SHORT REPORT

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Evaluation of Anti-inflammatory and Immunosuppressive Properties of *Cynanchum ascyrifolium* Matsum. and its Active Secondary Metabolites

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Abstract: The ethanol extract of *Cynanchum ascyrifolium* Matsum. showed significant anti-inflammatory and immunosuppressive activities with dose dependent manner using in vitro experiments. Then, the bioassay-guided ethanol extract lead to identification of four active compounds, namely ursolic acid (**1**), maslinic acid (**2**), cynanoside A (**3**) and syringaresinol (**4**). Among these compounds, cynanoside A (**3**), a C21 steroid, exhibited the highest anti-inflammatory and immunosuppressive effect on the Con-A or LPS activated lymphocytes at the concentration of 1μM ($P<0.01$ vs model group). The results provided experimental evidence for the traditional use of *C. ascyrifolium* Matsum. in treating various disease associated with inflammation.

Keywords: *Cynanchum ascyrifolium* Matsum.; anti-inflammation; immunosuppression; cynanoside A.

1. Plant Source

Cynanchum ascyrifolim (Franch. et Sav.) Matsum., a perennial herbaceous plant belonging to the family of Asclepiadaceae, is distributed in southwestern of China. There are about 57 species from *Cynanchum* widely distributed from the Northeast to the Southwest in China [1-2].

The roots of *C. ascyrifolium* were collected from Jilin province, China in August 2012, and identified by professor Mian Zhang. A voucher specimen (CFC-201205) has been deposited at the Herbarium of China Pharmaceutical University.

2. Previous Studies

The roots of *C. ascyrifolium* Matsum. are folk origins of the Chinese drug Radix Cynanchi Atrati., which has been used as an antifebrile and diuretic for its anti-inflammatory properties [2]. Up to now, four C_{21} steroidal glycosides have been isolated and identified from this plant [3-4]. As a kind of characteristic and bioactive constituents, more than 50 ones have been isolated from Radix Cynanchi Atrati [5]. With our continued search for anti-inflammatory and immunosuppressive agents

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in Asclepiadaceae [6], the 80%-ethanol extract of *C. ascyrifolium* Matsum. (EXT) was found with higher anti-inflammatory and immunosuppressive activities.

3. Present Study

Effects on LPS-induced NO production: To investigate the involvement of EXT and its different fractions in inflammatory mediator, the levels of secreted NO by were measured using Griess reagent [7]. EXT and its acetyl acetate extract (EXT-E) significantly reduced LPS (5μg/mL) - induced production of NO, the IC_{50} value of EXT-E is 54.61±2.13 μg/mL. Then, EXT-E was subjected to silica gel CC to give six sub-fractions (SFr. A-F) for activity screening. Among of them, SFr. B showed higher anti-inflammatory activity with inhibition rate of 89.36%, SFr. C and SFr. D showed inhibition rate of 56.54 and 50.67%, respectively (Table 1). Finally, the bioassay-guided isolation of SFr.B lead to four active compounds, namely, ursolic acid (**1**) [8], maslinic acid (**2**) [9], cynanoside A (**3**) [10] and syringaresinol (**4**)[11], especially Cynanoside A (**3**) showed the best properties at the dose-dependent manner (Table 1, Figure. 1).

Table 1. Effects of the ethanol extract from *C. ascyrifolium* and its fractions or compounds on cell viability and LPS-induced production of NO in RAW264.7 cells.

Groups	Concentration	Cell viability (%)	Inhibition rate (%)
Ethanol extract (EXT)	100μg/mL	89.86±2.14	80.45±1.88
EtOAc fraction (EXT-E)	100μg/mL	83.16±2.64	86.18±2.17
n-BuOH fraction (EXT-B)	100μg/mL	81.45±3.63	64.31±2.34
Water fraction（EXT-H）	100μg/mL	90.34±2.85	33.23±3.18
SFr. A	50μg/mL	101.54±2.15	20.43±2.46
SFr. B	50μg/mL	88.36±2.23	89.36±2.37
SFr. C	50μg/mL	80.15±2.57	56.54±1.46
SFr. D	50μg/mL	83.26±2.46	50.67±2.27
SFr. E	50μg/mL	80.47±2.73	9.65±2.36
SFr. F	50μg/mL	87.42±2.05	8.46±2.28
Comp. 1	10μM	103.05±3.57	41.43±1.26
Comp. 2	10μM	102.26±1.38	33.23±1.58
Comp. 3	10μM	98.17±2.07	48.65±1.64
Comp. 4	10μM	98.42±1.75	30.05±2.48

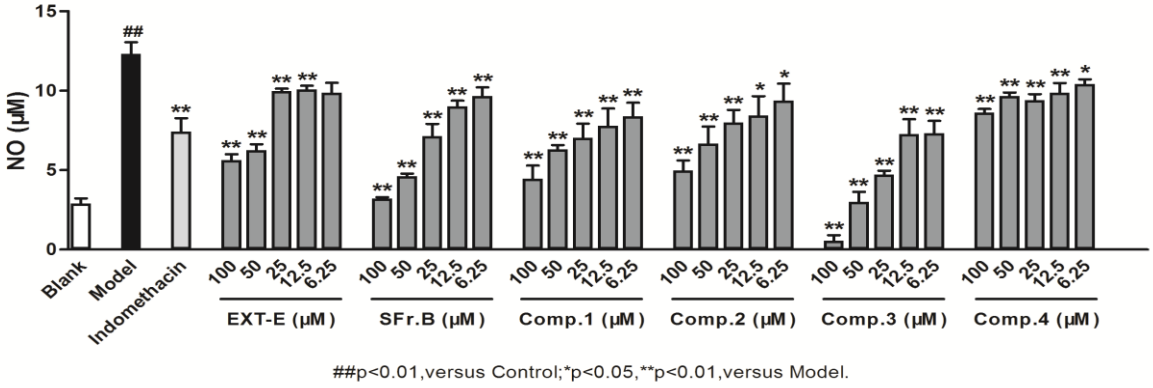

Figure 1. The ethyl acetate extract (EXT-E), sub-fraction (SFr. B) and four compounds (Comp.1-4) dose-dependently inhibited NO production in RAW264.7 cells. The concentration of NO in the supernatant was estimated using Griess reagent. Data are expressed as means ± S.D. from three replicates and two such independent experiments were carried out.

Effects on proliferation of activated lymphocytes: Spleen-derived lymphocytes isolated from BALB/c mice were incubated in medium or in the presence of Con-A (5μg/mL) or LPS (10 μg/mL) for 24 h. Then cells were further incubated with or without different concentrations of EXT (1, 10 and 100 μg/mL) for 24h, As shown in Figure 2A, EXT dose-dependently inhibited the proliferation of Con A or LPS-activated lymphocytes cells without influencing quiescent lymphocytes. Then, the inhibition effects of different solvent extracts from EXT were also screened, EXT-E showed higher but EXT-B

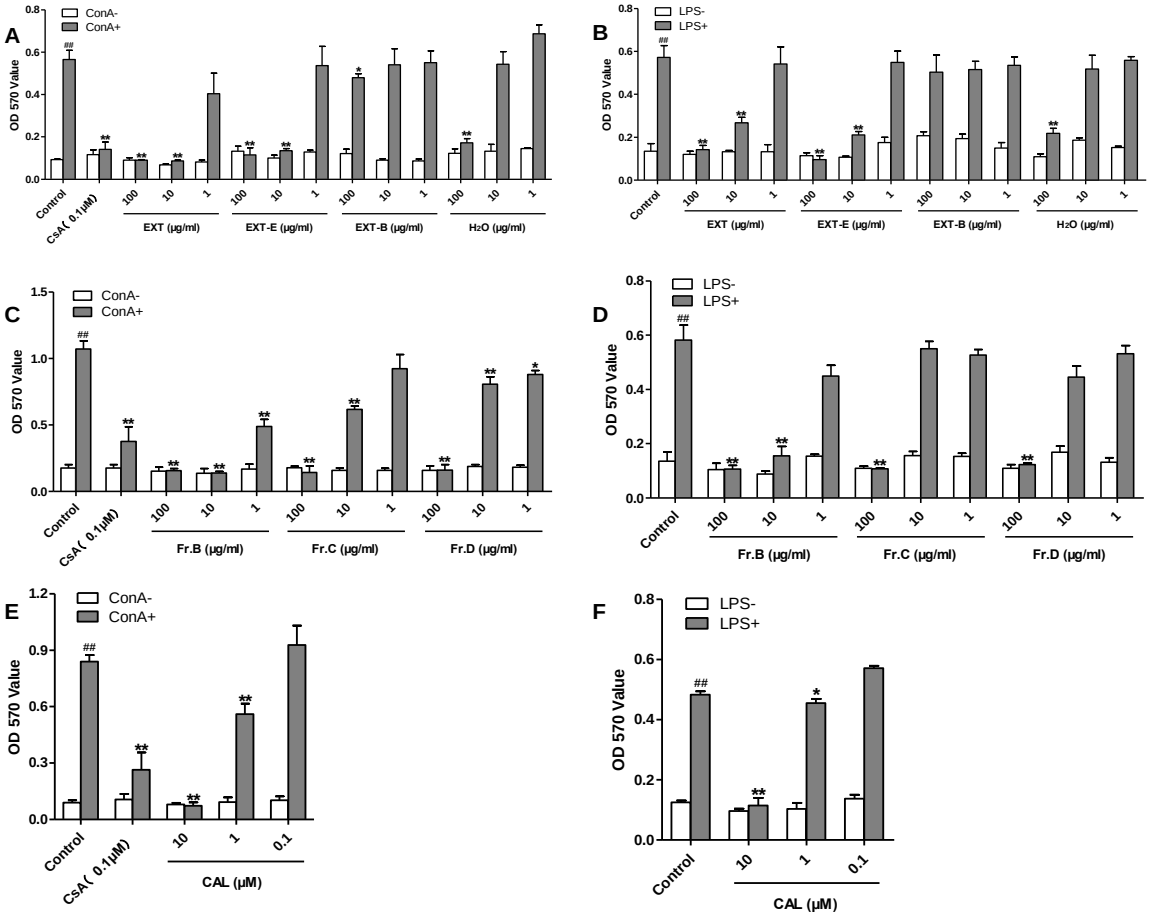

Figure 2. Inhibition of the different extracts of *C. ascyrifolium* Matsum. and sub-fractions from EXT-E on proliferation of Con-A or LPS activated lymphocytes by MTT assay. CSA (0.1μM) was used as positive drug. Resluts were presented as means ± S.D. in triplicate. *P < 0.05, **P < 0.01 vs LPS alone.

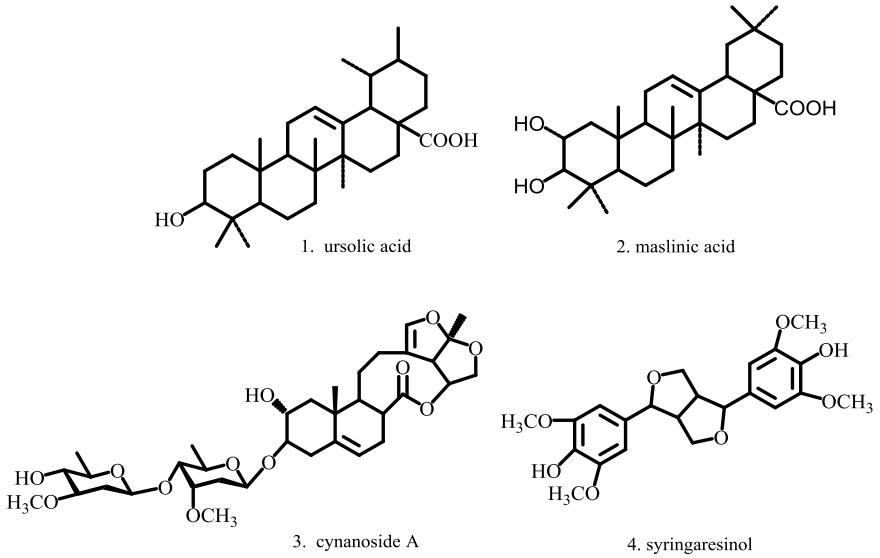

Figure 3. Structures of four compounds from active sub-fraction of SFr. B.

and water extracts showed inactive or weak immunosuppressive effects. Meanwhile, up to a concentration of 100μg/mL, no significant cytotoxicity of EXT-E could be observed using MTT assay (Fig.2). So, the most prominent anti-inflammatory activity of *C. ascyrifolium* Matsum. may be also focused in the extract of EXT-E.

As the further screening results, SFr. B-D from EXT-E showed dose-dependent inhibition on activated lymphocyte proliferation, especially, SFr. B displayed better inhibition on Con A activated but not on LPS-stimulated lymphocytes at the concentration of 1 μg/mL ($P < 0.01$, Fig. 2C&2D). Among isolated four compounds from SFr. B, cynanoside A (**3**) at the concentration of 1 μM showed the higher immunosuppressive activities on the activated T or B cells with 93.75 ± 2.79 and 70.67 ± 2.73%, respectively ($P<0.01$, Figure 2E&2F).

4. Conclusion

The acetyl acetate extract (EXT-E) of *C. ascyrifolium* Matsum. showed better anti-inflammation and significant immunosuppressive effects in vitro, which provide research for its application in autoimmune disorders, such as rheumatoid arthritis and inflammatory bowel disease. The anti-inflammatory activity-guided fractionation and structural characterization of EXT lead to four active compounds. Then, these four compounds were tested for inhibition on activation of lymphocytes by Con-A or LPS, LPS is an activator of B cells, Con-A is a T-cell mitogen, cynanoside A (3) may be used as a new leading compound, which exhibited higher selective inhibitions on activated T cells at the concentration of 1μM. So, the anti-inflammatory of *C. ascyrifolium* Matsum. is possibly involved immunosuppressive properties of these four compounds in activated lymphocytes and macrophages. These results might be responsible, at least in part, for the treatment of various disease associated with inflammation.

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