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ORIGINAL ARTICLE

Identification of bioactives from *Astragalus chinensis* L.f. and their antioxidant, anti-inflammatory and anti-proliferative effects

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Abstract This work was designed to obtain the valuable compounds with antioxidant, anti-proliferative and anti-inflammatory activities from *Astragalus chinensis*. Ethyl acetate fraction obtained from *A. chinensis* L.f. had significant antioxidant, anti-proliferative and anti-inflammatory activities. Subsequently, five single compounds were separated and purified, which were identified as formononetin (1), rhamnocitrin (2), calycosin (3), β-daucosterol (4), rhamnocitrin-3-*O*-β-D-glucoside (5). The results displayed that formononetin and rhamnocitrin exhibited significant cytotoxicity actions against tumor cell lines. Calycosin exerted the strongest anti-inflammatory effect of inhibition effects on NO production in macrophages.

Keywords *Astragalus chinensis* L.f. · Antioxidant · Antitumor · Anti-inflammatory · Formononetin · Calycosin

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Introduction

Astragalus chinensis L.f., the dried ripe seed of *Astragalus complanatus* Bunge, which belongs to Leguminosae, has been regarded as raw material of health food in China. It is extensively distributed in northern China and called shayuan-zi in Chinese. *China Pharmacopoeia* (1990 edition) recorded *Astragalus chinensis* L.f. with its healthcare functions and the clinical usage of its prescription (Zhang et al. 2013).

In the past 10 years, much research has been conducted to identify the chemical constituents from *Astragalus chinensis* L.f. and investigate their biological activities and pharmacological effects (Li et al. 2005). Reports indicated that *Astragalus chinensis* L.f. had the functions of tonifying kidney, protecting hepato and restraining urination (Xue et al. 2008). Besides, it also has been used as antihypertensive drugs in clinic (Liu et al. 2005). These results showed that many of these pharmacological activities are attributed to its main constituents such as complanatoside A, complanatoside B, astragalin, myricomplanoside, neocomplanoside and so on. Favonoids from *Astragalus chinensis* L.f. also showed good biological activity (Zhang et al. 2005; Zhang et al. 2010; Ou et al. 2007). Nowadays, many types of food products involved in *Astragalus chinensis* L.f. have been developed, such as commercial *Astragalus chinensis* L.f. extracts, candies and so on.

The present research investigated the effective ingredients in *Astragalus chinensis* L.f., which possess the antioxidant, anti-proliferative and anti-inflammatory activities that used to evaluate the potential nutritive value of *Astragalus chinensis* L.f., especially the individual compounds from it. It is hoped that, with the report of the present research, *Astragalus chinensis* L.f. may become a

more available material in the development of functional foods.

Materials and methods

Plant material

The dried *Astragalus chinensis* L.f., identified as the dry ripe seeds of *Astragalus complanatus* Bunge, was bought from Guangzhou medicamentarius, and were authenticated by professor Hao Gang of South China Agricultural University, where voucher specimens (voucher specimen number 38276) were kept. Samples were crushed in a cutting mill to powder for further use.

Reagents

1, 1-diphenyll-2-2-pricylhydrazyl (DPPH), vitamin C (VC), trichloroacetic acid (TCA), penicillin, streptomycin, MTT, 5-fluorouracil, lipopolysaccharide (LPS) were obtained from Sigma. Fetal bovine serum (FBS), DMEM medium and trypsin–EDTA were obtained from Gibco.

Extraction, isolation and purification of bioactive constituents

Firstly, *Astragalus chinensis* L.f. was extracted with 95% ethanol and the extracting solution was concentrated and evaporated. Next, four fractions were obtained by organic solvent extraction. Ethyl acetate fraction exhibited the best effects, which was chosen to be further isolated and purified.

Ethyl acetate fraction was directly loaded to a silica gel column and eluted with different concentrations of chloroform–methanol solution (100:0, 99:1, 98:2, 95:5, 90:10, 50:50, 0:100, v/v). The eluates were divided into several fractions on the basis of qualitative analysis. Fraction 99:1 was further loaded on a Sephadex LH-20 column and eluted with 100% methanol to yield compound 1 (natural crystal), and compound 2 was obtained by semi-preparative HPLC. Fraction 98:2 was further purified by a ODS column chromatography to obtain four fractions (30, 60, 90 and 100% methanol fraction, respectively). Compound 3 was obtained from the 30% methanol fraction, which was further purified by semi-preparative HPLC. Compound 4 was obtained from the fraction 95:5 through recrystallization method. Fraction 90:10 was applied on a ODS column to obtain four fractions (40, 70, 90 and 100% methanol fraction, respectively). The 70% methanol fraction was further purified by semi-preparative HPLC, and then compound 5 was obtained. Figure 1 illustrates the above separation and purification process of *Astragalus chinensis* L.f. in a flow diagram.

Chemical structure analysis

The chemical structures of isolated pure compounds were analyzed and identified according to EI-MS, ^{1}H-NMR, ^{13}C-NMR and DEPT135-NMR spectra analyses.

DPPH radical scavenging activity

The effects of four fractions on DPPH radical scavenging were performed on the basis of previous method with small changes (Prathapan et al. 2011; Muniz-Marquez et al. 2013). 0.5 mL of different samples at a concentration of 30, 50, 100, 300 and 500 μg/mL were added to 150 μM DPPH· solution. Then incubate the samples in dark for 30 min, and the absorbance was obtained at 517 nm.

Total reducing power ability

The effects of samples on the reducing power ability were performed on basis of previous method (Kapoor et al. 2013). Firstly, different samples at a concentration of 50, 100, 200, 400 and 800 μg/mL were added to phosphate buffer and $K_3Fe(CN)_6$. Then the reaction mixture was incubated at 50 °C for 20 min. Next, 10% TCA was mixed with the mixture and it was centrifuged for 10 min. After that, the upper layer solution was added to 0.1% $FeCl_3$. Then the optical density was obtained at 700 nm. The increase of absorbance value reflected the reducing power ability.

Inhibition activity on human cervical carcinoma cell HeLa

The anti-proliferative activities of four fractions and four pure compounds were tested by MTT assay with human cervical carcinoma cell HeLa in vitro, respectively, in which 5-FU was considered as the positive control. Briefly, exponential growth phase HeLa cells were cultured in complete DMEM medium, and they were put in 96-well plates and incubated at 37 °C overnight. Then the supernatant media were discarded and 100 μL complete medium containing different concentrations of samples were added and incubated at 37 °C for 24 h. After then, 100 μL of media containing 10% MTT (5 mg/mL) was added to each well, followed by incubation for 4 h at 37 °C. Then, the supernatant was discarded and 150 μL of DMSO was added to dissolve formazan crystals. The optical density (OD) was measured at 490 nm. Cell proliferation inhibition rate was calculated using the following formula (Wijesinghe et al. 2013):

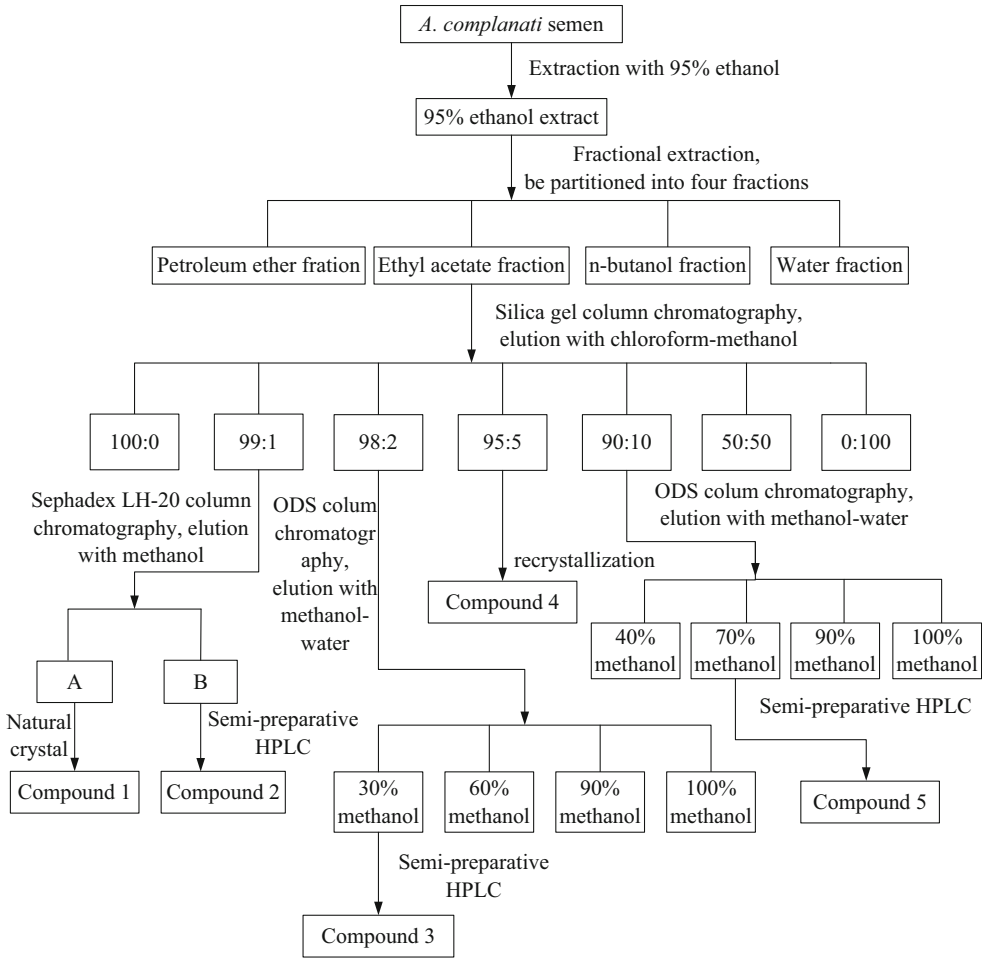

Fig. 1 The isolation and purification process of *Astragalus chinensis* L.f.

$$\text{Cell proliferation inhibition } (\%) = \left(1 - \frac{OD_{\text{sample}}}{OD_{control}}\right) \times 100\%$$

Anti-inflammatory assays

The effects of samples on anti-inflammatory activities were investigated with macrophages. Cells were cultured in complete DMEM medium. The Griess reaction was used to measure NO production, and the cells were activated with LPS on basis of Hsu et al. (2013). After that, absorbance was measured at 540 nm and nitrite levels in the samples were calculated from a standard curve with known concentrations of sodium nitrite. NO inhibition rate was calculated using the following formula:

$$\text{NO inhibition } (\%) = \frac{OD_{LPS} - OD_{\text{LPS+sample}}}{OD_{LPS} - OD_{blank}} \times 100\%$$

Statistical analysis

Data were analyzed by ANOVA and presented as mean ± SD. Significant differences were performed by variance-Duncan's multiple range test ($p \leq 0.05$). The letters in the figures indicated the significant differences between different fractions or compounds.

Results and discussion

DPPH radical scavenging activity

In this study, DPPH radical scavenging activities of four fractions were tested and the results were compared. As seen in Fig. 2a, the DPPH radical-scavenging capacity of all samples change in a dose-dependent manner. The scavenging ability of ethyl acetate fraction significantly increased with the increase in concentration and was stronger than that of other three fractions at all

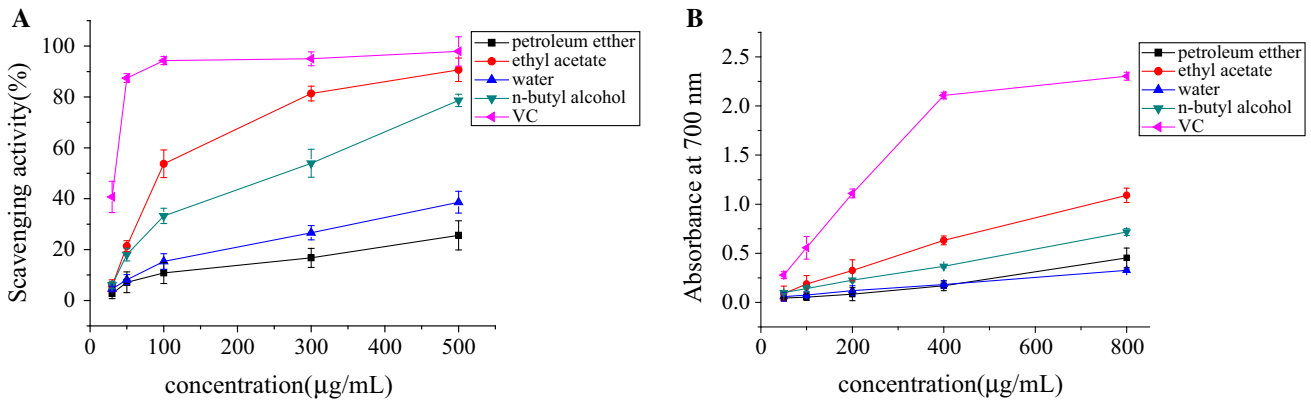

Fig. 2 Antioxidant activity of the four fractions from *Astragalus chinensis* L.f. **a** DPPH radical scavenging ability. **b** Total Reducing power

concentrations. The scavenging effect of ethyl acetate reached about 90% at the concentration of 500 μg/mL, which was on the verge of VC. In addition, *n*-butyl alcohol fraction showed a certain scavenging effect, while petroleum ether and water fraction showed low scavenging activity. Besides, the estimated IC_{50} values of the four fractions extracted from *Astragalus chinensis* L.f. and the positive control were obtained. VC exhibited remarkable IC_{50} value (31.96 μg/mL), and the IC_{50} values of four fractions (in the order of petroleum ether fraction, ethyl acetate fraction, water fraction and *n*-butyl alcohol fraction) were > 500, 95.69, > 500 and 229.55 μg/mL, respectively, which could be seen that ethyl acetate fraction displayed a better effect among the four fractions.

Total reducing power

The reducing power of chemical compounds may be used as an available indicator of the potential antioxidant activity. Figure 2b shows the reducing power of four fractions. The higher absorbance reflects the stronger reducing power. VC has very strong antioxidant ability that used as positive control. In this work, the value of all samples was much lower than that of VC. However, ethyl acetate fraction showed the best effect among the four fractions, which were higher than that of the other three fractions at various concentrations. According to reducing power assays, the activities of the four fractions decreased in the order of ethyl acetate fraction > *n*-butyl alcohol fraction > water fraction > petroleum ether fraction, which was similar to that observed for the DPPH assays.

The results mentioned above demonstrated that ethyl acetate fraction had a noticeable effect on inhibiting the formation of DPPH free radical and a better reducing power ability among these four fractions. Although none of the samples had stronger activities than VC at the same concentration, it implied that ethyl acetate fraction isolated from *Astragalus chinensis* L.f. was valuable for further separation and purification.

Structure identification

The chemical structures of five compounds isolated from *A. chinensis* L.f. were identified according to EI-MS and NMR spectra analyses, which are listed below and displayed in Fig. 3.

Compound 1 was white powder, molecular formula $C_{16}H_{12}O_4$, ESI–MS (m/z) $[M\text{-}H]^-$ 267. On the basis of ^{1}H-NMR spectral, four hydrogen atoms conducted a form of AA′XX′ spin coupling system, which at δ_H 7.5 (2H, d, $J = 8.6$ Hz) and δ_H 6.98 (2H, d, $J = 8.5$ Hz), showed that there was a benzene ring structure. It showed 2-H signal of isoflavone and $-OCH_3$ signal, which were at δ_H 8.30 (1H, s) and δ_H 3.77 (3H, s), respectively. It was identified as formononetin with a purity of 98% (Fig. 3a) (Wang et al. 2014).

Compound 2 was yellow acicular crystal, m.p. 221-222 °C. ESI–MS m/z: 323 $[M+Na]^+$, 301 $[M+H]^+$, 299 $[M\text{-}H]^-$, indicated that its molecular weight was 300. In the ^{1}H-NMR spectral, it showed kaempferol signals, which were at δ_H 12.47 (1H, s), δ_H 8.09 (2H, d, $J = 8.9$ Hz), δ_H 6.95 (2H, d, $J = 8.9$ Hz), δ_H 6.73(1H, d, $J = 1.6$ Hz) and δ_H 6.35 (1H, d, $J = 1.6$ Hz). There was an extra $-OCH_3$ signal in comparison with kaempferol, which was at δ_H 3.86 (3H, s). There were 14 carbon signals in ^{13}C-NMR spectral. It could also be concluded to have symmetrical structure fragments combined with ^{1}H-NMR and DEPT135-NMR spectral, which were at δ_C 129.5 (C–H) and δ_C 115.4 (C–H). Its molecular formula was $C_{16}H_{12}O_6$. Comparing the data with the literature, the compound was identified as rhamnocitrin with a purity of 98% (Fig. 3b) (Omosa et al. 2014).

Compound 3 was obtained as white crystal. It showed brownish red macula at UV254 nm, fluorescent at UV365 nm, but no color in an atmosphere of sulfuric acid

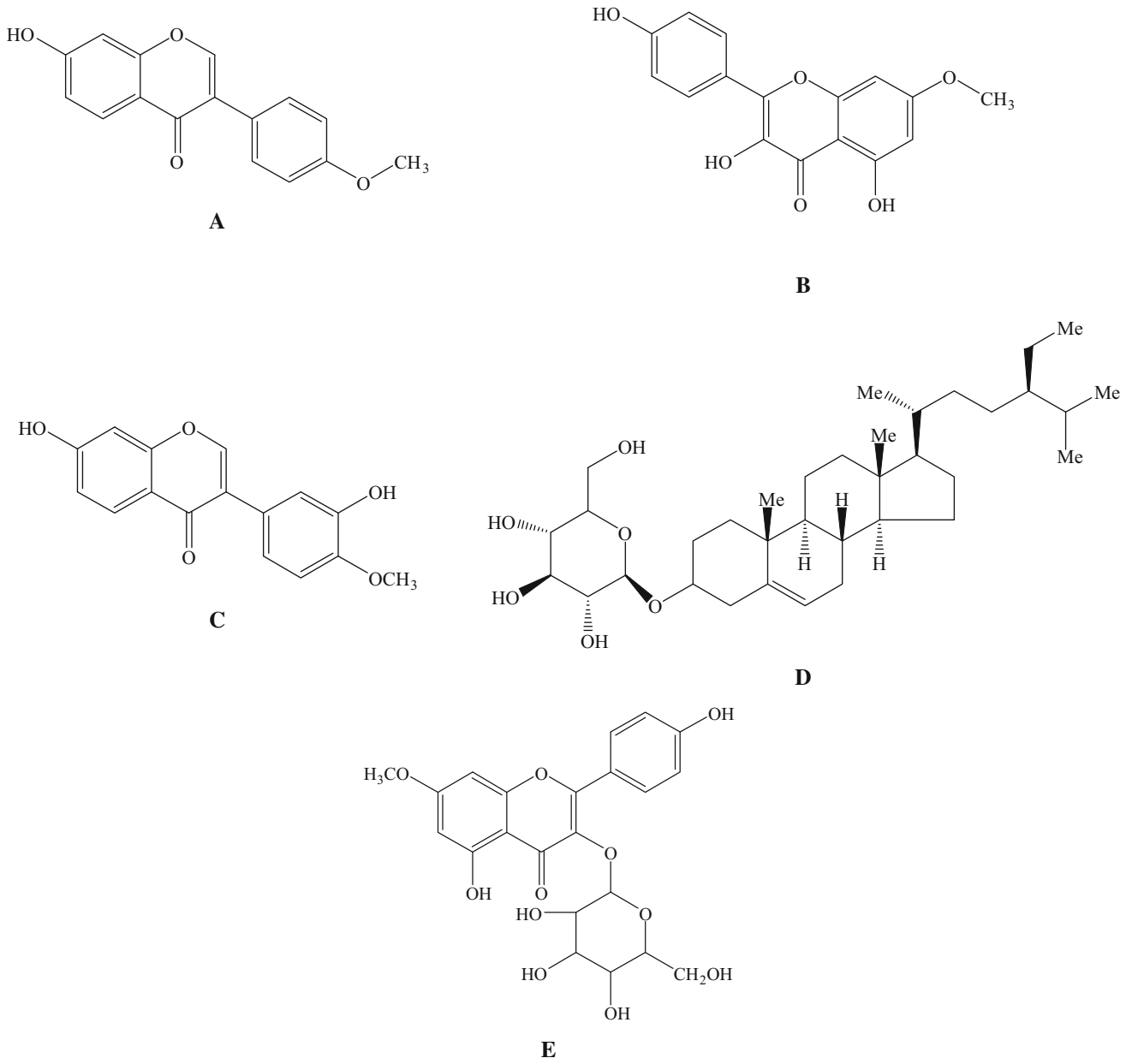

Fig. 3 Chemical structures of the compounds isolated from *Astragalus chinensis* L.f. **a**: rhamnocitrin-3-*O*-*β*-D-glucoside; **b**: calycosin; **c**: formononetin; **d**: rhamnocitrin; **e**: *β*-daucosterol

agent. It can be concluded to have 12 hydrogen atom and 16 carbon atom on the base of the NMR spectral. Combined with ESI–MS (m/z) $[M\text{-}H]^-$ 283, the molecular formula was $C_{16}H_{12}O_5$. In the ^{1}H-NMR spectral, it showed 2-H signal of isoflavone and $-OCH_3$ signal, which were at δ_H 8.28 (1H, s) and δ_H 3.79 (3H, s). In addition, there was a trisubstituted aromatic ring protons signals at δ_H 7.95 (1H, d, $J = 8.8$ Hz), δ_H 6.94 (1H, dd, $J = 8.8$, 2.0 Hz) and δ_H 6.92 (1H, d, $J = 2.0$ Hz) that were ABX system, which stand for H-5, H-6, H-8, respectively. There were 7 methyne carbons, 4 quaternary carbons attached to the oxygen, 1 carbonyl carbons, 3 quaternary carbons and 1 carbon attached to methoxyl. It showed the 4-C of isoflavone signal at δ_C 174.55. It was identified as calycosin with a purity of 96% (Fig. 3c) (Jiang et al. 2014a, b).

Compound 4 was obtained as white powder, m.p. 283–283 °C. It was soluble in chloroform. ESI–MS (m/z) $[M\text{-}H]^-$ 576. The color was amaranth when placed in 10% sulfuric acid–ethanol condition. On the basis of the rate of flow (Rf) value in the application of thin layer chromatography with various developing solvent systems (chloroform/methanol 85:15, Rf = 0.43; BAW 4:1:1, Rf = 0.57; chloroform/methanol/water 9:1:0.1, Rf = 0.45), and color rendering together with reference substance *β*-daucosterol, the data were

the same (Zhang et al. 2014a, b). The compound was identified as β-daucosterol with a purity of 99% (Fig. 3d).

Compound 5 was obtained as yellow acicular crystal, m.p. 198–200 °C. There was no fluorescence at 365 nm in UV lamp, while it appeared yellow under an atmosphere of $AlCl_3$-MeOH solution and sulfuric acid–ethanol solution, which show it belongs to flavonoids. ESI–MS m/z: 947 $[2M+Na]^+$, 485 $[M+Na]^+$, 461 $[M-H]^-$, indicated that its molecular weight was 462. In the secondary dissociation mass spectrum, m/z: 299 $[M-H-162]^-$, it shows that there may be hexose fragment in the structure. It displayed the similar signals in the ^{1}H-NMR spectra compared with the compound 2 (rhamnocitrin), and the difference is that there is saccharous terminal hydrogen signal at δ_H 5.47 (1H, s, $J = 7.2$ Hz) and other hydrogen signals of saccharide at δ_H 5.0–2.0. Combined with mass spectrum, it was concluded to be glucoside of rhamnocitrin. There were a series of carbon signals of glucose in the ^{13}C-NMR spectra, which were at δ_C 100.9 (C–H, anomeric carbon), 77.5 (C–H), 76.4 (C–H), 74.2 (C–H), 69.9 (C–H), 60.0 ($-CH_2$), and further confirmed that this compound was the glucoside of rhamnocitrin. The molecular formula was speculated as $C_{22}H_{22}O_{11}$ according to the EI-MS, ^{1}H-NMR, ^{13}C-NMR and DEPT135 NMR spectra. Comparing the data with the literature, the compound was identified as rhamnocitrin-3-*O*-β-D-glucoside (RHG) with a purity of 96% (Fig. 3e) (Zhou et al. 2009).

Formononetin, rhamnocitrin, calycosin, RHG are important compounds that could be found in several plants. There were some biological activities of these isolated compounds that were reported before. Formononetin had significant antioxidant and estrogenic effects, and the estrogenic effect was not in a dose-dependent manner (Mu et al. 2009). Tu et al. indicated that rhamnocitrin not only protect low-density lipoprotein (LDL) from oxidation but also prevent atherogenesis through suppressing macrophage uptake of oxLDL (Tu et al. 2007). Guo et al. suggested calycosin protected endothelial cells from hypoxia-induced barrier impairment, as well as improving cytoskeleton remodeling (Fan et al. 2003). However, no systematic report regarding its antitumor activity against the proliferation of HeLa cells and anti-inflammatory activity could be found. Besides, β-daucosterol is a very common compound widely distributed in plants, and much work about its biological activities have been investigated, so we make no further study on this compound (Jiang et al. 2014a, b; Choi et al. 2012; Lee et al. 2007).

Antitumor activity of various factions and purified compounds

The anti-proliferative effect of samples was performed by MTT assay. Different fractions showed different inhibition effects on HeLa cells (Fig. 4). Petroleum ether and *n*-butanol demonstrated moderate anticancer activity with the highest inhibition rate 67.60 and 57.36% at 200 μg/mL (Fig. 4a, c). In particular, ethyl acetate fraction exhibited a better antitumor activity, which could significantly inhibit the HeLa cells at concentrations of 12.5–200 μg/mL (Fig. 4b). The inhibition rate of ethyl acetate fraction significantly increased with the concentration increasing and was stronger than that of 5-fluorouracil (positive control) at each concentration. Whereas, water fraction showed low inhibition effect (Fig. 4d). Generally speaking, petroleum ether fraction contains much oil and fatty acid, which are not the main components we desired. Ethyl acetate fraction is rich in medium polar components such as flavonoids, alkaloids,

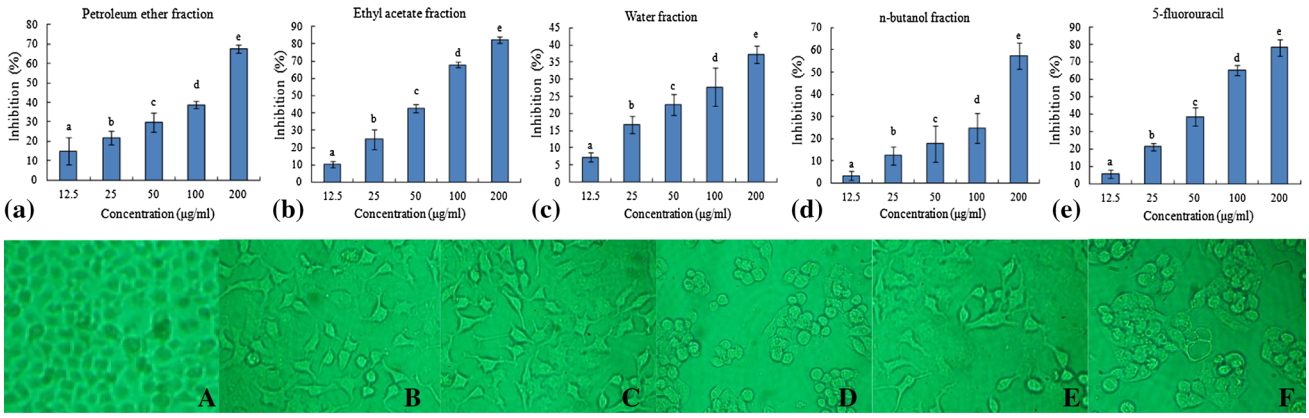

Fig. 4 Effects of four fractions on the growth and survival of HeLa cells. The column graphs (**a**–**e**) showed the in vitro inhibition ratio of HeLa cells by four fractions at different concentrations for 24 h. The cell images (**A**–**F**) showed the cell growth morphology of **A** control group, **B** petroleum ether fraction (200 μg/mL), **C** ethyl acetate fraction (200 μg/mL), **D** *n*-butanol fraction (200 μg/ml), **E** water fraction (200 μg/mL) and **F** 5-fluorouracil (200 μg/mL), respectively, observed with invert microscope. Letters **a**–**e** refer to significant differences within a fraction/compound by one-way analysis of variance-Duncan's multiple range test ($p \leq 0.05$)

coumarins and organic acids, which are the main bioactive ingredients contained in medical plants and exerts the anti-proliferation activity on HeLa cells. The experimental results showed that the active ingredients on antitumor were concentrated in the Ethyl acetate fraction after 95% ethanol was partitioned into four fractions. As a consequence, the ethyl acetate fraction was made for further isolation and purification to obtain bioactive compounds.

Among these four compounds isolated and purified from ethyl acetate fraction, formononetin and rhamnocitrin showed a stronger inhibition effect on the HeLa cells, of which formononetin exhibited a closer inhibition activity to 5-fluorouracil at the concentration of 25–100 μg/mL (Fig. 5). Calycosin showed moderate activities at 100 μg/mL with the inhibition rate was 45.14%. RHG did not display a significant inhibition effect on the HeLa cells, and its inhibition rate increased slowly with the increase of the concentration.

Formononetin is a natural isoflavone that can be found in the roots of many medical plants. The in vitro and in vivo studies showed that formononetin inhibited the cell migration and invasion of breast cancer through the decreasing expression of MMP-2 and MMP-9 (Zhou et al. 2014). A similar result indicated that formononetin, isolated from the red clover, exerted the anticarcinogenic effect on prostatic adenocarcinoma cell line (Zhang et al. 2014a, b). In the present study, formononetin showed an obvious inhibition of HeLa cells, suggesting that it would be a novel drug carrier for carcinoma of uterine cervix. It is considered that higher concentrations of formononetin caused cell cycle arrest at certain phase and inhibited the proliferation of HeLa cells by up-regulating the expression of cell apoptotic protein and down-regulating the expression of cell proliferation protein within signaling pathway in a dose-dependent manner, which resulted in induced apoptosis in HeLa cells.

Anti-inflammatory activity of various factions and purified compounds

As seen in Fig. 6a, some significant toxic effects were observed on the cells treated with these fractions. Petroleum ether fraction and *n*-butanol fraction both exhibited a regular change of toxic effect on cells with increasing concentration. The highest cell viability was about 80% at 12.5 μg/mL, and the lowest cell viability was only about 50% with the concentration was 200 μg/mL. Therefore, these two fractions should not be assayed for the anti-inflammatory effect. The ethyl acetate fraction and water fraction both improved the cell viability. When treated with ethyl acetate fraction and water fraction, the highest cell viability were 91.20% and 89.07%, respectively. The same method was used to analyze the purified compounds (Fig. 6b). Results suggested that the growth of RAW 264.7 cell lines had been a little affected by ethyl acetate fraction, water fraction, RHG, calycosin, formononetin, and the cell viability was almost not influenced by them, which could be applied to the anti-inflammatory activity.

The anti-inflammatory activity of samples was quantitatively analyzed on the basis of the NO^{2-}concentration that in order to reflect the inhibition of NO production. Ethyl acetate fraction and water fraction dose-dependently inhibited NO production with the inhibition rate reached 73.10 and 55.39% at the concentration of 200 μg/mL, indicating that these two fractions both had a certain anti-inflammatory activity at concentrations of 12.5-200 μg/mL, especially for the ethyl acetate fraction (Fig. 6c). Among the three purified compounds, RHG and formononetin did not display a stronger inhibitory activity, but

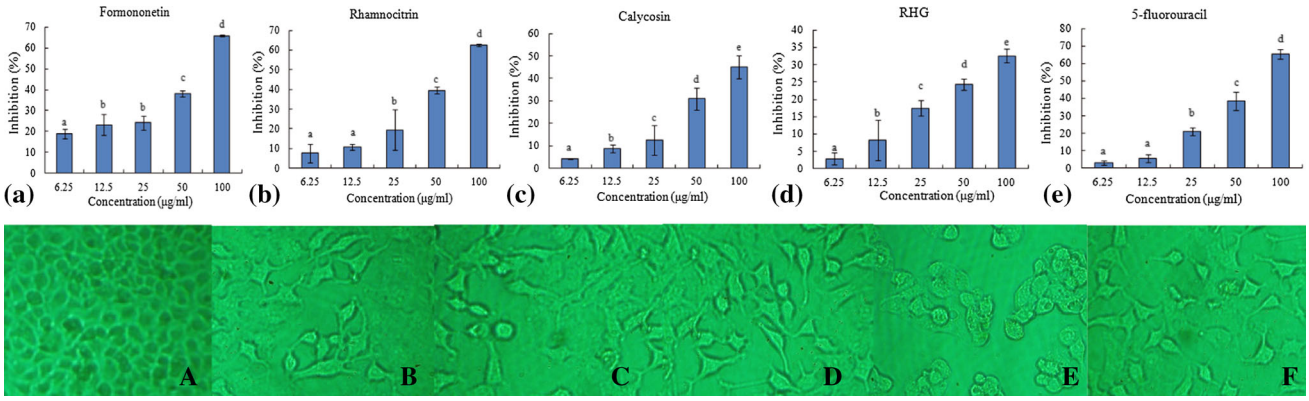

Fig. 5 Effects of four purified compounds on the growth and survival of HeLa cells. The column graphs (**a**–**e**) showed the in vitro inhibition ratio of HeLa cells by four purified compounds at different concentrations for 24 h. The cell images (**A**–**F**) showed the cell growth morphology of **A** control group, **B** RHG (100 μg/mL), **C** calycosin (100 μg/mL), **D** formononetin (100 μg/mL), **E** rhamnocitrin (100 μg/mL) and **F** 5-fluorouracil (100 μg/mL), respectively, observed with invert microscope. Letters a-e refer to significant differences within a fraction/compound by one-way analysis of variance-Duncan's multiple range test ($p \leq 0.05$)

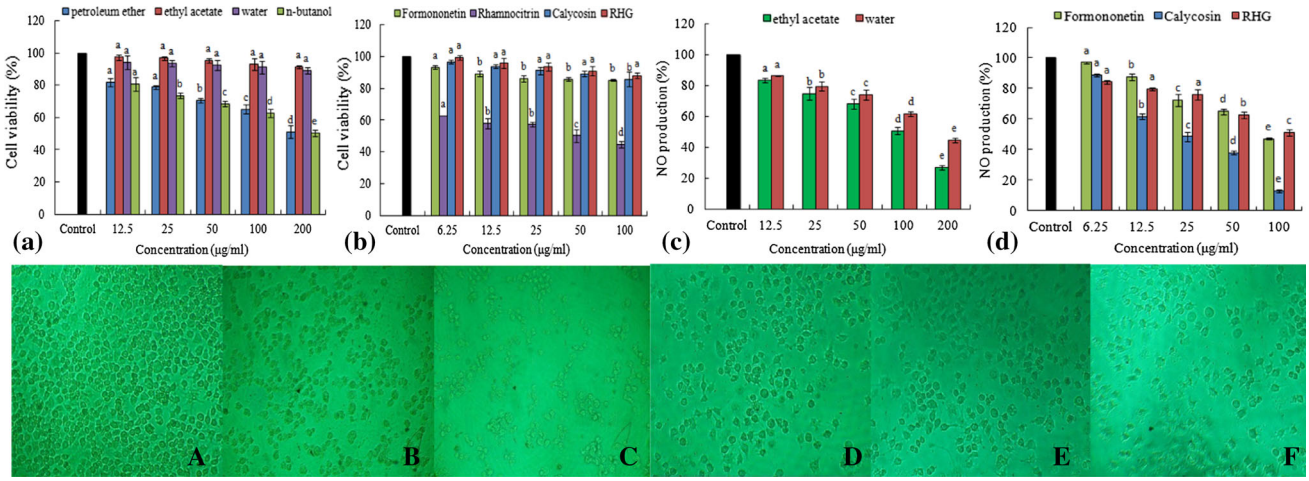

Fig. 6 Effects of four fractons and four purified compounds on macrophages. The column graphs (**a**–**d**) showed the effect of samples on the vitality of macrophages and the inhibitory effect on NO production of samples at different concentrations. The cell images (**A**–**F**) showed the cell growth morphology of **A** control group, **B** ethyl acetate fraction (200 μg/mL), **C** water fraction (200 μg/mL), **D** Formononetin (100 μg/mL), **E** Calycosin (100 μg/mL) and **F** RHG (100 μg/mL), respectively, observed with invert microscope. Letters a–e refer to significant differences within a fraction/compound by one-way analysis of variance-Duncan's multiple range test ($p \leq 0.05$)

a moderate anti-inflammatory activity with the increasing concentration. Calycosin showed the strongest inhibitory effect at 100 μg/mL with the lowest inhibition rate of 12.80% (Fig. 6d).

Calycosin, a bioactive compound isolated from *Radix astragali*, showed significant anticancer and anti-inflammatory activities. It could regulate and control cell cycle and apoptosis, as well as show a certain effect on the treatment of leukemia (Jin et al. 2010; Zhang et al. 2012). Although this study showed that calycosin had a fine anti-inflammatory activity, exhibiting its great potentiality as a therapeutic drug, the mechanism of its anti-inflammatory activity should be further investigated in future.

Conclusion

The antioxidant activity of the ethyl acetate fraction from *A. chinensis* L.f. were stronger than other fractions, which may be due to the most abundant flavonoids compounds in the fraction. Subsequently, five pure compounds were identified from ethyl acetate fraction, and then their anti-proliferative and anti-inflammatory activities were compared. Petroleum ether and ethyl acetate both exhibited effectively anti-proliferative activity. Water fraction and ethyl acetate fraction exerted a better anti-inflammatory activity. Among the four pure compounds, formononetin and calycosin were the bioactive compounds, which had obvious cytotoxicities against HeLa cells, as well as effective anti-inflammatory activities. The results obtained in this work might be beneficial to the application of *Astragalus chinensis* L.f. in food and drug enterprises.

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