ACE and platelet aggregation inhibitors from *Tamarix hohenackeri* Bunge (host plant of Herba Cistanches) growing in Xinjiang

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ABSTRACT

Background: Tamarix hohenackeri Bunge is a salt cedar that grows widespread in the desert mountains in Xinjiang. T. hohenackeri has not been investigated earlier, although there are many reports of phytochemical work on other Tamarix species. **Materials and Methods:** To find out natural angiotensin-converting enzyme (ACE) inhibitor and platelet aggregation inhibitors, the bioactive extract (ethyl acetate [EtOAc] fraction) from the dried aerial parts of T. hohenackeri were investigated. The active fraction was purified by repeated column chromatography, including silica gel, Sephadex LH-20 column, medium-pressure liquid chromatography (MPLC) (polyamide column) and high-performance liquid chromatography (HPLC). The isolated major constituents were tested for their anti-platelet aggregation activity. **Results:** Bioassay-directed separation of the EtOAc fraction of the 70% ethanol extract from the air-dried aerial parts of T. hohenackeri led to the isolation of a new triterpenoid lactone (1), together with 13 known compounds (2-14). It was the first time to focus on screening bioactive constituents for this plant. The chemical structures were established on the basis of spectral data (ESI-MS and NMR). The results showed that the flavonoid compounds (7 and 8) and phenolic compounds (9, 10, 11, and 14) were potential ACE inhibitors. And the flavonoid compounds (5 and 7) showed significant anti-platelet aggregation activities. **Conclusion:** On the basis of the chemical and biological data, the material basis of ACE inhibitory activity for the active part was the phenolic constituents. However, the flavonoid compounds were responsible for the anti-platelet aggregation. The primary structure and activity relationship were also discussed respectively.

Key words: ACE inhibitors, bioassay-directed separation, new triterpenoid lactone, platelet aggregation inhibitors, Tamarix hohenackeri Bunge

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INTRODUCTION

Tamarix L. is an important member of Tamaricaceae family, consisting of 20 species and one variety of Tamarix growing in China. They usually distribute in arid or semiarid desert area and saline-alkali fields in the various Northwest provinces of China.[1] Tamarix species are traditional medicinal plants used for the treatment of leukoderma, spleen troubles, and eye diseases,[2] as well as an astringent, aperitif, stimulus of perspiration, and diuretic.[3] The pharmacological and biological activities study revealed that the extract of some species of Tamarix plants showed hepatoprotective,[4-6] antioxidant,[7] antibacterial,[8] anti-inflammatory,[9] antineoplastic,[10] and inhibitive on α-glucosidase[11] effects. The plants of this genus are also famous as the main host plants of valuable and rare traditional Chinese herbal medicine Herba Cistanches, which has obvious effects of supplementing kidney. Now, Tamarix hohenackeri Bunge and Tamarix ramosissima Ledeb are widely used as host plants in large-scale cultivation of Herba Cistanches in Xinjiang. The phytochemical research has revealed that some species of Tamarix plants are rich in flavonoids, triterpenes, phenylpropanoids, organic acids, steroids, tanins, and lignans.[12,13] However, there are few reports on T. hohenackeri Bunge.

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As we know, angiotensin I-converting enzyme (ACE) plays a critical physiological role in the regulation of blood pressure.[14] ACE can increase blood pressure by converting an inactive form of decapeptide (angiotensin I) into a potent vasoconstrictor angiotensin-II (anoctapeptide) and inactivating catalytic function of bradykinin, which has depressor action.[15]

Blood platelets are implicated in the hemostatic process and also in thrombus formation, which is one of the most important contributors to pathogenesis of many circulatory diseases and inflammatory conditions.[16-19] Thus, anti-platelet compounds have wide therapeutic potential for various circulatory diseases.

In our previous research on screening ACE inhibitory and anti-platelet aggregation active extracts from herbs distributing in Xinjiang, the 70% EtOH extract and EtOAc-soluble part of T. hohenackeri showed significant ACE inhibitory and anti-platelet aggregation activities. In order to reveal the natural ACE and platelet aggregation inhibitors from T. hohenackeri, the bioactivity-guided fractionation and chemical identification were carried out. In the present study, we just want to discuss the separation and characterization of main constituents including a new triterpenoid lactone (1), together with 13 known compounds 2-14. It is the first time to focus on the biochemical constituents of this plant, and all the compounds described in this manuscript were reported firstly. Furthermore, the ACE inhibitory and anti-platelet aggregation activities of the extracts and purified compounds from the active fraction (EtOAc extract) were evaluated systematically and the material–activity relationship and structure–activity relationship were discussed according to the result.

MATERIALS AND METHODS

Mass spectra were measured using Shimadzu QP-2010 Plus (Japan). NMR spectra were recorded on Bruker ARX-300 and ARX-600 spectrometers, used $CDCl_3$ or DMSO-d_6 as solvents with TMS as the internal standard. HR-ESI-MS spectra were obtained using Bruker APEX 7.0 Tesla FT-MS apparatus; in m/z (rel. %). Silica gel (SiO_2: 200-300 mesh, Qingdao Marine Chemical Company, P. R. China), Sephadex LH-20 (Pharmacia, Co., Switzerland), macroporous resin HPD-100 (Cangzhou Bonchem Co., Ltd., P. R. China), polyamide (Taizhou City Luqiao Sijia Biochemical Plastic Factory, P. R. China) were used for column chromatography. Prep. semi-preparative HPLC was carried out on a YMC ODS C-18 column (250 mm × 10 mm, 10 μm, ODS-A, Co., Ltd. Japan) and Shimadzu SPD-10A UV detector. Silica gel GF_{254} was used for TLC (SiO_2: 200-300 mesh, Qingdao Marine Chemical Company, P.R. China). The chromatograms were visualized under UV light (at 254 and 365 nm) before and after spraying with sulfuric acid-ethanol (10%) spray reagent, ferric chloride-ethanol spray reagent and bromocresol green-ethanol-sodium hydroxide spray reagent. Other chemicals and solvents in this experiment were all analytical grade or higher and from Shandong Yuwang Co., Ltd. Chemical Engineering Branch and Tianjin Damao Chemical Reagent Co.

Plant material

The aerial parts of T. hohenackeri Bunge were collected from Hetian district of Xinjiang province in China, in June 2011. The plant material was identified by Prof. Xiaoguang Jia. (Xinjiang Institute of Chinese Materia Medica and Ethnodrug). A voucher specimen (No. 20110613) is deposited in School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University.

Extraction and isolation

The air-dried aerial parts (6.8 kg) were powdered and refluxed with 70% ethanol (3 × 9 L) to yield the ethanol extract (1850 g). The residue (1800 g) was suspended in distilled water and partitioned with petroleum ether, ethyl acetate, and n-butanol (saturated with water). The solvents were evaporated separately under reduced pressure to yield petroleum ether extract (56 g), ethyl acetate extract (75 g), n-butanol extract (235 g), respectively. The ethyl acetate fraction (73 g) was subjected to column chromatography on silica gel (200-300 mesh, 130 × 8 cm, 662 g) gradiently eluted with CH_2Cl_2/MeOH (100:0-0:1) to give 12 fractions (F_1-F_{12}). Compound 1 (16.8 mg) was recrystallized in acetone from F_2 (1.5 g). F_3 (2.0 g) was subjected to Sephedax LH-20 (100 × 2 cm) eluted with MeOH to give compound 3 (18.6 mg). F_5 (7.5 g) was purified on silica gel column (60 × 3.5 cm, 85 g) eluted with PE/acetone (in gradient, 100:0-0:1) to yield subfractions (Subfr. 5-1-Subfr. 5-8). Compound 9 (13.3 mg) was isolated from Subfr. 5-3 using recrystallization from acetone. Subfr. 5-4 was separated over MPLC (MeOH/H_2O 10:90-100:0) to give compound 4 (32.1 mg). Subfr. 5-6 was subjected to column chromatography over Sephadex LH-20, eluting with gradient MeOH/H_2O to obtain compound 5 (11.8 mg). Compound 6 (18.0 mg) and 7 (25.6 mg) were isolated from F_6 (8.6 g) by chromatography on macroporous resin eluted with MeOH/H_2O (6:4 and 9:1), and silica gel eluted with PE/acetone (10:3). F_7 (14.7 g) was applied to CC on silica gel column (80 × 5 cm, 150 g) with PE/acetone gradient system, MPLC (MeOH/H_2O 8:2) and then purified by Sephedax LH-20 to give compound 2 (17.3 mg). F_8 (2.3 g) was separated by macroporous resin column chromatography using MeOH/H_2O (0:1, 3:7, 6:4, 9:1) to obtain compound 10 (201.3 mg). F_9 (5.5 g) was chromatographied on macroporous resin column eluted with MeOH/H_2O (3:7, 6:4, 9:1) and then purified by Sephadex

LH-20 (MeOH) to give compound 8 (37.6 mg). F_{10} (7.2 g) was subjected to MPLC on polyamide column (MeOH/H_2O 10:90-100:0), silica gel (CH_2Cl_2/MeOH, 25:1-1:1), Sephadex LH-20 (MeOH), was purified by HPLC (RP-18, 11:89 MeOH/H_2O + 0.05% HAc) to give compound 12 (tR = 47 min, 12.4 mg) and 14 (tR = 115 min, 10.1 mg). F_{11} (4.9 g) was subjected to polyamide column (MeOH/H_2O 10:90-100:0) and purified by Sephadex LH-20 (MeOH) to give compound 11 (253.1 mg).

3α-hydroxyolean-28,13-olide (1): White amorphous powder; 1H-NMR (600 MHz, DMSO-d_6) and 13C-NMR (150 MHz, DMSO-d_6) spectral data, see Table 1; HR-ESI-MS: m/z 479.3496 [M + Na]+ (calcd. for $C_{30}H_{48}O_3Na$, 479.3475).

Angiotensin I-converting enzyme assay

For the assay, 1U ACE was dissolved in 5 mL borate buffer (pH 8.3), and each 0.5 mL of enzyme solution was added 1 mL of borate buffer (pH 8.3) before working. Hip-His-Leu (7.6 mmol/L) were prepared in borate buffer (0.2 mol/L, pH 8.3, containing 608 mmol/L of NaCl). Briefly, 15 μL ACE extract was incubated with 5 μL samples for 30 mins at 37°C in 0.5 mL centrifuge tube. Then, 25 μL HHL was added and incubated for 15 mins at 37°C. Afterward, the action was terminated by adding 5 μL 10% TFA.[20] The reaction buffer was directly analyzed in HPLC autoinjection system (mobile phase: 30% methol-1 mL TFA-0.5 mL glacial acetic acid (pH 3-3.3), 70% methol-1 mL TFA-0.5 mL glacial acetic acid (pH 3-3.3) eluted in gradient, AR; detection wavelength: 228 nm; flow rate: 1 mL/min). The resulting hippuric acid was quantitative analyzed by HPLC. The results are listed in Figure 1 and Table 2.

Blood collection and preparation of platelet rich plasma (PRP) and platelet poor plasma[21]

Platelets were drawn from healthy male rat (215 g) into 15% (v/v) acid–citrate–dextrose (ACD: 1.5 mM citric acid, 75 mM sodium citrate, 124 mM dextrose). platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 800 g for 10 min. Platelets were washed and resuspended in buffer A (130 mM NaCl, 10 mM sodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 9 mM $MgCl_2$, 0.81 mM KH_2PO_4 3 mM KCl, 10 mM Tris [tris (hydroxymethyl) aminomethane], pH 7.4), adjusted to 3 × 105/μl, and supplemented with 1.8 mM $CaCl_2$.

Inhibition ratio for platelet aggregation

Platelets were resuspended in buffer A to a consistent concentration (OD value: 1.02) and were allowed to rest for 15 min prior to use. Calcium (1.8 mM) and ADP (20 mM) were added just prior to assay. Platelet aggregations were performed at 37°C. The platelet suspension was added to the 96-well plate with the amount of 100 μl/well. The tested samples were dissolved in DMSO and diluted with buffer A to different concentration. Aspirin was used as positive control and the buffer A (0.1% DMSO) was used as negative control. The OD values were recorded in Flexstation® Enzyme-labelled meter (molecular

Table 1: ^{1}H-NMR (600 MHz), ^{13}C-NMR (150 MHz) spectral data in $CDCl_3$ of compound 1

δ_C	δ_H
33.6/33.7	1.42 (m),1.20 (m)
27.6	1.58 (m)
76.4	3.38 (br. s)
37.8	
48.9	1.18 (m)
17.9	1.38 (m)
33.6/33.7	1.42 (m),1.20 (m)
42.5/42.6	
50.4	1.27 (m)
37.2	
18.2	1.65 (m), 1.46 (m)
25.6	1.93 (m), 1.52 (m)
92.1	
42.5/42.6	
26.7	1.15 (m), 1.80 (m)
21.2	2.03 (m), 1.21 (m)
44.3	
50.8	1.78 (m)
37.7	1.73 (m), 1.31 (m)
31.7	
34.4	1.32 (m), 1.58 (m)
31.6	1.89 (m), 1.51 (m)
28.4	0.92 (s)
22.3	0.81 (s)
16.0	0.86 (s)
18.6	1.12 (s)
20.0	1.04 (s)
180.7	
33.5	0.96 (s)
24.1	0.84 (s)

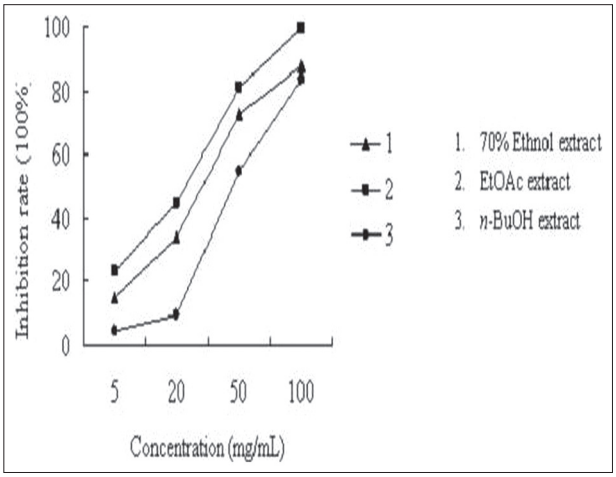

Figure 1: The ACE inhibiting activities of different extracts of Tamarix hohenackeri Bunge (1. 70% ethanol extract; 2. EtOAc extract; 3. n-BuOH extract)

Table 2: The ACE inhibiting activities of extracts and compounds isolated from the active fraction (EtOAc extract)[a]

Sample name	C (mg/mL)	Inhibition rate (%)
70% ethanol extract	5	14.6
	20	33.7
	50	72.8
	100	87.3
EtOAc extract	5	23.5
	20	44.8
	50	81.2
	100	99.2
n-BuOH extract	5	4.6
	20	9.7
	50	54.9
	100	83.2
Compound 1	10	−8.7
	20	−9.7
Compound 2	10	−18.0
	20	−13.9
Compound 3	10	−9.9
	20	−19.9
Compound 4	10	−24.4
	20	−60.1
Compound 5	10	−19.7
	20	−8.0
Compound 6	10	−16.4
	20	−13.9
Compound 7	10	19.5
	20	57.6
Compound 8	10	2.2
	20	48.6
Compound 9	10	−21.4
	20	30.6
Compound 10	10	36.9
	20	46.2
Compound 11	10	15.3
	20	43.1
Compound 12	10	−13.3
	20	10.2
Compound 13	10	8.9
	20	13.3
Compound 14	10	12.9
	20	35.7

n-BuOH extract = *n*-butanol extract, EtOAc: Ethyl acetate. [a]Captopril was used as positive control with IC50 value 6.95 mg/

device) at 405 nm. All the samples were pre-incubated at 37°C for 10 min by sustained oscillation. Then 50 μl antagonists (containing ADP and calcium) were added to the samples. Aggregation was monitored for 30 min after the addition of agonist. The OD values were recorded every 45 s in Flexstation® Enzyme-labelled meter (molecular device) at 405 nm and the aging kinetic curves were expressed. Platelet aggregation rate was calculated by the calculation formula as follows:

$$A_t = (OD_0 - OD_t / OD_t \times 100\%$$

The maximal platelet aggregations of test compounds were selected to calculate the inhibition ratio, which expressed as percentages maximal platelet aggregation of negative normalized to 100% by the calculation formula as follows:

$$\text{Inhibition rate} = (A_{Nmax} - A_{Tmax}) / A_{Nmax} \times 100\%$$

RESULTS AND DISCUSSION

The EtOAc extraction was identified as active part because of its significant ACE inhibitory activity (100 mg/mL, 99.2% inhibiting rate) and anti-platelet aggregation activity (100 μg/mL, 99.2% inhibiting rate). In turn, the active part was purified for ACE and platelet aggregation inhibitors. Fourteen main constituents were isolated and elucidated. Meanwhile, the potential activities were investigated systematically.

Compound 1 was obtained as white amorphous powder. The positive HR-ESI-MS of compound 1 showed a molecular ion peak at m/z 479.3496 [M + Na]+, which indicated a molecular formula of $C_{30}H_{48}O_3$, in conjunction with the ^{13}C-NMR data. The ^{1}H and ^{13}C-NMR spectra of 1 showed characteristics of the oleanane skeleton. The ^{1}H-NMR spectrum displayed seven singlets for δ 0.81, 0.84, 0.85, 0.92, 0.96, 1.04, 1.12 (7 × 3H, s) and 30 carbon signals were observed in the ^{13}C-NMR spectrum. The presence of a quaternary carbon signal at δ 180.7 (C-28) suggested a carboxyl ester or γ-lactone. While the signal at δ 92.1 in the ^{13}C-NMR spectrum showed that the oxygen-bearing carbon in the lactone group was tertiary carbon on either C-13 or C-18. Heteronuclear multiple-bond correlation (HMBC) [Figure 2] between δ 1.04 (3H, s, H-27), 1.15 (1H, m, H-15), 1.78 (1H, m, H-18), and δ 92.1 (C-13); δ 2.03 (1H, m, H-16) and δ 180.7 (C-28) indicated the presence of 28,13-lactone. In the 1H-NMR spectrum, the significant downfield were observed in the methyl region at δ 1.12 (m, H-26) due to the strong anisotropic effect of the lactone group at C-28, while the C-27 methyl appeared at δ 1.04. The facts provided further evidence for the 28, 13-lactone.[22,23] The HMBC spectrum [Figure 2] showed the long-range correlations between methyl proton signals at δ 0.92 (3H, s, H-23), 0.81 (3H, s, H-24), and δ 76.4 (C-3), 48.9 (C-5). The relative configuration of 1 was established by the NOESY spectrum [Figure 3], and a comparison with pertinent reference values.[24-26] The configuration of the hydroxyl group at C-3 could be determined as α-oriented on the basis of the coupling pattern of H-3 (δ 3.38, br. s). In the NOESY spectrum, the correlations of H-1β/H-3, H-9/H-27, H-18/H-29, H-5/H-23 indicated H-3, H-9, H-18 were β-oriented, while OH-3, H-5 were α-oriented. Based on above data, the structure of 1 was elucidated as 3α-hydroxyolean-28, 13-olide. The assignment of ^{1}H and

^{13}C-NMR spectroscopic data of 1 was based on HSQC, HMBC spectra and listed in Table 1.

By comparison their physical and spectral data (ESI-MS, ^{1}H-NMR, ^{13}C-NMR) with those reported, the known compounds were characterized to be isoaleuritolic acid 3-(3',4'-dihydroxy) caffeate (2),[27] 7, 4'-dimethoxykaemferol (3),[28] 4'-methoxykaempferol (4),[10] dillenetin (5),[29] tamarixetin (6),[30] chrysoeriol (7),[31] querceti (8),[32] isoferulic acid (9),[33] 3,5-dihydroxy-4- methoxybenzoic acid (10),[34] gallic acid (11),[35] protocatechuic acid (12),[36] gallic acid 3-methyl ether (13),[37] methyl gallate (14).[38] Structures of the isolated compounds are shown in Figure 4.

Then, the ACE inhibitory activity of the extracts and compounds isolated was investigated at different concentrations. The results are listed in Figure 1 and Table 2. The flavonoid compounds 7 and 8, and phenolic compounds 9, 10, 11, 14 showed strong ACE inhibitory activity. The phenolic compounds 12 and 13 exhibited moderate ACE inhibiting activity. However, the isolated triterpene did not give any ACE inhibitory activity. Considering the concentration of the isolated compounds, it is obvious that 3, 5-dihydroxy-4-methoxybenzoic acid (10) and gallic acid (11) make sense in ACE inhibiting activity. By analyzing the structures of tested compounds, the primary structure and activity relationship was concluded. First, comparing the activities of flavonoid compounds, it is suggested that 4'-hydroxy substitution might play an important role in flavonoid compounds for ACE inhibitory activity. Second, according to the activity result of compounds 10–14, we can summarize that the benzoic acids with 3,5-dihydroxy substitution exhibit better activity. Third, by comparing compounds 11 and 14, it can also be conferred that the benzoic acids showed stronger activity than benzoate.

Meanwhile, the anti-platelet aggregation activities were also investigated systematically Table 3. Dillenetin (5) and chrysoeriol (7) showed significant anti-platelet aggregation activity, respectively by 40.05% and 50.36% at a concentration of 200 μM/mL, which is much better than that of aspirin as a positive control in our experiment. However, the compounds 6, 8, 11, and 14 showed moderate inhibiting activities. Compounds 1, 9, 10, 12, and 13 exhibited

Table 3: The anti-platelet aggregation activities of extracts and compounds isolated from the active fraction (EtOAc extract)

Sample name	C (μg/mL for extracts and μM/mL for compounds)	Inhibition rate (%)
70% ethanol extract	25	14.13
	100	87.25
	400	84.56
EtOAc extract	25	46.88
	100	80.66
	400	97.21
n-BuOH extract	25	−23.2
	100	58.6
	400	81.7
Compound 1	200	5.90
Compound 2	200	−35.44
Compound 3	200	−8.66
Compound 4	200	−3.86
Compound 5	200	40.05
Compound 6	200	14.26
Compound 7	200	50.36
Compound 8	200	22.06
Compound 9	200	4.00
Compound 10	200	2.22
Compound 11	200	13.66
Compound 12	200	5.00
Compound 13	200	7.46
Compound 14	200	18.97
Aspirin[b]	1650	34.01

n-BuOH extract = *n*-butanol extract, EtOAc: Ethyl acetate. [b]Aspirin was tested as the positive control

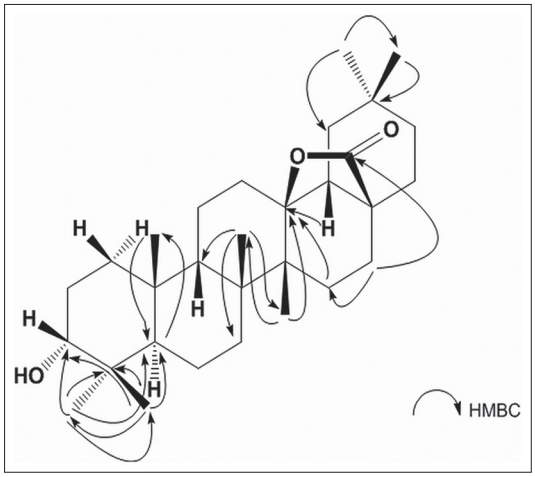

Figure 2: Selected correlations observed in the HMBC spectra of 1

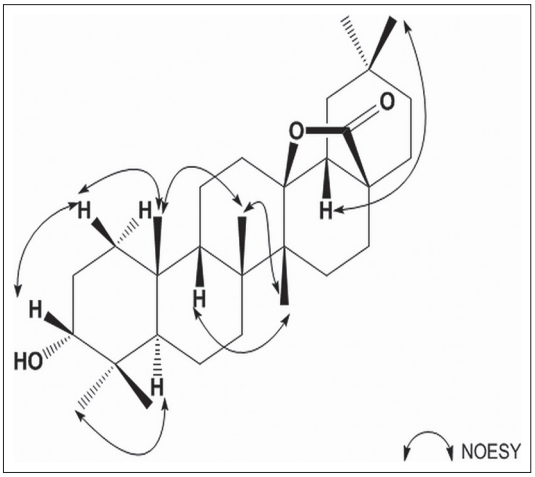

Figure 3: Key correlations observed in the NOESY spectra of 1

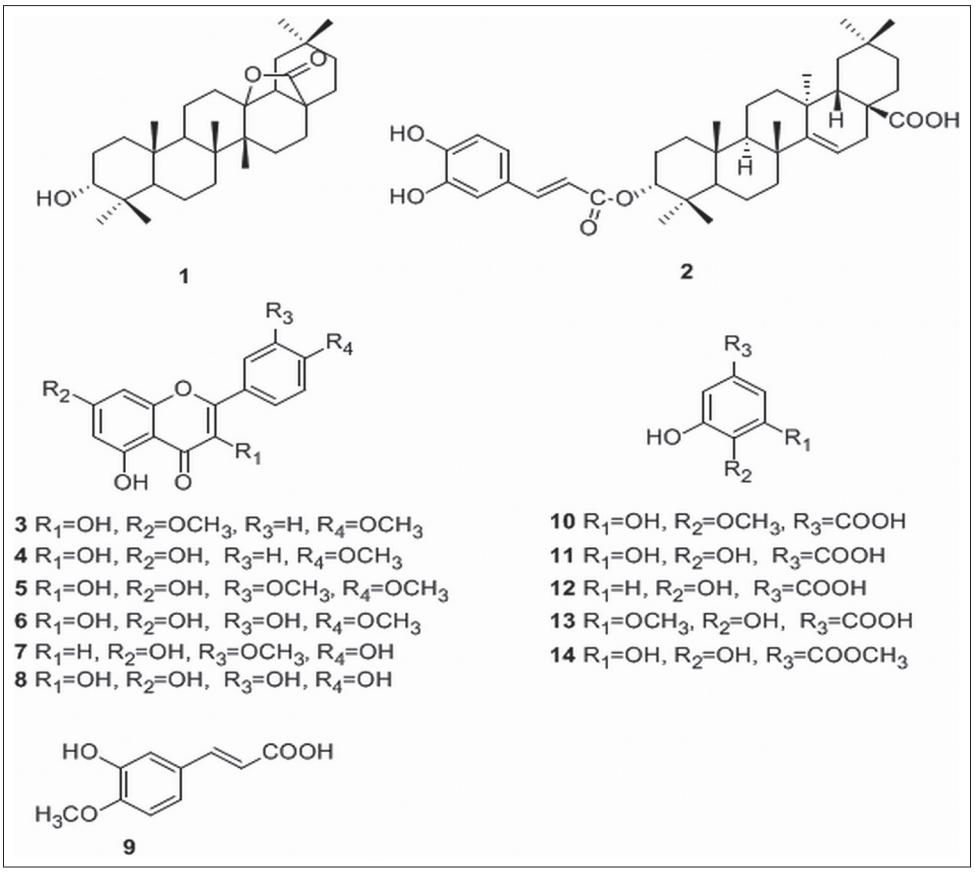

Figure 4: Structures of compounds 1-14

weak activities. Obviously, the flavonoid compounds are responsible for the anti-platelet aggregation activities of this plant. The analysis of structure and activity relationship revealed that the 3'-methoxyl substitution might play a significant role in flavonoid compounds for anti-platelet aggregation activities. Moreover, according to the activities of compounds 10-14, we can conclude that benzoic acids with 3, 4, 5-trihydroxy substitution exhibit better activity.

Above all, compounds 7, 8, and 11 are both natural potential ACE and platelet aggregation inhibitors.

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