11

With multiple chromatographic methods, eleven compounds (Figure 1), including a new compound, were isolated from the EtOAc fraction and *n*-BuOH fraction of the fruits of *C. dahurica*. All of these compounds were measured for ^{1}H and ^{13}C NMR data. Compound 1 was obtained as a white powder. Its HR-ESI MS revealed a pseudomolecular ion peak $[M+Na]^+$ at m/z 699.35740 (calcd. 699.35678) established the molecular formula of compound 1 as $C_{33}H_{56}O_{14}$ which showed six degrees of unsaturation. Its IR spectrum displayed characteristic bands for hydroxyl, carbonyl groups and double bonds at 3401, 1736 and 1645 cm^{-1}, respectively. The ^{13}C-NMR spectrum of 1 displayed six signals of unsaturated double bonds carbons, together with six sp2 hybridization protons signals in ^{1}H-NMR spectrum, indicating the presence of three double bonds in the molecule. In addition, a quaternary carbon at δ_C 173.0 in the ^{13}C-NMR spectra demonstrated the presence of an ester carbonyl group. In the ^{1}H-NMR spectrum of 1, two anomeric protons $[\delta_H$ 4.67 (1H, d, J =3.4 Hz) and δ_H 4.09 (1H, d, J = 3.6 Hz)] and their corresponding carbons (δ_C 99.5 and 104.0) together with multiple oxygenated carbons signals from δ_C 60.6 to δ_C 73.1 indicated that there were two sugar units. After hydrolysis of 1, the sugar was identified as D-galactose by co-TLC with the control of D-galactose. Additionally, two molecules of D-galactose liberated from the hydrolysate by GC analysis also confirmed the result. The configuration of D-galactose was determined as α-orientation by their coupling constants of the anomeric protons. In the ^{1}H-^{1}H COSY spectrum, the correlations of three protons connected to oxygenated carbons attributed by HMQC spectra, from H-1 (δ_H 3.96, 4.04, δ_C 65.5) to H-2 (δ_H 3.80, δ_C 67.4) and H-2 (δ_H 3.80, δ_C 67.4) to H-3 (δ_H 3.55, δ_C 66.5) suggested the presence of a glycerol moiety in the molecule (Figure S1). One of the galactoses was linked to the left position of the glycerol which was determined by the HMBC correlations from H-1″ (δ_H 4.67) to C-3 (δ_C 66.5) and H-3 (δ_H 3.60, 3.55) to C-1″ (δ_C 99.5). And the right position of the glycerol linked to the carbonyl group by the formation of the ester bond with the hydroxyl group of the glycerol, which was confirmed by the observation of the cross peak of H-1 (δ_H 3.96, 4.04) to C-1′ (δ_C 173.0). After extensive analysis of the ^{1}H-^{1}H COSY and HMBC spectra of 1 (Figure S1), it was found that the carbonyl group was the carboxyl carbon of a long chain multiple unsaturated fatty acid. Compound 1 was hydrolyzed with 5% NaOMe-MeOH to give a methyl 9Z, 12Z, 15Z-octadecatrienoate identified by GC with an authentic sample. The another *α*-D-galactose linked to the 6 position of the inner galactose confirmed by the correlations in HMBC spectra of 1 from H-1‴ (δ_H 4.09) to C-6″(δ_C70.4) and H-6″ (δ_H 3.66, 3.43) to C-1″(δ_C 104.0). Therefore, compound 1 was determined as 1-*O*-(9Z, 12Z, 15Z-octa decatrienoyl)-3-*O*-[α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl] glycerol, which was a previously undescribed compound.

The structures of the known compounds were characterized based on the physicochemical properties and compare their data with those reported in related literature. These compounds were identified as 2-oxo pomolic acid (2) [4], cecropiacic acid (3) [5]. nikoenoside (4) [6], garcimangosone D (5) [7], ampelopsisionoside (6) [8], saussureosides B (7) [9], 6-*O*-[(2*E*, 6*S*)-2, 6-dimethyl-6-hydroxy-2, 7-octadienoyl]-α-D-glucopyranoside (8) [10], 6-*O*-[(2*E*, 6*S*)-2, 6-dimethyl-6-hydroxy-2, 7-octadienoyl]-β-D-glucopyranoside (9) [10], betulalbuside A (10) [11], quercetin (11) [12], respectively. All of these compounds were isolated from the fruit and the whole plant of *C. dahurica* for the first time.

All isolated compounds were subjected to evaluate the inhibitory activity on TG in HepG2 cells. And the results showed that compound 6 could significantly inhibit the accumulation of TG in cells without induction of cytotoxic activity, compared to the control group. Meanwhile, compounds 1 and 7 also moderately reduced TG concentrations in HepG2 cells after 4 days of co-culture (Table 1 and S1). In addition, as shown in Table 1, although compound 2 and 3 also significantly inhibited TG accumulation in cells (inhibition rate: 35.9 ± 2.8% and 19.6 ±1.9% at 100 μM, respectively), however, the inhibitory activity on TG was induced mainly by their cytotoxic activity (Table S1). Actually, this kind of triterpenoids was frequently reported to exhibit cytotoxicity in different cell lines [13].

Compounds	Inhibition (%) in TG levels				
	0	3	10	30	100 (μM)
1	0.0 ± 1.6	5.9 ± 1.4	11.7 ± 1.3	15.6 ± 2.1*	18.1 ± 2.0*
2	0.0 ± 1.6	8.8 ± 1.2	18.6 ± 1.4*	26.4 ± 1.2**	35.9 ± 2.8**
3	0.0 ± 1.6	-2.5 ± 1.1	-4.6 ± 1.5	15.8 ± 2.1*	19.6 ± 1.9*
4	0.0 ± 2.1	3.2 ± 1.8	5.6 ± 1.4	-8.2 ± 2.3	3.1 ± 1.4
5	0.0 ± 2.1	2.7 ± 1.6	6.8 ± 1.5	7.9 ± 2.1	15.6 ± 2.6*
6	0.0 ± 2.1	4.2 ± 2.3	8.1 ± 0.8	12.4 ± 0.6*	29.4 ± 1.2**
7	0.0 ± 1.3	5.1 ± 2.0	-6.9 ± 1.9	16.9 ± 2.6*	15.8 ± 2.3*
8	0.0 ± 1.3	2.5 ± 0.7	5.2 ± 1.3	3.2 ± 0.8	6.1 ± 0.6
9	0.0 ± 1.3	1.9 ± 1.5	2.3 ± 0.8	4.2 ± 1.3	3.6 ± 0.4
10	0.0 ± 0.6	3.9 ± 0.6	5.4 ± 1.1	6.8 ± 1.3	4.6 ± 1.4
11	0.0 ± 0.6	4.2 ± 1.4	-1.3 ± 0.6	12.6 ± 2.9	10.2 ± 1.2
Fenofibrate	0.0 ± 0.6	15.6 ± 1.2*	28.1 ± 0.9**	32.5 ± 2.1**	—

Each value is represented as mean ± SEM (n=3)
Table 1: The antihyperlipidemic activity of the compounds from the fruits of *C. dahurica* in HepG2 cells

Acknowledgment

Supplementary Material

^{1}H-^{1}H COSY, main HMBC correlations, NMR, HR-MS and cytotoxicity of compounds are available alongside Figures S1 to S8, Table S1.

References

1. Gao X, Li S, Wang B, Qiu Y (2007) Study on extraction process of ursolic acid from Crataegus dahurica Koehne. Journal of Beihua University 8: 543-7.

2. Zhang J, Zhou L, Li J (2006) Actuality and exploitation of officinal plants resource in Great Xing`an Mountains area.¬¬ Territory & Natural Resources Study 93-94.

3. Wang X, Zhang C, Peng Y, Zhang H, Wang Z, et al. (2018) Chemical constituents, antioxidant and gastrointestinal transit accelerating activities of dried fruit of Crataegus dahurica. Food Chem 246: 41-7.

4. Thuong PT, Jin W, Lee J, Seong R, Lee Y, et al. (2005) Inhibitory effect on TNF-α-induced IL-8-production in the HT29 cell of constituents from the leaf and stem of Weigela subsessilis. Archives of Pharmacal Research 28: 1135-41.

5. Huang X, Ma G, Zhong X, Zhou Y, Dai H, et al. (2014) Triterpene constituents from Rosa cymosa Tratt. China Journal of Chinese Materia Medica 39: 4637-41.

6. Morikawa T, Tao J, Ueda K, Matsuda H, Yoshikawa M (2003) Medicinal foodstuffs. XXXI. Structures of new aromatic constituents and inhibitors of degranulation in RBL-2H3 cells from a Japanese folk medicine, the stem bark of Acer nikoense. Chemical & Pharmaceutical Bulletin 51: 62-7.

7. Huang YL, Chen CC, Chen YJ, Huang RL, Shieh BJ (2001) Three xanthones and a benzophenone from Garcinia mangostana. J Nat Prod 64: 903-6.

8. Marino S, Borbnoe N, Zollo F, Ianaro A, Paola D et al. (2004) Megastigmane and phenolic components from Laurus nobilis L. leaves and their inhibitory effects on nitric oxide production. J Agric Food Chem 52: 7525-31.

9. Liao S, Wang Z, Li J, Liu Y, Li Y, et al. (2012) Cytotoxic sesquiterpene lactones from Vernonia bockiana. Chin J Nat Medicines 10: 230-23.

10. Ye X, Huang K, Ding L (2004) Spectral characterization of three new aromatic acyi monosaccharoses. Journal of Wenzhou Medical College 34: 161-2.

11. Morikawa H, Kasai R, Otsuka H, Hirata E, Shinzato T, et al. (2004) Terpenic and phenolic glycosides from leaves of Breynia officinalis HEMSL. Chemical & Pharmaceutical Bulletin 52: 1086-90.

12. Lu Y, Xin Q, Li X, Ren M, Zhao Y, et al. (2019) Chemical constituents from Saussurea obvallata. Chinese Traditional Patent Medicine 41: 1065-9.

13. Bai L, Zhang H, Liu Q, Zhao Y, Cui X, et al. (2016) Chemical characterization of the main bioactive constituents from fruits of Ziziphus jujuba. Food Funct 7: 2870-7.

14. Gao Y, Su Y, Huo Y, Mi J, Wang X, et al. (2014) Identification of antihyperlipidemic constituents from the roots of Rubia yunnanensis Diels. J Ethnopharmacol 155: 1315-21.

15. Jiang Z (2010) Study on preparative isolation of glycoglycerolipid and its bioactivity [D]. Zhejiang Gongshang University, China.

16. Kiem PV, Minh CV, Nhiem NX, Cuong NX, Tai BH, et al. (2012) Inhibitory effect on TNF-alpha-induced IL-8 secretion in HT-29 cell line by glyceroglycolipids from the leaves of Ficus microcarpa. Arch Pharm Res 35: 2135-42.