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Ramosissimin, a new flavonol from *Tararix ramosissima*, induces apoptosis in rheumatoid arthritis fibroblast-like synoviocytes

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Tamarix ramosissima is a traditional Chinese herbal medicine used for rheumatoid arthritis (RA) treatment in Northwest China. Chemical investigation of EtOH/H_2O extracts of *T. ramosissima* led to the discovery of a new flavonol, ramosissimin (**1**), together with the known flavonoids compounds quercetin (**2**), quercetin-3'4'-dimethylether (**3**) and kaempferol (**4**). By means of high resolution electrospray ionization mass spectroscopy (HRESIMS) and 1D and 2D-NMR experiments, and after comparison with literature data, the structures of the compounds were determined. The effect of compound **1** on the viability of RA fibroblast-like synoviocytes (RA-FLS) was evaluated by MTT assay. Apoptosis-inducing effect of compound **1** in RA-FLS was further investigated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and activated caspase-3/7 level assessment using luminescent assay. The results revealed that ramosissimin displayed remarkable proliferation inhibitory effect in RA-FLS. Furthermore, compound **1** could significantly induce cellular apoptosis of RA-FLS and increase activated caspase-3/7 levels. It is suggested that ramosissimin may inhibit the proliferation of RA-FLS by inducing apoptosis.

1. Introduction

Tamarix ramosissima Ledeb., which is a shrub or dungarunga belonging to the family Tamaricaceae, is commonly known for its strong adaptability to the arid desert environment, saline and alkaline soil. They are excellent plants for sand-fixation and afforestation in the desert area and saline-alkali land (Devitt et al. 1997). Many different species of *Tamarix* have been used for the treatment of inflammation, leucoderma, spleen troubles, and eye diseases as herbal medicines (Sharma and Parmar 1998). Tender branches and leaves of *T. ramosissima* have been used for the treatment of rheumatoid arthritis (RA) in the Ningxia Hui Autonomous Region, Northwest China, for a long time. Previous pharmacological studies showed that the extract of *T. ramosissima* has antimicrobial and antioxidant activities (Sultanova et al. 2001). In phytochemical studies, it was found to be rich in polyphenolic compounds like phenolic acids, tannins, flavonoids and coumarins (Zhang and Tu 2006; Li et al. 2014; Liao et al. 2012).

The chemical investigation of *T. ramosissima* was carried out in order to find new natural compounds effective against rheumatoid arthritis (RA). In our previous study, a new phenolic aromatic ring substituted lactam which could inhibit the proliferation of RA fibroblast-like synoviocytes (RA-FLS) through apoptosis-inducing effect was found (Yao et al. 2017). In the continued study, four flavonoids including a new flavonol, ramosissimin (**1**), together with quercetin (**2**), quercetin-3'4'-dimethylether (**3**) and kaempferol (**4**), were isolated and identified from the tender branches and leaves of *T. ramosissima*. The anti-proliferation effect and the apoptosis-inducing effect of the new compound ramosissimin were further assessed on RA-FLS.

2. Investigations, results and discussion

2.1. Purification of compounds 1-4

Tender branches and leaves of of *T. ramosissima* were extracted with a mixture of EtOH/H_2O (7:3). The crude extract was further extracted with petroleum ether, EtOAc and n-BuOH successively. The EtOAc extraction was subjected to chromatographic separation on normal SiO_2 columns, ODS RP-18 columns, and prep-HPLC to provide the new flavonol, ramosissimin (**1**), quercetin (**2**), quercetin-3'4'-dimethylether (**3**) and kaempferol (**4**) (Fig. 1).

2.2. Structure elucidation of compounds 1-4

Compound **1** was obtained as a yellow crystalline material. A dark spot could be observed under 254 nm on thin layer plate after development, while a yellow spot could be observed under sunlight. Its molecular formula was determined as $C_{25}H_{22}O_9$ on the basis of the positive high resolution electrospray ionization mass spectroscopy (HRESIMS) pseudomolecular ion peak at m/z 467.1340 $[M+H]^+$ (calcd. for 467.1342) and ^1H and ^{13}C NMR spectral analyses. The ^1H NMR spectrum (400 MHz, DMSO-d_6) of compound **1** displayed two methoxyl signals δ_H 3.81 (3H, s) and 3.58 (3H, s). A methyl signal δ_H 1.64 (3H, d, J = 7.2 Hz) and a coupling methylene signal δ_H 4.70 (3H, q, J = 7.2 Hz), which suggested the existence of -CH-CH_3 fragment. Six aromatic proton signals δ_H 7.06 (1H, d, J = 8.4 Hz), 7.56 (2H, dd, J = 8.4, 8.4 Hz), 7.43 (1H, d, J = 1.6 Hz), and 6.61 (1H, d, J = 8.0 Hz), 6.53 (2H, dd, J = 8.0, 8.0 Hz), 6.62 (1H, d, J = 1.6 Hz are typical signals belonging to two ABX coupling systems, which suggested two triple-substituted benzene rings in the structure. In low field area there are six singlet proton

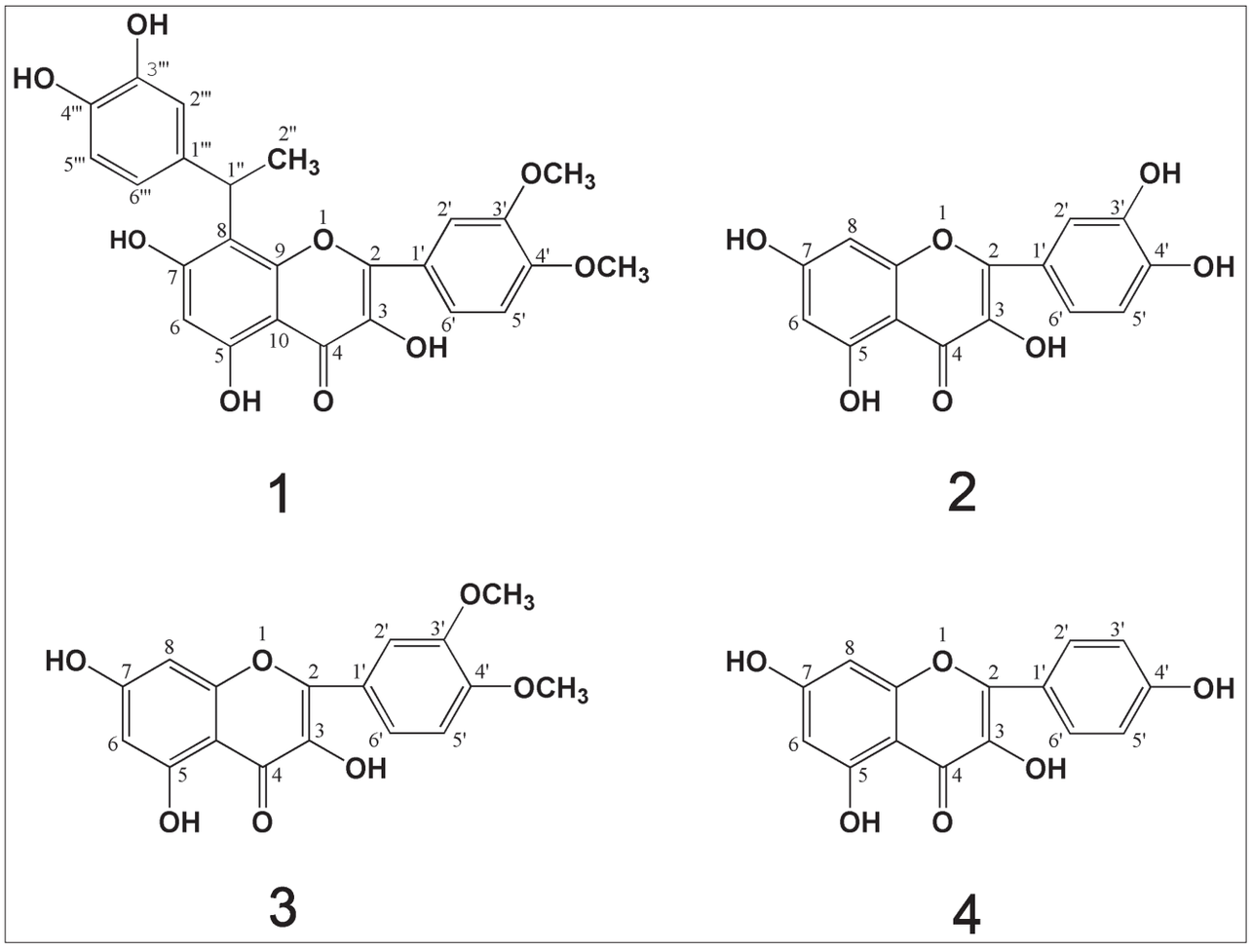

Fig. 1: Structures of compounds **1-4**

signals δ_H 12.56 (1H, s), 10.75 (1H, s), 9.48 (1H, s), 8.63 (1H, s), 8.53 (1H, s) and 6.33 (1H, s). In heteronuclear multiple-quantum correlation (HSQC) NMR spectrum analysis, the signal δ_H 6.33 was determined to be a sp^2 hybridized CH proton signal by the correlation with δ_C 98.2. While the others five singlets should be active hydrogen signals, which have no correlation with carbon signals in the HSQC spectrum.

The ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of compound **1** displayed nances for 25 carbon signals. The δ_C 18.8 and 31.7 are the methyl and attached methylene carbon signals. It could be inferred that methylene is not connected with oxygen by the chemical shift of methylene carbon. δ_C 55.5 and 55.2 were two methoxyl carbon signals, while δ_C 176.2 is a carbonyl carbon signal. There were 20 carbon signals between δ_C 98.1 and 161.5, which could be inferred to be signals belonging to three benzene rings and one double bond by comprehensive analysis of ^1H NMR spectrum. Comprehensive analysis of the HSQC and the heteronuclear multiple bond correlation (HMBC) NMR spectrums led to the assembly of a 5,7-dihydroxy flavonol fragment, including the ring A proton signal δ_H 6.33 (1H, s) and carbon signals δ_C 161.5, 158.6, 154.0, 110.5, 103.5, 98.2, ring B proton signals δ_H 7.06 (1H, d, J = 8.4 Hz), 7.56 (2H, dd, J = 8.4, 8.4 Hz) and 7.43 (1H, d, J = 1.6 Hz) and the carbon signals δ_C 150.2, 148.3, 123.4, 121.7, 111.4, 110.5, and ring C carbon signals δ_C 176.3, 146.4, 135.3. The heteronuclear long range correlations between methoxyl signal δ_H 3.82 and ring B carbon signal δ_C 150.2, methoxyl signal δ_H 3.59 and ring B carbon signal δ_C 148.3 suggested that both methoxyl group were attached to the ring B, which provide a quercetin-3', 4'-dimethyl ether structure. There is only one proton (δ_H 6.33) on ring A, which suggests the substitution on C-5 or C-8.

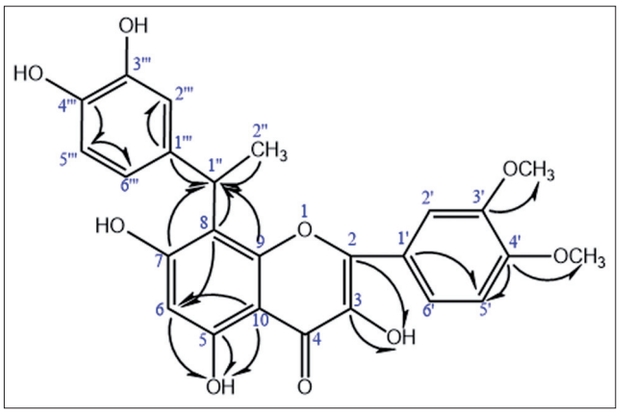

Fig. 2: Selective HMBC correlations of compound **1**

Further analysis of the two-dimensional NMR spectra led to the confirmation of the fragments of 3,4-dihydroxy substituted phenyl and -CH-CH_3. The connection of these fragments was based on HMBC correlation between methylene signal $\delta_{H\text{-}1''}$ 4.70 (3H, q, J = 7.2 Hz) and aromatic carbons C-8 (δ 110.5) and C-1'''(δ 135.9), so the 3,4-dihydroxy substituted phenyl group should be connected to the quercetin-3', 4'-dimethyl ether on C-8 through -CH-CH_3. Therefore, the structure of the compound 1 was finally identified as 8-(1-(3,4-dihydroxyphenyl)ethyl)-3',4'-dimethoxy-3,5,7 – trihydroxyflavone as shown in Fig. 2. Compound **1** was generically named ramosissimin.

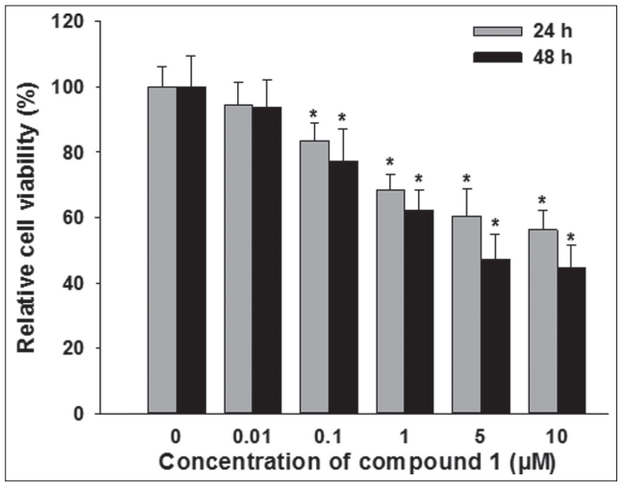

Fig. 3: Inhibition effect of compound **1** on RA-FLS cell viability. Cell viability was measured with 3-(4,5-dimethy1-2-thiazoly1)-2,5-dipheny1-2-H-tetrazolium bromide (MTT) assay after treatment with compound 1 (0, 0.01, 0.1, 1, 5, and 10 μM) for 24 or 48 h. Data were shown as means±S.E.M. of three independent experiments (*$p < 0.05$).

The other three compounds (Fig. 1) were identified as quercetin (**2**) (Abouzid and Sleem 2011), quercetin-3'4'-dimethylether (**3**) (Assiri 2006) and kaempferol (**4**) (Allen and Yi 2013) by extensive analysis of their spectroscopic data, including ESIMS, ^{1}H and ^{13}C NMR data, as well as by comparison with literature data.

2.3. In vitro anti-RA activity of compound 1

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation, which ultimately leads to the destruction of cartilage and bone in the affected joints (Firestein 2003; Wachsmann and Sibilia 2011). Synovial hyperplasia is a hallmark of RA pathology, which causes marginal bone erosion and joint destruction (Ruiz-Heiland et al. 2013; Ziswiler et al. 2009). RA-FLS exhibit aggressive features including hyperproliferation, apoptosis resistance, and high invasiveness, which suggest that RA-FLS play a pivotal part in the pathological process of joint destruction and synovitis in RA (Lefevre et al. 2009; Huber et al. 2006). Therefore, the *in vitro* anti-RA effect of compound **1** was investigated on cultured RA-FLS.

RA-FLS were incubated with various concentrations of compound **1** (0.01, 0.1, 1, 5, and 10 μM) for 24 or 48 h, and then cell viability was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tet-

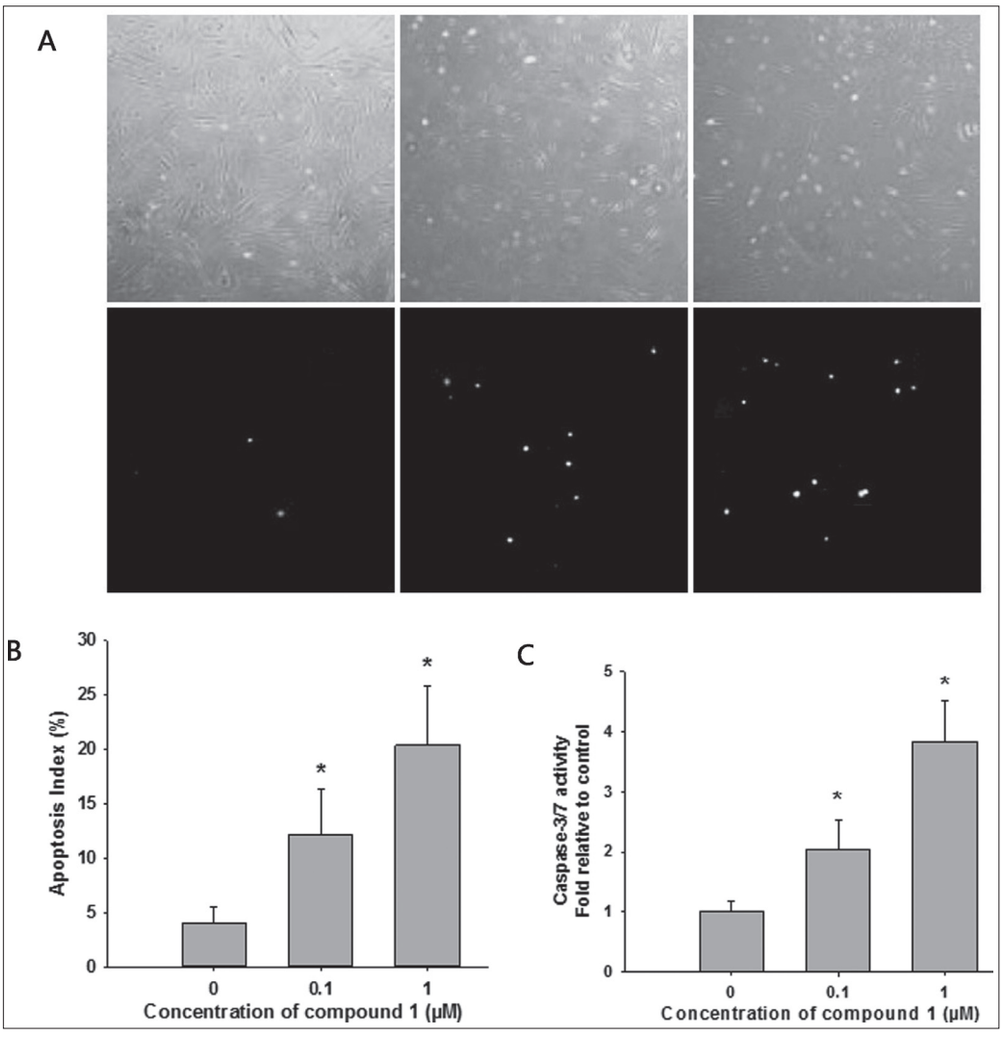

Fig. 4: Effects of compound 1 on RA-FLS apoptosis. (A) After treatment with compound 1 (0.1 or 1 μM) or dimethyl sulphoxide (DMSO) vehicle for 48h, apoptotic cell death measured by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (×100). Bright spots in the lower panels are TUNEL-positive apoptotic cells. Corresponding phase contrast microscopy images are shown in the upper panels; (B) The apoptosis index of RA-FLS treated with 0.1 or 1 μM of compound 1 or DMSO vehicle. Data were shown as means ± S.E.M. of three independent experiments (*$p < 0.05$); (C) Effects of compound 1 on the levels of active caspase-3/7. RA-FLS cells were treated with 0.1 or 1 μM of compound 1 for 48 h and then processed to measure using the Caspase-Glo kit. Data were shown as means ± S.E.M. of three independent experiments (*$p < 0.05$).

Table: NMR spectral data of 1 (DMSO-d_6, 400 and 100 MHz)

NO.	δ_H [Mult., J (Hz)]	δ_C
1	-	-
2	-	146.4 CH
3	-	135.3 C
4	-	176.3 C
5	-	158.6 C
6	63.3s	98.2 CH
7	-	161.5 C
8	-	110.5 C
9	-	154.0 C
10	-	103.5 C
1’	-	123.4 C
2’	7.43 d (1.6)	110.5 CH
3’	-	150.2 C
4’	-	148.3 C
5’	7.06 d (8.4)	111.4 CH
6’	7.56 dd (8.4, 1.6)	121.7 CH
1”	4.70 q (7.2)	31.7 CH
2”	1.64 d (7.2)	18.8 CH
1”’	-	135.9 C
2”’	6.62 d (1.6)	114.4 CH
3”’	-	143.0 C
4”’	-	144.8 C
5”’	6.61 d (8.0)	115.2 CH
6”’	6.53 dd (8.0, 1.6)	117.3 CH
3-OH	9.48 s	-
5-OH	12.56 s	-
7-OH	10.75 s	-
3’-OCH3	3.81 s	55.5 CH_3
4’-OCH3	3.58 s	55.2 CH_3
3”’-OH	8.63 s	-
4”’-OH	8.53 s	-

razolium bromide (MTT) assay (Gerlier and Thomasset 1986). The results revealed that 0.1~1.0 μM compound **1** treatment could significantly inhibit the cell viability of RA-FLS in both time- and dose-dependent manners ($p < 0.05$) compared with vehicle-treated control cells; 24 h treatment with 0.01, 0.1, 1, 5, and 10 μM of compound **1** decreased cell survival by 95%, 83%, 68%, 61%, and 56%, respectively, and by 94%, 77%, 62%, 47%, and 45% of the control viability level after 48 h, respectively (Fig. 3). Concentrations of 0.1 and 1 μM of compound **1** were used for subsequent apoptosis inducing assessments based on these results.

To evaluate whether compound **1** treatment has an effect on apoptotic cell death of RA-FLS, a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performanced (Byrne et al. 1999). The results showed that treatment with 0.1 and 1 μM of compound **1** for 48 h significantly increased the number of TUNEL-positive cells compared to vehicle control cells (Figs. 4A, B; 12.17±4.08 % and 20.34±5.47 %, respectively). Meanwhile, it was found that compound **1** could significantly increase activated caspase-3/7 levels (Fig. 4C; 0.1 and 1 μM are about 2.03- and 3.83-fold higher than untreated vehicle, respectively), which were examined with a Caspase-Glo kit, suggesting the potent pro-apoptotic effect of compound **1** (Lakhani et al. 2006).

RA is a systemic autoimmune disease characterized by chronic synovial inflammation, which ultimately leads to marginal bony erosions and bone in the affected joints (Yu-Jung et al. 2011). Synovial hyperplasia is recognized as one of the major pathological characteristics of RA, which have been linked most prominently to the progressive destruction of articular cartilage (Mor et al. 2005). Some evidence suggested that rheumatoid synovia shows tumor-like expansion attributed to the resistance of RA-FLS to the apoptotic process (Baier et al. 2003; Korb et al. 2009). Therefore, compounds which could exert apoptosis-inducing activity on RA-FLS may provide a potent therapeutic approach for the treatment of RA. Our investigations showed that compound **1** had a remarkable effect of apoptosis-inducing effect on RA-FLS *in vitro*, but the *in vivo* anti-RA effect and related mechanisms need further investigation.

2.4. Conclusion

In the phytochemical investigation of the 70% EtOH/H_2O extract of the traditional herbal medicine *T. ramosissima*, a new flavonol, ramosissimin (**1**), together with three previously reported flavonoids quercetin (**2**), quercetin-3’4’-dimethylether (**3**) and kaempferol (**4**) were isolated. These compounds were identified by HRESIMS and 1D and 2D-NMR experiments, as well as comparison with literature data. Compound **1** showed remarkable apoptosis-inducing effect on RA-FLS, suggesting the potential for RA treatment.

3. Experimental

3.1. General procedures

High resolution electrospray ionization mass spectroscopy (HRESIMS) data were recorded with an APEX II HR-TOF spectrometer (Bruker, Bremen, Germany). Melting points were measured on a XT4 microscopic melting-point apparatus (Shanghai Jingke Instruments Company, Shanghai, China). NMR spectra were obtained in DMSO-d_6 on a Bruker Avance DRX 400-MHz spectrometer at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. Precoated silica gel GF_{254} plates (Merck, Darmstadt, Germany) were used for TLC. For column chromatography, SiO_2 (100-200 mesh, Qingdao Marine Chemical Factory, Qingdao, China) and Rp-C_{18} (ODS-A, 50μm, YMC, Yantai, China) were used. HPLC purifications were performed on HPLC columns (YMC-Pack Pro C18, 5μm, 250 mm × 4.6 mm and 250 mm × 10 mm, YMC, Kyoto, Japan) with a L-2000 HPLC system (Hitachi, Tokyo, Japan).

3.2. Biological materials

Tender branches and leaves of *T. ramosissima* were collected from sandy land near Yellow River, Yinchuan, China. The samples were identified by Dr. Yunsheng Zhao, School of Pharmacy, Ningxia Medical University. A voucher sample was kept at Department of Medical Chemistry, School of Basic Medical Science, Ningxia Medical University under the registration code No. 2014050201.

3.3. Isolation and purification of the compounds

Air-dried branches and leaves of *T. ramosissima* (5.0 kg) were smashed and extracted with a mixture of EtOH/H_2O (7:3, 3 times, each for 2 h, under reflux). The successive extracts were combined and evaporated under reduced pressure to afford a crude extract (412 g), which was partitioned in 1 L H_2O and extracted with petroleum ether (PE), EtOAc, and n-BuOH successively (4 × 1 L). Then these combined extracts were evaporated under reduced pressure separately to give three different crude extracts. The EtOAc extract (43 g) was subjected to a silica gel column with gradient elution (CH_2Cl_2-MeOH) to give 11 fractions (Fr. 1 to Fr. 11). Fr. 7 (1.93 g), which was eluted with 15% MeOH in CH_2Cl_2, was fractionated on a Rp-C18 column using MeOH-H_2O gradient eluent, affording 9 subfractions (Fr.7-1 to Fr.7-9). Fr.7-8 was subjected to the Sephadex LH-20 gel column using methanol as the eluent and further purified by recrystallization from MeOH to yield compound **1** (21 mg). Compounds **2** and **3** were isolated and purified from 1.92 g fraction Fr. 8 by applying Rp-C18 column fractionation using MeOH-H_2O gradient eluent and Sephadex LH-20 column fractionation with MeOH-H_2O (40:60). Compound **4** was separated from fraction Fr.6 (2.55g) through fractionation on Rp-C18 column using MeOH-H_2O gradient eluent and Sephadex LH-20 column with MeOH.

3.4. Characterization of compounds 1-4

Ramosissimin (**1**): Yellow crystalls; HRESIMS m/z: 467.1340 $[M+H]^+$ (calcd. for 467.1342); NMR spectral data, see Table.

Quercetin (**2**): Yellow amorphous powder; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 12.48(1H, s), 10.76 (1H, brs), 9.57 (1H, brs), 9.34 (2H, brs), 7.68 (1H, d, J = 2.0 Hz, H-2’), 7.54 (1H, dd, J = 8.4, 2.0 Hz, H-6’), 6.88 (1H, d, J = 8.4 Hz, H-5’), 6.40 (1H, d, J = 2.0 Hz, H-8), 6.18 (1H, d, J = 2.0 Hz, H-6).

Quercetin-3’4’-dimethylether (**3**): Yellow amorphous powder; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 12.42 (1H, s), 10.78 (1H, s), 9.51 (1H, s), 7.74 (1H, d, J = 2.0 Hz, H-2’), 7.79 (1H, dd, J = 8.4, 2.0 Hz, H-6’), 7.13 (1H, d, J = 8.4 Hz, H-5’), 6.49 (1H, d, J = 2.0 Hz, H-8), 6.20 (1H, d, J = 2.0 Hz, H-6), 3.84 (3H, s), 3.83 (3H, s).

Kaempferol (**4**): Yellow amorphous powder; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 12.47 (1H, s), 10.21 (2H, brs), 9.61 (1H, brs), 8.04 (2H, d, J = 8.4 Hz, H-2’, H-6’), 6.92 (2H, d, J = 8.4 Hz, H-3’, H-5’), 6.43 (1H, d, J = 2.0 Hz, H-8), 6.19 (1H, d, J = 2.0 Hz, H-6); ^{13}C NMR (DMSO-d6, 100MHz): δ_C 163.9 (C-7), 160.8 (C-5), 159.2 (C-2), 156.2 (C-4’), 146.8 (C-9), 135.7 (C-3), 129.6 (C-2’, C-6’), 121.7 (C-1’), 115.5 (C-3’, C-5’), 103.1(C-10), 98.2 (C-6), 93.5 (d, C-8).

3.5. Biological assays

3.5.1. RA-FLS cell culture

Human RA-FLS were obtained from Cell Applications, Inc. (San Diego, CA, USA) and cultured with a synoviocyte growth medium (Cell Applications). RA-FLS obtained from the 3rd to 5th passages were used for experiments. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Ningxia Medical University (Project identification code: 2014-036).

3.5.2. Assessment of cell viability using MTT assay

Compound **1** was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Co. St. Louis, MO, USA). RA-FLS were seeded in 48-well plates at a density of 3×10^4 cells/well, and were treated with 0.01, 0.1, 1, 5, and 10 μM of compound **1** or DMSO vehicle only in a serum free medium. After 24 or 48 h incubation, 2.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (Sigma-Aldrich) were added to the wells, and the cells were then incubated for 2 h. The absorbance of each well was measured using a Bio-Rad 680 microplate reader (Bio-Rad laboratories, Hercules, CA, USA) at 570 nm.

3.5.3. TUNEL assay

Apoptosis in RA-FLS cells was measured by TUNEL assay (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. Briefly, RA-FLS were incubated with 0.1 and 1 μM of compound **1** for 48 h and then fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with phosphate buffer saline (PBS), permeabilization solution (0.1% sodium citrate, 0.1% Triton X-100) was added for 2 min reaction and cells were incubated with terminal deoxynucleotidyl transferase (TdT) and biotin-11-dUTP for 1 h at 37 ºC. The nuclear morphology was observed by fluorescence microscopy IX71 (Olympus, Tokyo, Japan). The apoptosis index was calculated based on the percentage of TUNEL-positive cells in 1000 RA-FLS.

3.5.4. Measurement of caspase-3/7 activity

The levels of activated caspase-3/7 in RA-FLS were further assessed with a Caspase-Glo kit (Promega, Madison, WI, USA) (Xu et al. 2015), according to the manufacturer's protocol. Briefly, cells were plated at 1×10^4 cells/well in 96-well plates and treated with 0.1 and 1 μM of compound **1** for 48 h. After incubation, cells were treated for 2 h with reconstituted Caspase 3/7-Glo reagent. Then the luminescence signal generated after cleavage of DEVD-aminoluciferin substrate by caspase 3/7 was detected using a luminometer plate reader (Luminoskan Ascent, Thermo Electron, Helsinki, Finland).

3.6. Data analysis

The level of statistical significance was determined by using one-way analysis of variance (ANOVA). All statistical tests were performed using SPSS statistical software, version 12.0 (SPSS, Chicago, IL, USA). Results were expressed as the mean±S.E.M. of three independent experiments performed in triplicate. Differences were considered to be significant when $p < 0.05$.

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Conflicts of interest: None declared.

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