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Compositional Analysis of Major Saponins and Anti-inflammatory Activitiy of Steam-Processed Platycodi Radix under Pressure

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Abstract – Platycosides are the saponins in Platycodi Radix and they have several beneficial effects such as anti-inflammatory and anti-obesity activities. This study was designed to determine the changes in the saponin composition in Platycodi Radix (platycosides) after being processed under steam and pressure and to investigate the anti-inflammatory effects of their extracts. The change of the platycoside compositions was investigated after 1, 2, 3, 6 and 9h heat processing of Platycodi Radices by using HPLC coupled with an evaporative light scattering detection (ELSD) system. After heat treatment (125 ºC, 1, 2, 3, 6 and 9 h), the contents of several platycosides such as platycoside E, platycodin D_3, platycodin D, polygalacin D, and platycodin A decreased as the processing time was longer. While the total contents of the saponins decreased, the contents of deapi-forms of deapi-platycoside E, deapi-platycodin D_3, and deapi-platycodin D increased relatively. These results indicate that the linkage between apiose and xylose located at C-28 is labile to heat and pressure. The LPS-induced iNOS inhibitory activities of the samples treated for 1 and 2 hours were enhanced and after then, the activities were reduced. These results suggested that heat treatment of the samples affect the content of the total saponins and the saponin content may be the important criteria representing the anti-inflammatory activity.
Keywords – Platycosides, Platycodi Radix, HPLC-ELSD, compositional analysis, steam-processed, anti-inflammatory activity

Introduction

Traditionally, the root of *Platycodon grandiflorum* called Plaltycodi Radix has been used in Oriental medicine. Platycodi Radix has several bioactivities such as an expectorant and anti-tussive to treat cough, cold, sore throats, tonsillitis, bronchitis and chest congestion (Takagi and Lee, 1972b; Arai, *et al.*, 1997; Kim, *et al.*, 2001). Platycosides, saponins in Platycodi Radix which are the major effective constituents, include several potent effects such as anti-tumor, antioxidant and anti-obesity activities (Lee, *et al.*, 2004; Kim, *et al.*, 2005; Zhao, *et al.*, 2005; Kim, *et al.*, 2006; Kim, *et al.*, 2006). Platycosides also have anti-inflammatory activities by the expression of inducible NOS (Ahn, *et al.*, 2006; Wang, *et al.*, 2004). Several natural products have been processed using steam, heat, and pressure to improve their activities or reduce toxicities. Some changes in the chemical structures and biological effects of those have been investigated. Those changes of products have resulted in increase of beneficial compounds and effects and decrease of side effects (Keum, *et al.*, 2000; Dewanto, *et al.*, 2002a; Dewanto, *et al.*, 2002b; Yoo, *et al.*, 2006; Kang, *et al.*, 2006). Chemistry and their pharmacological activities of platycosides have been reported in the literature since 1972 (Takagi and Lee, 1972a; Nikaido, *et al.*, 1999). The crude platycosides and each compound have been studied and investigated by high-performance liquid chromatography (HPLC) (Saeki, *et al.*, 1999; Ha, *et al.*, 2006). However, there have been no attempts to analyze platycosides of processed Platycodi Radix and also to estimate their activities. In this study, Platycodi Radix was processed varying the time under heat and pressure and the compositional changes of the platycosides were determined using HPLC and their anti-inflammatory effects such as lipopolysaccharide (LPS)-induced iNOS and cell viability was obtained.

Experimental

Materials and reagents – All solvents used were purchased from J. T. Baker (USA) and they were all analytical grades. The Platycodi Radix was purchased from a local market in Seoul, Korea. The standards for

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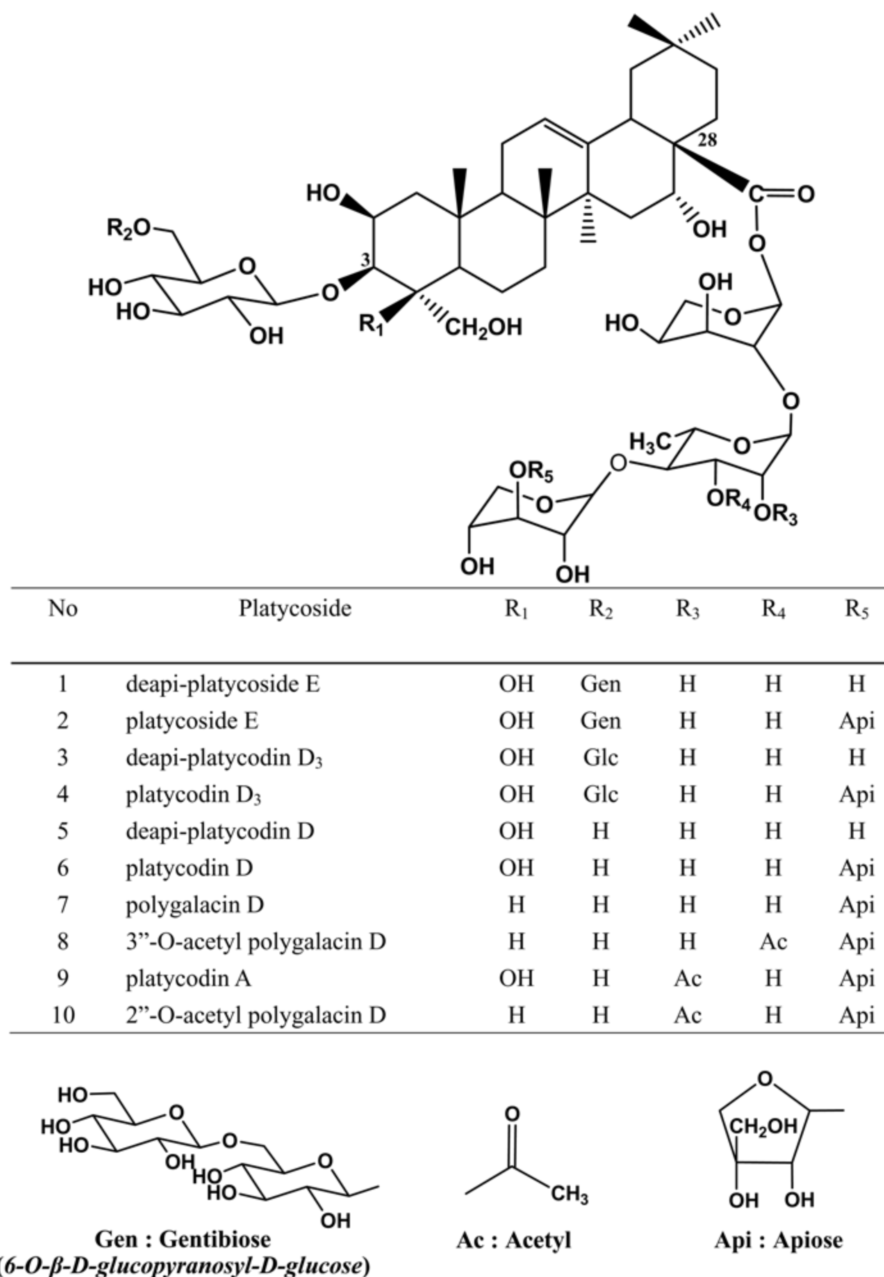

No	Platycoside	R_1	R_2	R_3	R_4	R_5
1	deapi-platycoside E	OH	Gen	H	H	H
2	platycoside E	OH	Gen	H	H	Api
3	deapi-platycodin D_3	OH	Glc	H	H	H
4	platycodin D_3	OH	Glc	H	H	Api
5	deapi-platycodin D	OH	H	H	H	H
6	platycodin D	OH	H	H	H	Api
7	polygalacin D	H	H	H	H	Api
8	3”-O-acetyl polygalacin D	H	H	H	Ac	Api
9	platycodin A	OH	H	Ac	H	Api
10	2”-O-acetyl polygalacin D	H	H	Ac	H	Api

Fig. 1. Structures of ten major platycosides.

pure saponins were isolated from the aqueous extract from Platycodi Radix as previously described (Zhao*, et al.*, 2005; Ha*, et al.*, 2006). The purity of these saponins (Fig. 1) was more than 98% as confirmed by HPLC and their structures are shown in Fig. 1. LPS (*Escherichia coli* serotype 0127:B8), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 2-amino-5-mercapto-1,3,4-thiadiazole (AMT), and penicillin-streptomycin solution purchased from Sigma were used for the in vitro assay.

Sample preparation – One hundred grams of dried Platycodi Radix was transferred into a 2 L flask. The raw samples of Platycodi Radix were processed at 125 °C for 1, 2, 3, 6, and 9 h in the autoclave (Daihan Scientific, Korea). Those were lyophilized to obtain the dry samples and then the processed samples were extracted with a 1 L volume of distilled water for 1 h at 90 °C with a water bath. The solution was filtered through the filter paper (Advantec Toyo, Japan) and the filtrate was collected. This step was repeated three times. After water extraction, the residue was re-extracted with a 1 L volume of 100% methanol as described above. The filtrates were

concentrated with a rotary evaporator at 50 °C under the reduced pressure. The residues were then lyophilized to obtain the dried extracts. One hundred grams of the raw sample which was not processed was also extracted and treated as described above. As a result, six samples including one unprocessed and five processed samples for 1, 2, 3, 6, and 9 h were obtained.

The extracts were fractioned with Diaion-HP20 column (10 cm × 100 cm) according to the following method. The water/methanol gradient was used for the elution system. The extracts were dissolved in water. The dissolved sample was then loaded onto the column and eluted with water, 30, 50, 60 and 100% methanol, sequentially. The 100% methanol fraction containing a saponin concentrate was evaporated, lyophilized and stored in a refrigerator for the subsequent HPLC analysis and the activity test.

For the HPLC analysis, 4 mg of the fractionated extract was dissolved in 1 ml of 50% methanol in water. The sample solution was filtered through a 0.45 μL PTFE syringe filter (Whatman, USA). Each 25 μL of the filtrate was injected to the HPLC system. For the *in vitro* anti-inflammatory assay, the extract was dissolved in DMSO.

HPLC instrumentation and quantitative analysis – The analysis of platycosides was performed by HPLC-ELSD as previously described (Ha*, et al.*, 2006). HPLC of the enriched saponins fractions of the extracts obtained at each different process time were carried out on a Hitachi L-6200 instrument equipped with a Sedex 75 evaporative light scattering detection (ELSD) system and SIL-9A auto injector (Shimadzu, Japan). All separations were carried out on a Zorbax SB-Aq C18 column (150 mm × 4.6 mm I.D., 5 μm particle size) from Agilent Technologies (Palo Alto, CA, USA). The gradient elution system consisted of water (solvent A) and acetonitrile (solvent B) and the gradient system for separation was as follows. The initial gradient was 10% solvent B and linearly increased to 15% B for 6 min. It was changed to 25% B for 44 min, to 70% B for 20 min, and to 100% B for 2 min and held for 8 min. Finally, it was equilibrated with 10% B for 8 min. The flow rate was set to 1 ml/min.

Ten major saponins (Fig. 1) of the extract samples were identified by their retention times on the chromatogram and compared with their contents of saponins in each sample.

Cell culture – RAW264.7 murine macrophages were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained at sub-confluence in a 95% air and 5% CO_2 humidified atmosphere at 37 °C. The medium used as the routine subculture was DMEM and RPMI1640 respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (100 unit/mL) and streptomycin (100 μg/mL).

Cell viability – The effects of the saponin extracts of raw and processed Platycodi Radices on cell viability were evaluated using the Cell Counting Kit-8 (CCK-8) manufactured by Dojindo Laboratories (Tokyo, Japan). Briefly, RAW264.7 cells were plated at a density of 1×10^4 per well in a 96-well plate, and incubated at 37 °C for 24 h. The cells were treated with various concentrations of samples or vehicle alone, and incubated at 37 °C for an additional 24 h. After incubation, 10 μl of CCK8 solution was added to each well and incubated under the same conditions for another 3 h. Water-soluble tetrazolium salt [WST8-[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] in CCK8 produces a water-soluble formazan dye upon reduction, and the resulting color was assayed at 450 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA).

Nitric oxide determination – Nitrite concentration in the medium was measured as an indicator of nitric oxide production according to the Griess reaction method as previously described (Ahn*, et al.*, 2005). In brief, 1×10^5 RAW264.7 cells were plated in 24-well plates, incubated for 24 h and pre-treated with the extracts of raw and processed Platycodi Radix for 2 h and incubated with LPS (1 μg/ml) for 24 h. Equal volumes of cultured medium and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in distilled water) were mixed, and the absorbance at 540 nm was determined with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA, USA), and the absorption coefficient was calibrated using a sodium nitrite solution standard. For this experiment, 2-amino-5, 6-dihydro-6-methyl-4H-1,3-thiazine (AMT) was used as a positive control.

Data analysis – The results, unless otherwise stated, were expressed as means ± standard deviation (S.D.) from three different experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was applied to assess the statistical significance of the differences among the study groups (SPSS version 10.0, Chicago, USA). A value of $P < 0.05$ (*) was chosen as the criterion of statistical significance.

Results and Discussion

Comparison of platycosides in raw and steam-processed Platycodi Radices – The extracts of processed Platycodi Radices were analyzed using HPLC-ELSD. The

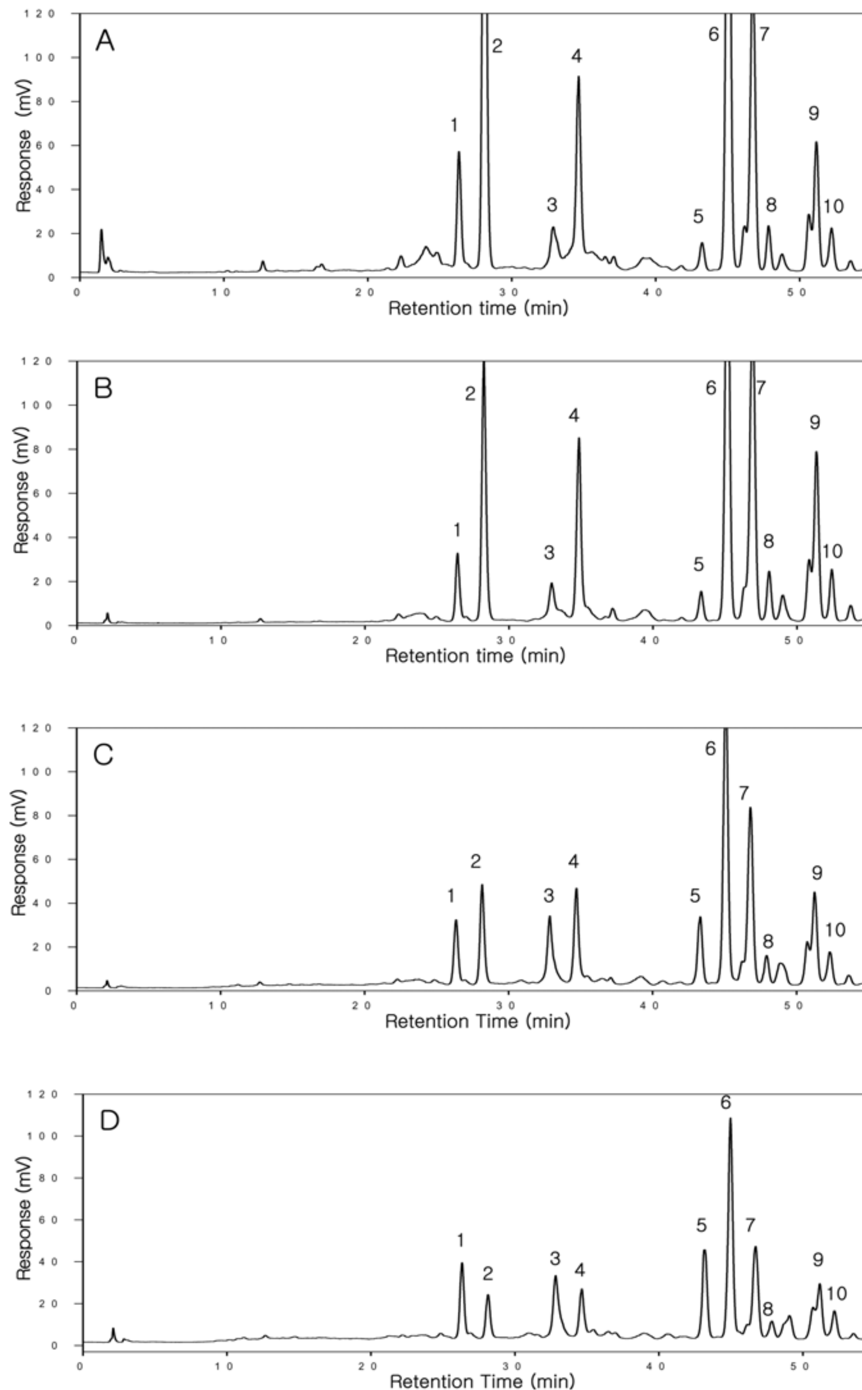

Fig. 2. HPLC chromatograms of the extracts of Platycodi Radix. Column; Agilent Zorbax SB-Aq C18 column, detector; ELSD. (A) the extracts of Platycodi Radix unprocessed; (B) processed for 1 h; (C) processed for 2 h; (D) processed for 3 h. The numbers indicate each platycoside in Fig. 1.

representative chromatograms for the crude extract of unprocessed and processed Platycodi Radices for 1, 2, and 3h are shown in Fig. 2 (HPLC chromatograms A-D). The samples were processed for 6 and 9 h, but the results are not shown here because of the degradation of total platycosides. The chromatograms indicate that the contents of saponins from Platycodi Radix (platycosides) were changed according to the processing time. In Fig. 2A, platycosie E and platycodin D are main contents of platycosides. The level of platycoside E decreased as the processing time was getting longer. Instead, deapi-platycoside E gradually increased in an opposite way. The

Table 1. The contents of ten saponins in the extract of Platycodi Radix with different processing time

	Compound	A (mg/1g)	B (mg/1g)	C (mg/1g)	D (mg/1g)
1	deapi-platycoside E	18.2	12.9	13.0	14.4
2	platycoside E	73.6	34.1	15.9	10.2
3	deapi-platycodin D_3	11.6	10.1	14.3	15.6
4	platycodin D_3	23.5	23.8	15.1	10.4
5	deapi-platycodin D	8.9	9.1	13.9	17.8
6	platycodin D	95.7	69.8	49.5	39.0
7	polygalacin D	80.4	85.2	48.4	29.0
8	3''-O-acetyl polygalacin D	14.6	15.4	12.9	10.6
9	platycodin A	23.3	28.2	19.7	15.2
10	2''-O-acetyl polygalacin D	16.3	16.8	14.9	13.0
	Total	365.9	305.3	217.6	175.3

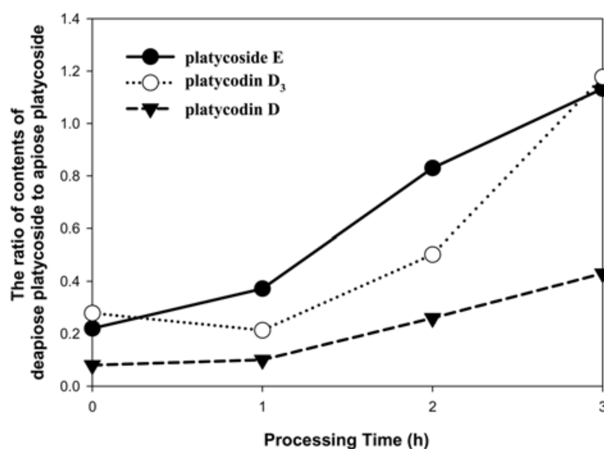

Fig. 3. The ratio of content of deapiose platycoside to apiose platycoside. The ratio of the content of deapi-platycoside E to platycoside E (●); the ratio of the content of deapi-platycodin D_3 to platycodin D_3 (○); the ratio of the content of deapio-platycodin D to platycodin D (▼) for 0 h, 1 h, 2 h, and 3 h processing time.

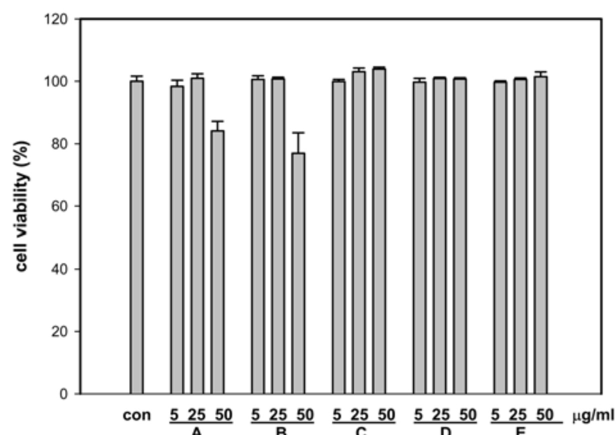

Fig. 4. Cell viability by the saponin extracts from 0 h, 1 h, 2 h, 3 h, and 6 h processed Platycodi Radix. Sample A-E; Saponin content in Platycodi Radix processed at 125 °C for 0 h (A), 1 h (B), 2 h (C), 3 h (D), and 6 h (E). Cell viability was determined and expressed as a percentage of the control.

level of deapi-playcodin D_3 also increased relatively as shown in Fig. 2. The total contents of platycosides slightly decreased for the 1, 2, and 3h processing time, which are shown in Table 1. When the Platycodi Radix was processed for 6 h and 9h, platycosides in extracts significantly decreased and few platycosides were detected. During the processing time, the content of platycoside E decreased 34.1, 15.9, and 10.2 mg; platycodin D_3 decreased 23.8, 15.1, and 10.4 mg, and platycodin D decreased 69.8, 49.5, and 39.0 mg, respectively. By contrast, deapi-platycoside E increased 12.9, 13.0, and 14.4 mg; deapi-platycodin D_3 increased to 10.1, 14.3, and 15.6 mg; deapi-platycodin D increased 9.1, 13.9, and 17.8 mg as shown in Table 1. The major saponins that contain the triterpenoid backbones are linked with two side chains (Fig. 1). One side chain is a 3-*O*-glucose linked by a glycosidic bond and the other is a 28-*O*-arabinose-rhamnose-xylose-apiose linked by an ester bond (Wie, *et al.*, 2007). The ratio of deapiose-formed to apiose-formed saponins increased as shown in Fig. 3. The results suggest that the apiose residue in the side sugar chain linked to C-28 be eliminated by heat and pressure treatments.

Effect on cell viability – RAW264.7 cells were incubated with the saponin extracts of unprocessed or processed Platycodi Radices for 24 hours at 37 °C. The viable cells were detected at 450 nm using a UV-microplate reader and the data were presented as the ratio of sample treated/control in percentage. The results showed that none of the extracts after processing did not caused cell death except a 1 h processed sample (Fig. 4). At the highest tested concentration of 50 μg/mL, the percentage of viable cells was 84.1 ± 3.1% for the unprocessed extract and 76.9 ± 6.6% for the 1 h processed

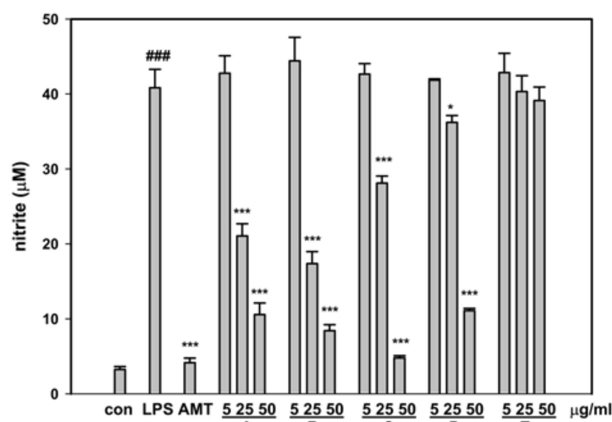

Fig. 5. Effect of the saponin extracts obtained from the 0 - 6 h processed Platycodi Radices on LPS-induced nitrite production in RAW 264.7 Cells; The treatment concentrations of saponin extracts with various processing times were 5, 25, and 50 μg/ml. The samples A-E represent the processing time for 0 h (A), 1 h (B), 3 h (D), and 6 h (E) at 125 °C.

extract. However, the cell viability of other extracts was not changed at all.

Effect on NO production in LPS-stimulated RAW 264.7 cells – RAW264.7 cells were pre-treated with Platycodi Radix saponin extracts for 2 h, followed by co-incubating with LPS (1 μg/ml) for an additional 24 h. The tested results demonstrate that the Platycodi Radix extracts inhibited LPS-induced NO production except for the 6 h processed sample (Fig. 5). The NO inhibition rates were 48.4 ± 3.7% at 25 μg/ml and 74.1 ± 10.7% at 50 μg/ml for the extract of the unprocessed sample. The treatment of the 1 h processed sample showed an additional inhibitory activity, which represents 57.5 ± 5.3% at 25 μg/ml and 79.4 ± 7.5% at 50 μg/ml. They showed the inhibitory activity in a dose-dependent way. The positive control AMT decreased NO production 89.8 ± 12.9 at 10 μM (Fig. 5). However, two extracts prepared after a longer period processing (e.g. 6 and 9 h) caused the loss of NO inhibitory activity as well as reduction of the total amounts of saponins (Fig. 2 and 5). Furthermore, as shown in Table 1, the observed NO inhibitory effects of the saponin preparations do not seem to correlate with their total amounts of saponins to a certain extent. For example, 3 h steam-processing of Platycodi Radix lost half of its total saponins from 365.9 mg/g to 175.3 mg/g, still showed a potent NO inhibition.

Taken together, the contents of platycoside E, platycodin D_3 and platycodin D were markedly decreased accordingly to the prolonged processing time, and the deapiose forms of platycosides such as deapi-platycoside E, deapi-platycodin D_3 and deapi-platycodin D were increased only slightly. Triterpenoid saponins of Platycodi Radix are known to be the active components for NO inhibition, among which platycoside E, platycodin D_3 and platycodin D are thought to be the possible candidates for the representing the inhibitory activity. The additional studies should be continued to shed light on the relationship among the characteristics of the compositional transformation of platycosides and their biological activities.

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