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Anti-inflammatory Activities of Lupane-triterpenoids *In Vitro* and Their Phytochemical Fingerprinting from Leaves of *Acanthopanax gracilistylus*

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Abstract – The activities on the inhibition of NO on LPS-induced RAW 264.7 macrophages were investigated in this work. A simple and sensitive method has been developed and validated for fingerprinting analysis of leaves of *Acanthopanax gracilistylus* W.W. Smith (AGS). The cytotoxicity and inhibition of NO on LPS-induced RAW 264.7 cells of the extract and triterpenoids were determined. Optimal conditions of HPLC analysis were established as follows. The separation was performed with an ODS-C_{18} column at 30 ºC, the detected wavelength was 210 nm, the flow rate was 1 mL/min, and the mobile phase consisted of acetonitrile (0.05% phosphoric acid) –0.05% phosphoric acid solution with gradient elution. Our results showed that impressic acid and acankoreaogenin was more effective on the inhibition of NO than the methanol extract and other compounds. There were seventeen peaks coexisted with similarities above 0.95 and nine lupane-triterpenoids including acankoreaogenin and impressic acid detected and identified. The result of anti-inflammatory activities provides a potential explanation for the use of AGS leaves as a herbal medicine in the treatment of inflammatory diseases. Our results also show that acankoreanogenin and impressic acid may be potentially useful in developing new anti-inflammatory agents. In addition, the fingerprint chromatography clearly illustrated and confirmed the material basis for the anti-inflammatory activities of this plant.
Keywords – *Acanthopanax gracilistylus* W. W. Smith, HPLC fingerprinting, lupane-triterpenoids, cytotoxicity, anti-inflammatory activity

Introduction

Acanthopanax gracilistylus W.W. Smith (AGS) belonging to *Acanthopanax* spp. (Araliaceae) is widely distributed in China, especially in provinces of Hunan, Anhui and Hubei. The dried roots and stem barks are officially listed in the Chinese Pharmacopoeia as "Wujiapi", used as traditional Chinese medicine for many centuries in the treatment of paralysis, arthritis, rheumatism, lameness, and high blood pressure. They were also extensively used as tonic.[1] The leaves of AGS were recorded in the textbook of Traditional Chinese Medicine.[2] In fact, the whole plant including leaves, stems and roots has been also widely taken as health supplements and folk medicine. Nevertheless, no report has been seen in the literature on the study of medical effectiveness of AGS leaves except for some of the chemical constituents detected from them. In our previous papers, for lupane-triterpenoids as the main constituents of AGS leaves,[3-5] we reported their structure determination[6-8] and anti-inflammatory and suppressive effects on human lymphocytes. Moreover, we have shown that acankoreaogenin and its analogues from AGS leaves could significantly attenuate the release of high mobility group box chromosomal protein-1 (HMGB-1), one of new and key anti-inflammation factors appeared in the later period of the anti-inflammation response.[9]

It is well known that nitric oxide (NO) is also a key anti-inflammation factor and appeared in the earlier period compared to HMGB-1,[10] and that its overproduction contributes to the development of various inflammatory diseases. Moreover, the down-regulation of NO has been used to treat such diseases in the clinic. In order to evaluate anti-inflammatory effects of these compounds isolated from the leaves of AGS and provide theoretical values for finding lead compounds, we report for the first time the activities of nine compounds and the MeOH extract on the inhibition of NO production in LPS-induced RAW 264.7 macrophages in this work.

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Based on the rich resources and excellent activities from our previous studies, there is an urgent need to look for a satisfactory method for the comprehensive evaluation and subsequent medicinal development of the leaves of AGS. Although there have been some reports concerning the quality control of AGS, most of them have been limited to qualitative and quantitative analyses of selected components rather than using chemical fingerprinting. Among a variety of quality control methods, chromatographic fingerprinting has gained more and more attention and been recognized as a rapid, reliable method, where a chromatogram is used to represent the chemical characteristics of each compound from herbal medicine. Generally speaking, samples with similar chromatographic fingerprint have similar properties. As a result, chromatographic fingerprinting has the potential to determine the identity, authenticity, and lot-to-lot consistency of herbal medicines. Up to now, there have been lots of chromatographic fingerprints reports of herbal medicines such as the quality evaluation of *Picrasma quassioides*, *Semen zizyphi spinosae*, Actaea racemosa L., *etc*.[11-15]

Experimental

Materials and Chemicals – Ten samples of AGS leaves were collected from different collection periods and different acquisition places of Hunan province in China, which is one of the major distributed areas, and all of them were carefully identified by one of the authors (X.Q.L.). The voucher specimens, with the voucher specimen numbers corresponding to the sample number shown in Table 1, were deposited at Herbarium of School of Pharmacy, Hunan University of Chinese Medicine. Griess reagent (G4410) was provided by Sigma. MTT, DMSO, Dulbecco's modified Eagle's minimum essential medium (DMEM) and Fetal bovine serum (FBS) were purchased from Life Technologies Inc. (Grand Island, NY, USA). RAW 264.7 macrophage cells were supplied by Korea Cell Line Bank (Seoul, Korea). Acetonitrile (CH_3CN, HPLC grade), methanol (CH_3OH, HPLC grade) and ethanol (C_2H_5OH, HPLC grade) were both purchased from Tianjin Kermel Chemical Reagent Co. Ltd; double-distilled water for the chromatography was purified by using the automatical double pure water distillatory. Acetic acid (CH_3COOH) and phosphoric acid (H_3PO_4) were purchased from Chengdu Kelong Chemical Reagent Factory. The solvents were filtered through 0.45 μm membranes and degassed in an ultrasonic bath before use.

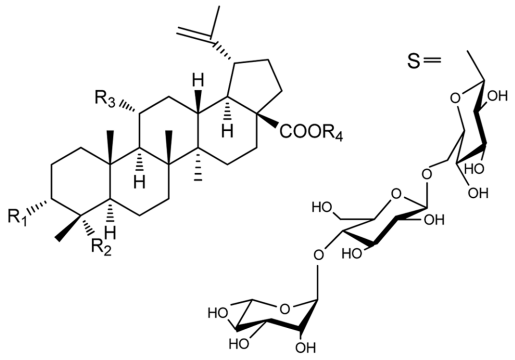
Reference compounds – Reference compounds of acankoreoside B, acankoreoside D, acantrifoside A, acankoreoside A, 3-O-β-D-glucopyranosyl-3α,11α-dihydroxylup-20(29)-en-28-oic acid, 3α,11α,23-trihydroxy-lup-20(29)-en-28-oic acid, 3α,11α-dihydroxy-23-oxo-lup-20(29)-en-28- oic acid, impressic acid, and acankoreaogenin were internally prepared in the laboratory (isolated from the leaves of AGS with their purities and structures determined by HPLC, NMR, MS, *etc* and their purities above 98.5%). Their structures are shown in Fig. 1.

Preparation of standards solution – The mixed standard solution of impressic acid, 3α,11α-dihydroxy-23-oxo-lup-20(29)-en-28-oic acid, 3α,11α,23-trihydroxy-lup-20(29)-en-28-oic acid, acankoreanogenin, 3-O-β-D-glucopyranosyl-3α,11α-dihydroxylup-20(29)-en-28-oic acid, acantrifoside A, acankoreoside D, acankoreoside B and acankoreoside A were prepared in methanol. After preparation, the solution was stored in the refrigerator until analyzed.

Preparation of samples – The leaves of AGS were powdered to a homogeneous size in a mill, and sieved through 40 mesh. The dried power (4.0 g) was accurately weighed and extracted by 50% MeOH reflux for 90 min after degreased with petroleum ether, and then filtered.

Table 1. Raw herbs that were studied in this work

No.	Sample source	Batch number	Collection time	Description
S1	Changsha, Hunan	110601	June, 2011	Dried
S2	Changsha, Hunan	120802	August, 2012	Dried
S3	Changsha, Hunan	111003	October, 2011	Dried
S4	Ningxiang, Hunan	110804	August, 2011	Dried
S5	Ningxiang, Hunan	120605	June, 2012	Dried
S6	Changsha, Hunan	120606	June, 2012	Dried
S7	Changsha, Hunan	111107	November, 2011	Dried
S8	Xinhua, Hunan	110908	September, 2011	Dried
S9	Xinhua, Hunan	121009	October, 2012	Dried
S10	Yuanling, Hunan	120710	July, 2012	Dried



No.	R_1	R_2	R_3	R_4
XZ1	OH	CH_3	OH	H
XZ2	OH	CHO	OH	H
XZ3	OH	CH_2OH	OH	H
XZ4	OH	COOH	H	H
XZ5	-O-β-D-Glc	CH_3	OH	H
XZ6	OH	CH_3	OH	S
XZ7	OH	CHO	OH	S
XZ8	OH	CH_2OH	OH	S
XZ9	OH	COOH	H	S

Fig. 1. Chemical structures of nine reference compounds [XZ1. impressic acid; XZ2. 3α,11α- dihydroxy-23-oxo-lup-20(29)-en-28-oic acid; XZ3. 3α,11α,23-trihydroxy-lup-20(29)-en-28-oic acid; XZ4. acankoreanogenin; XZ5. 3-O-β-D-glucopyranosyl-3α,11α-dihydroxylup-20(29)-en-28-oic acid; XZ6. acantrifoside A; XZ7. acankoreoside D; XZ8. acankoreoside B; XZ9. acankoreoside A].

The resulting solution was evaporated to dryness in vacuum. The residue was dissolved in 10 mL of methanol using a volumetric flask. All samples were filtered through a 0.45 μm millipore filter before 10 μL is injected for HPLC analysis. Because these triterpenoids were insoluble in petroleum ether, leaves were treated with petroleum ether to remove pigments in order to carry out HPLC.

Cell culture and preincubation of drugs – The RAW 264.7 macrophage cell lines were grown at 37 °C in DMEM supplemented with 10% FBS, penicillin (100 units·mL^{-1}), and streptomycin sulfate (100 μg·mL^{-1}) in a humidified 5% CO_2 atmosphere. Cells were incubated with 50% methanol extract and nine lupane-triterpenoids at the indicated concentrations or with the positive chemicals respectively, and then stimulated with LPS (1 μg·mL^{-1}) for the indicated time.

MTT assay for cell viability – The RAW 264.7 cells viability after 24 h of continuous exposure to the tested compounds was measured with a colorimetric assay based on the ability of the mitochondria in viable cells to reduce MTT. The percentage of cell viability was calculated as the absorbance of treated cells/control cells × 100. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells (1.0×10^4 cells·mL^{-1}) were cultured in 96-well plates for 24 h, followed by treatment with LPS (1 μg·mL^{-1}) in the presence of various concentrations (0, 20, and 40 μg·mL^{-1}) of 50% methanol extract (the extract from sample of No.120605) and nine lupane-triterpenoids, respectively. After 24 h incubation, MTT stock solution (50 μL; 2 mg·mL^{-1} in PBS) was added to the medium, and the medium was incubated for 4 h. The supernatant was removed, and the obtained formazan crystals were dissolved in of dimethylsulfoxide (DMSO) (200 μL). Absorbance was measured at 540 nm. The percentage of cells showing cytotoxicity was determined relative to the control group.

Determination of Nitrite in the LPS-stimulated RAW 264.7 cells – RAW 264.7 cells were plated at 4×10^5 cells/well in 24-well plates and then incubated with or without LPS (1 μg·mL^{-1}) in the absence or presence of various concentrations (0, 20, 40 μg·mL^{-1}) of 50% methanol extract and nine lupane-triterpenoids for 24 h, respectively. Nitrite levels in culture media were determined using the Griess reaction and presumed to reflect NO levels. Briefly, cell culture medium (100 μL) was mixed with Griess reagent [equal volumes of 1% (W/V) sulfanilamide in 5% (V/V) phosphoric acid and 0.1% (W/V) naphtylethylenediamine-hydrochloride] (100 μL) and incubated at room temperature for 10 min. The absorbance was then measured at 540 nm using a microplate reader. Fresh culture media were used as blanks in all experiments. Nitrite levels in samples were read off a standard sodium nitrite curve.

Statistical analysis – The results were expressed as the mean ± S.D of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett's *post-hoc* test, and *P* values of less than 0.05 were considered to be statistically significant.

Apparatus and chromatographic conditions for HPLC Fingerprint analysis – A HPLC system (Shimadzu, Japan, LC-10AT) consisted of a SPD-10A UV-Vis detector and a CBM-102 chromatography workstation was used for analyses. Chromatography separation was achieved with an ODS-C_{18} column (250 mm × 4.6 mm, 5 μm) at 30 °C. The mobile phase consisted of solvent A (acetonitrile with 0.1% phosphoric acid, v/v) and solvent B (water with 0.1% phosphoric acid, v/v) with a gradient elution

(0 - 15 min, 78% - 70% A; 15 - 21 min, 70% A; 21 - 35 min, 70% - 60% A; 35 - 45 min, 60% - 54% A; 45 - 50 min, 54% A; 50 - 70 min, 54% - 39% A; 70 - 82 min, 39% - 78% A; 85 min, 78% A). The flow rate was kept constant at 1.0 mL/min. The UV detection wavelength was selected as 210 nm. The injection volume was 10 μL.

Validation of methodology – The sample of No.120605 (collected from Ningxiang, Hunan in June 2011) was used for the methodology evaluation. With the optimum chromatographic conditions, samples from the same batch of preparation were analyzed for six times to determine analysis precision according to the relative standard deviation (RSDs) of the relative retention times and the relative peak areas of the 9 characteristic peaks. Repeatability was evaluated by the successive analysis of six replicates of the same sample. The stability of sample solution at room temperature was studied at different time intervals, 0 h, 4 h, 8 h, 12 h, 24 h, 36 h and 48 h.[16,17]

Results and Discussion

Our results showed that the viability of acankoreanogenin on RAW264.7 cells was 104.64% and 100.65% at the concentration of 20 μg/mL and 40 μg/mL, respectively. And the viability of impressic acid on RAW264.7 cells was 109.63% and 108.74% at the concentration of 20 μg/mL and 40 μg/mL, respectively. Moreover, the NO inhibition production of acankoreanogenin and impressic acid were 57.48%, 43.32% and 64.11%, 54.52% at the concentration of 20 μg/mL and 40 μg/mL, respectively. However, the 50% methanol extract of AGS leaves and other compounds had strongest toxicity or weak inhibition on NO production, it could be deduced that mutual inhibitions of these triterpenoids leaded to the effect of the methanol extract. The results are shown as in Fig. 2.

Fig. 3 shows the results for the comparison of ultrasonic and reflux extraction methods investigated, in this work,

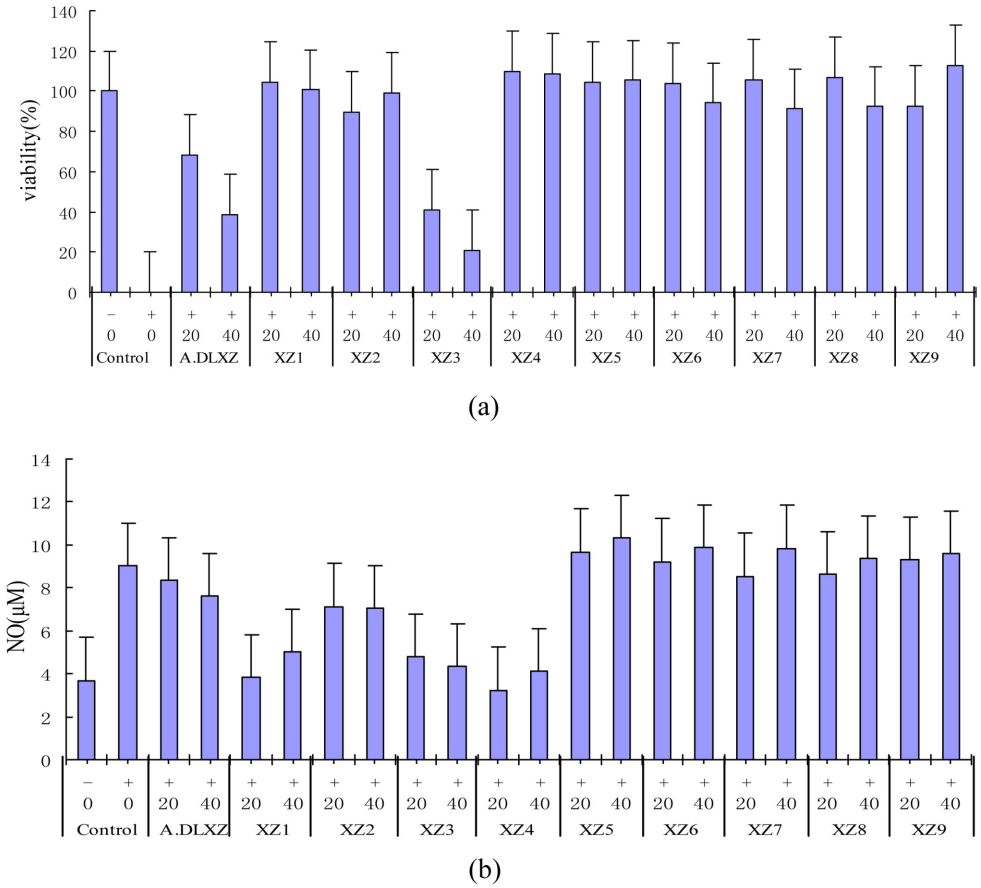
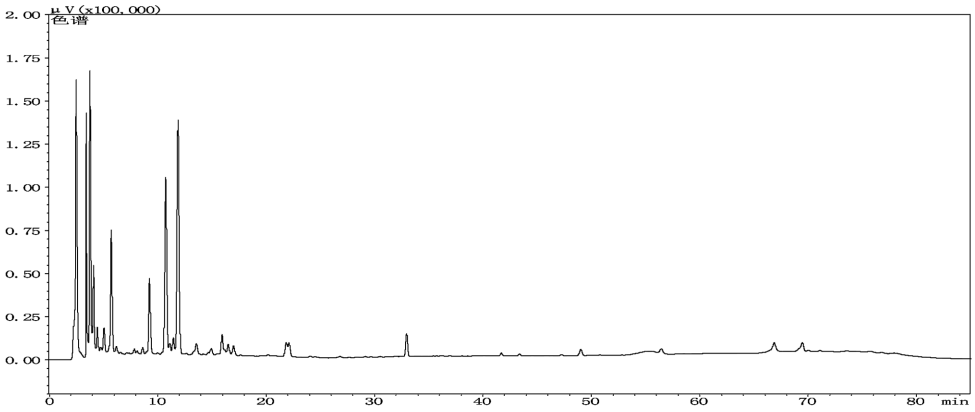
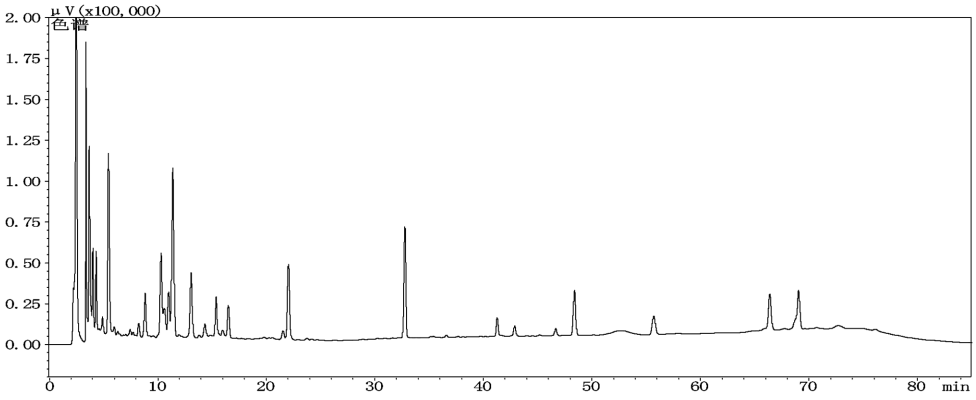


Fig. 2. a) The effects of 50% methanol extract and lupane-triterpenoids from leaves of AGS on the cell viability of RAW 264.7 macrophage (n = 3); b) The inhibitory effects of the 50% methanol extract and lupane-triterpenoids from leaves of AGS on NO production in LPS-stimulated RAW 264.7 cells (The cells were pre-incubated with drugs for 1 h, and then stimulated by LPS (1 μg·mL^{-1}) for 24 h. (n = 3)) (A.DLXZ. 50% methanol extract of leaves of AGS; XZ1. impressic acid; XZ2. 3α,11α- dihydroxy-23-oxo-lup-20(29)-en-28-oic acid; XZ3. 3α,11α,23-trihydroxy-lup-20(29)-en-28-oic acid; XZ4. acankoreanogenin; XZ5. 3-O-β-D-glucopyranosyl-3α,11α-dihydroxylup-20(29)-en-28-oic acid; XZ6. acantrifoside A; XZ7. acankoreoside D; XZ8. acankoreoside B; XZ9. acankoreoside A).



(a)



(b)

Fig. 3. Comparison of extraction conditions between ultrasonic extraction (a) and reflux extraction (b).

We found that reflux extraction was better than ultrasonic extraction. Therefore, reflux extraction was selected for the use in further experiments. Various extraction solvents (methanol and ethanol mixture with different concentrations in water) were investigated. According to the total content of 9 compounds, a reflux extraction with 50% methanol at 85 ºC for 90 min was chosen as the best extraction method. The results were shown in Fig. 3.

Different mobile phase systems (systems of methanol or acetonitrile with different ratios and different acids) were examined next. Several mobile phase additives, such as acetic acid and phosphoric acid, were used to achieve better resolution of the analytes. It was found that good signal intensity, resolution and peak shape can be achieved when phosphoric acid was added to both acetonitrile and aqueous solutions. Ultimately, a gradient elution system (0 - 15 min, 78% - 70% A; 15 - 21 min, 70% A; 21 - 35 min, 70% - 60% A; 35 - 45 min, 60% - 54% A; 45 - 50 min, 54% A; 50 - 70 min, 54% - 39% A; 70 - 82 min, 39% - 78% A; 85 min, 78% A) of mobile phase consisted of solvent A (acetonitrile with 0.1% phosphoric acid, v/v) and solvent B (water with 0.1% phosphoric acid, v/v) were chosen for the well baseline resolution and suitable duration for analysis. In order to obtain more detectable peaks with larger areas on the HPLC chromatogram, the UV spectra were investigated, and 210 nm was selected as the eventual detection wavelength. The chromatograms of standard mixture of nine active compounds and AGS sample are shown in Fig. 4.

For the precision testing, RSDs of relative retention time and relative peak area were found to be not exceeding 2%, respectively. The repeatability testing showed that RSDs of the relative retention time and relative peak areas were also below 3%, respectively. The sample stability test was determined with one sample over 48 h and the RSDs of the relative retention time and relative peak area were less than 3%, respectively. Based on the best test conditions, ten samples from different collection periods and different acquisition places were analyzed. The result of overlay chromatographs was shown in Fig. 5.

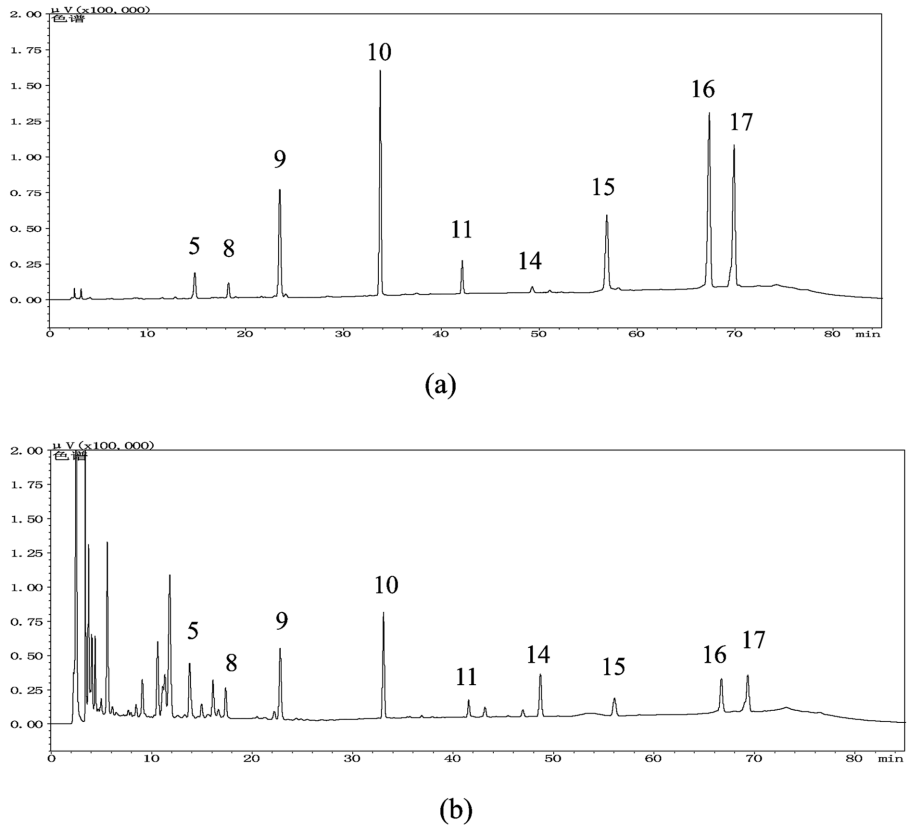


Fig. 4. Chromatograms of the Standard Mixture of nine active compounds (a) and AGS sample (b) [5---XZ8; 8---XZ7; 9---XZ6; 10---XZ9; 11---XZ5; 14---XZ3; 15---XZ2; 16---XZ4; 17---XZ1] [Chromatography separation was achieved with an ODS-C_{18} column (250 mm × 4.6 mm, 5 μm) at 30 °C. The mobile phase consisted of solvent A (acetonitrile with 0.1% phosphoric acid, v/v) and solvent B (water with 0.1% phosphoric acid, v/v) with a gradient elution (0 - 15 min, 78% - 70% A; 15 - 21 min, 70% A; 21 - 35 min, 70% - 60% A; 35 - 45 min, 60% - 54% A; 45 - 50 min, 54% A; 50 - 70 min, 54% - 39% A; 70 - 82 min, 39% - 78% A; 85 min, 78% A). The flow rate was kept constant at 1.0 mL/min. The UV detection wavelength was selected as 210 nm. The injection volume was 10 μL].

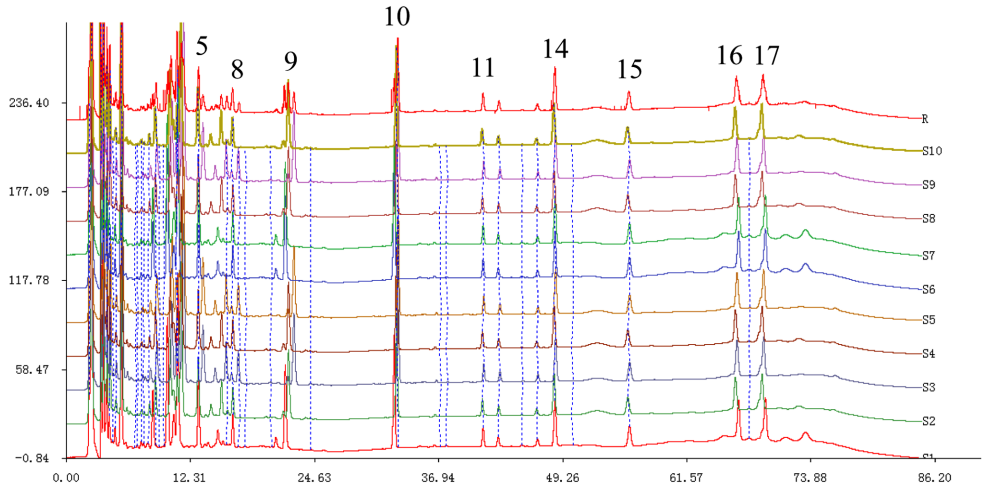


Fig. 5. Overlay chromatographs of ten samples of leaves of AGS [5---XZ8; 8---XZ7; 9---XZ6; 10---XZ9; 11---XZ5; 14---XZ3; 15---XZ2; 16---XZ4; 17---XZ1] [Chromatography separation was achieved with an ODS-C_{18} column (250 mm × 4.6 mm, 5 μm) at 30 °C. The mobile phase consisted of solvent A (acetonitrile with 0.1% phosphoric acid, v/v) and solvent B (water with 0.1% phosphoric acid, v/v) with a gradient elution (0 - 15 min, 78% - 70% A; 15 - 21 min, 70% A; 21 - 35 min, 70% - 60% A; 35 - 45 min, 60% - 54% A; 45 - 50 min, 54% A; 50 - 70 min, 54% - 39% A; 70 - 82 min, 39% - 78% A; 85 min, 78% A). The flow rate was kept constant at 1.0 mL/min. The UV detection wavelength was selected as 210 nm. The injection volume was 10 μL].

Data analysis was carried out by professional software of Similarity Evaluation System for Chromatographic Fingerprint (VERSION 2004A), recommended by SFDA of China, and used for evaluating similarities of different chromatograms by calculating the similarity between a sample chromatograph and standard chromatograph. Our results showed that there were seventeen common peaks, and nine lupane-triterpenoids peaks (acankoreoside B, acankoreoside D, acantrifoside A, acankoreoside A, 3-O-β-D-glucopyranosyl-3α,11α-dihydroxylup-20(29)-en-28-oic acid, 3α,11α, 23-trihydroxy-lup-20(29)-en-28-oic acid, 3α,11α-dihydroxy-23-oxo-lup-20(29)-en-28-oic acid, impressic acid, and acankoreanogenin) were confirmed. The similarity results were above 0.95, indicating that there were only minor differences between the ten kinds of AGS leaves from different collection periods and different acquisition places. It can be concluded that the content of main active components, nine lupane-triterpenoids, is consistent with each other from 10 leave samples of AGS from Hunan Province of China. Moreover, all leaves in this study were collected after the 6th month of the year, suggesting that the accumulations of these components might well have been accomplished before the month of June every year.

Conclusions

(1) Our results on the inhibition of NO on LPS-induced RAW 264.7 macrophages in *vitro* showed that the NO inhibitory effect of acankoreanogenin and impressic acid were better than other lupane-triterpenoids and 50% methanol extract, and their toxicity was the weakest among them, which were in good accordance with the results about HMGB-1 from our previous research. These results indicate that acankoreanogenin and impressic acid were better inhibitors of inflammation. Further studies on the action mechanism of anti-inflammation are necessary.

(2) This study also established HPLC fingerprint chromatography method for AGS leaves for the first time. The fingerprint chromatography showed that leaves of AGS from different times all contain the nine triterpenoids, and, moreover, all include acankoreanogenin and impressic acid, which have better anti-inflammatory activities. There was a little difference among leaves collected after June every year. Therefore, leaves of AGS may be as a good preparation source of acankoreanogenin and impressic acid. The established method has been proven to be sensitive, accurate, and reproducible, and could provide valuable qualitative information for the quality assessment of leaves of AGS.

(3) The results of anti-inflammatory activities reported in this work provide a potential explanation for the use of the leaves of AGS as a herbal medicine in the treatment of inflammatory diseases. Acankoreanogenin and impressic acid may be potentially useful in developing new anti-inflammatory agents. The fingerprint chromatography also clearly illustrates the material basis for the anti-inflammatory activities of this plant.

Acknowledgments

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