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UPLC-MS identification and anticomplement activity of the metabolites of *Sophora tonkinensis* flavonoids treated with human intestinal bacteria

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**Abstract**

Anticomplement activity played an important role in anti-inflammatory effects of traditional Chinese herbs. The total flavonoids of *Sophora tonkinensis* (TFST) were inactive on the complement system but showed obvious anticomplement activity after incubated with human intestinal bacteria *in vitro*. In order to discover the metabolic activation of TFST by intestinal flora, the constituents of TFST and its metabolites were identified by UPLC-ESI-LTQ/MS. Their anticomplement activities were evaluated through the classical and alternative pathway. As a result, eighteen flavonoids were identified, including seven flavonoid glycosides, five aglycones and six isoprenylated flavonoids. All the glycosides (daidzein-4'-glucoside-rhamnoside, sophorabioside, rutin, isoquercitrin, quercitrin, ononin, trifolirhizin) were metabolized into their corresponding aglycones in different extent by human intestinal bacteria, resulting in the contents of the five aglycones were highly increased in 24 h. However, no changes have occurred on the six isoprenylated flavonoids. Interestingly, three aglycones (quercetin, formononetin and maackiai) had significantly more potent anticomplement activities than their prototype glycosides. The results indicated that the enhancement of TFST anticomplement activity was attributed to the active aglycones, especially formononetin and quercetin, produced by human intestinal bacteria. These aglycones are likely to be among the potential active components of *S. tonkinensis* for its inhibiting inflammation effects.

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1. Introduction

The roots and rhizomes of *Sophora tonkinensis*, known as “Shandougen” in traditional Chinese medicine, have been recorded in Chinese Pharmacopoeia and widely used for the treatment of laryngopharyngeal inflammation and throat pain through oral administration [1,2]. Flavonoids and alkaloids are the two kinds of major components of *S. tonkinensis*. Pharmacological studies reported that the flavonoids in *S. tonkinensis* possessed significant bioactivities, such as antioxidant, anticancer, anti-gastric ulcer, and inhibiting gastric acid secretion effects [3–6]. The alkaloids of *S. tonkinensis* exhibited antiviral activity against hepatitis B virus (HBV), influenza virus A/Hanfang/359/95 (H3N2) and coxsackie virus B3 (CVB3) [7,8].

Most traditional Chinese medicines are given orally and the chemical compounds inevitably contact intestinal flora within the gastrointestinal tract. Moreover, those components were metabolized by intestinal bacteria before absorbed from the digestive tract [9,10]. Research on herbal components of metabolism with intestinal flora is greatly significant to understand their potential biological characteristics [11].

The system of complement plays a dominant role in regulating host defense. However, the pathogenesis of some diseases, such as severe acute respiratory syndrome (SARS) and acute respiratory distress syndrome (ARDS), may cause the complement system excessive activation and lead to systemic inflammation response [12,13]. It’s notable that, traditional Chinese medicine commonly had beneficial effects on infectious diseases by inhibiting inflammation and reducing fever, and the mechanism is probably related to inhibiting excessive activation of the complement system *in vivo* [14,15]. Hence, the discovery and application of complement inhibitors should become a potential therapeutic strategy for the treatment of these infectious diseases.

Interestingly, our previous study found that the total flavonoids of *S. tonkinensis* (TFST) were absence of complement inhibitory activity but showed obvious anticomplement activity after incubated with human intestinal bacteria. However, intestinal bacteria
had no distinct influence on the weak anticomplement activity of the total alkaloids of S. tonkinensis. We speculated that TFST might be the key effective components that exhibit anticomplement activity in vivo after metabolized with intestinal bacteria.

To find out how the structures and activities of TFST were changed by human intestinal bacteria, TFST was extracted and incubated with human fecal microflora in this study. Components of TFST and their metabolites were recognized and compared via UPLC-ESI-LTQ/MS, as well as their anticomplement activities in vitro.

2. Experimental

2.1. Materials and reagents

The dried roots of S. tonkinensis were purchased from Leiyunshang drug store (Shanghai), and authenticated by Prof. DaoFeng Chen at Fudan University. The voucher specimen (SDG-JX07) has been deposited at School of Pharmacy, Fudan University, Shanghai, People’s Republic of China. Sheep blood cells were prepared in Alsevers’ solution. Antisheep erythrocyte antibody was provided by Prof. Yunyi Zhang. Guinea pig serum was prepared by healthy guinea pigs and rabbit blood cells were prepared from the ear vein of New Zealand white rabbits (both purchased from Laboratory Animals Research Institute of Fudan University, Shanghai, China.). Normal human serum (NHS) was prepared from healthy male donors (at the age of 20–30 years old). Buffers: VBS2+, isotonic veronal buffered saline, containing 0.15 mmol/L CaCl2 and 0.5 mmol/L MgCl2. EGTA-VBS2+, veronal buffered saline, containing 5 mmol/L MgCl2 and 8 mmol/L ethylene glycol tetraacetic acid.

Reference substances (purity>98 %): ononin, formononetin, trifolirhizin, maackiain, rutin, quercitrin, isoorientin, quercetin, daidzein, genistin and sophorabioside were purchased from Meilun Biological Technology Co., Ltd (Shanghai, China). UPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultrapure water was purified by the Milli-Q water purification system from Millipore (Boston, MA, USA). General anaerobic medium broth (GAM broth) was purchased from Meilun Biological Technology Co., Ltd (Shanghai, China). All other reagents and chemical compounds were of analytical grade.

2.2. UPLC-MS analysis

Analyses of TFST and its metabolites were performed using a UPLC system (Dionex ultimate 3000) with a conditioned autosampler at 4 °C. The qualitative analysis and separation were performed on a C18 column (4.6 × 250 mm, i.d., 5 μm Thermo, Finnigan, San Jose, CA, USA). The temperature of the column was set at 25 °C using an UPLC system (Thermo, Finnigan, San Jose, CA, USA). General anaerobic medium broth (GAM broth) was prepared using Meilun Biological Technology Co., Ltd (Shanghai, China). All other reagents and chemical compounds were of analytical grade.

2.3. Preparation of total flavonoids

Dried roots of S. tonkinensis (50 g) were extracted three times using 80 % ethanol for 2 h by heating reflux method, after filtrated, the filtrate was collected and evaporated in vacuum. The 80 % ethanol extract was mixed in water and purified with an AB-8 macroporous resin column, eluted successively with distilled water 3 times column volume and then use 80 % ethanol to wash 6–8 times column volume [16]. 80 % ethanol elution was collected and evaporated in vacuum to yield TFST (4.18 g).

2.4. Metabolism of flavonoid samples by human intestinal bacteria in vitro

Fresh fecal samples were obtained from 8 healthy human volunteers (four males, four females, 20–40 years of age, had neither gastrointestinal disease history nor use of antibiotics for at least six months before the experiment), and homogenized with 20 times volume of general anaerobic medium broth immediately [17]. After removing residue using gauze filter, the intestinal bacterial suspension was cultured in an anaerobic system (nitrogen 85 %, carbon dioxide 10 %, hydrogen 5%) at 37 °C. 20 mg TFST was added to 20 mL human intestinal bacterial suspension and the mixture was anaerobically incubated at 37 °C for 24 h. The incubated solution was respectively taken out and extracted with water saturated n-butanol three times. The extract was centrifuged at 6000 rpm for 10 min at 4 °C. The supernatant was evaporated in vacuum to dryness. The residue was dissolved in 1 mL MeOH and centrifuged at 14,000 rpm for 8 min at 4 °C. The supernatant was then analyzed by UPLC-ESI-LTQ/MS. The chemical constituent change in TFST was also analyzed at incubation time points of 0, 1, 2, and 24 h.

Using the same method as above, 5 mg of each reference substance (sophorabioside, ononin, trifolirhizin, rutin, quercitin and isoorientin) was added in 10 mL human intestinal bacterial suspension and incubated anaerobically for 24 h at 37 °C. After extracted by n-butanol and purified through centrifugation, the final methanol solution was also analyzed using UPLC-ESI-LTQ/MS.

2.5. Anticomplement activity assay

The anticomplement activities through the classical pathway of sophorabioside, rutin, isoorientin, quercetin, ononin, trifolirhizin, daidzein, genistin, quercetin, formononetin, maackiain and heparin sodium (positive control) were evaluated according to Mayer’s modified method [18]. Their anticomplement activities through the alternative pathway were measured according to Klerx’s method [19].

2.6. Anti-inflammatory activity assay

To evaluate the effect of sophorabioside, rutin, isoorientin, quercitin, ononin, trifolirhizin, daidzein, genistin, quercetin, formononetin, maackiain on NO secretion, lipopolysaccharide (LPS) was used as initiators in RAW264.7 cells. The murine macrophage cell line RAW264.7 was maintained in DMEM (Gibco, NY, USA) containing 10% (v/v) fetal bovine serum (Gibco) and cultured in 96-well plates (1 × 105 cells per well), at 37 °C in a humidified atmosphere with 5% CO2. LPS (10 ng/ml) and test samples were added to the corresponding wells and incubated for 24 h. The NO content in the culture media was measured according to the colorimetric method, using Griess reagent [16].

3. Results and discussion

3.1. Biotransformation of TFST by human intestinal bacteria in vitro

Fig. 1 presents the UPLC chromatograms of TFST and its metabolites obtained by incubating with the human intestinal bacterial mixture for 24 h. The results showed that no obvious new peak
in the metabolites (Fig. 1B) was observed compared with TFST (Fig. 1A), but the peak height and peak area changed significantly.

### 3.2. Identification constituents of flavonoids

The chromatographic peaks 2–7, 1’, 2’, 3’, 6’ and 7’ of TFST, were identified by comparing the retention times, molecular ion peaks and UV spectra with reference substances by UPLC-ESI-LTQ/MS in negative ionization mode. The data were summarized in Table 1. The negative electrospray mass spectra of peaks 2–7 revealed [M-H]- ions at m/z 577.06, m/z 609.12, m/z 463.07, m/z 447.09, m/z 429.08 and m/z 445.08 were identified as six flavonoid glycosides, including sophorobioside, rutin, isoquercitrin, quercitrin, ononin and trifoliirhizin. The peaks 1’, 2’, 3’, 6’ and 7’ revealed [M-H]- ions at m/z 252.95, m/z 268.96, m/z 300.94, m/z 266.99 and m/z 283.09, and were identified as five flavonoid aglycones, including daidzein, genistein, quercetin, formononetin and maackiain.

Compound 1 gave a [M-H]- ion at m/z 561.09 and fragment ions at m/z 414.97, 396.92, 378.96 and 349.01. The ion at m/z 414.97 was corresponding to the loss of rhamnose [M-H-rha]-, the ions at m/z 396.92 and 378.96 were generated by losing one and two molecules of H2O (18 Da), the fragment ion at m/z 349.01 was corresponding to [M-H-rha-2H2O-2CH3]-. The fragmentation pathways were similar to those of compound 2, except for 16 Da (-OH) less than every fragment ion of compound 2. Besides, considering the molecular weight of compound 1 was just 308 DA (-glc-rha) more than that of 1’, we speculated that compound 1 and 2 have the same metabolic pathways. Thus, compound 1 was identified as daidzein-4’-glucoside-rhamnoside.

Compounds 8–13 showed typical fragmentation patterns of isoprenylated flavonoids, which could be distinguished by the characteristic 1,3A- ion and 1,3B ions according to the Retro Diels Alder (RDA) Cleavage (Fig. S1) and the characteristic fragments originated from the degradation of different isopentene groups in the MS/MS spectra (Fig. S2). The results were summarized in Table 2.

![Fig. 1. UPLC chromatograms of TFST (A) and its metabolites produced by human intestinal bacteria (B).](image-url)
Table 1
Retention time (RT), molecular ion peaks and peak area ratios of 7 flavonoid glycosides and 5 aglycones in TFST and its metabolites.

| NO. | Compound                     | RT (min) | [M-H] m/z     | Molecular Formula | Area (%) in TFST/Area (%) after incubation |
|-----|------------------------------|----------|---------------|-------------------|------------------------------------------|
| 1   | Daidzein-4′-glucoside-rhamnoside | 22.48    | 561.09        | C₂₂H₂₂O₁₃         | 19.47/14.21                              |
| 2   | Sophorabioside               | 27.74    | 577.06        | C₂₂H₂₄O₁₄         | 9.51/5.17                                |
| 3   | Rutin                        | 28.91    | 609.12        | C₂₂H₂₄O₁₅         | 3.17/0                                   |
| 4   | Isoquercitrin                | 30.23    | 463.07        | C₂₂H₂₄O₁₂         | 3.78/0                                   |
| 5   | Quercitin                    | 33.56    | 447.09        | C₂₂H₂₄O₁₁         | 5.74/0                                   |
| 6   | Ononin                       | 38.17    | 429.08        | C₂₂H₂₄O₃          | 5.89/0                                   |
| 7   | Trifolirhizin                | 41.81    | 445.08        | C₂₂H₂₄O₁₂         | 17.24/0                                  |
| 8   | Daidzein                     | 40.57    | 252.95        | C₁₅H₁₀O₄          | 3.21/0.74                                |
| 9   | Genistein                    | 59.19    | 268.96        | C₁₅H₁₀O₃          | 1.75/0.16                                |
| 10  | Quercetin                    | 42.24    | 300.94        | C₁₁H₁₀O₇          | 0.17/0.96                                |
| 11  | Formononetin                 | 54.63    | 266.99        | C₁₅H₁₀O₄          | 0.21/0.74                                |
| 12  | Maackiain                    | 56.37    | 283.09        | C₁₁H₁₀O₃          | 6.21/3.84                                |

In total, eighteen compounds of TFST and its metabolites were identified (Fig. S3), including seven flavonoid glycosides: daidzein-4′-glucoside-rhamnoside (1), sophorabioside (2), rutin (3), isoquercitrin (4), quercitin (5), ononin (6) and trifolirhizin (7); five flavonoid aglycones: daidzein (1′), genistein (2′), quercitin (3′), formononetin (4′), maackiain (5′) and six isoprenylated flavonoids: kurarinone (8), sophoradin (9), sophorachromene (10), 2-(3-Hydroxy-2,2-dimethyl-8-prenyl-6-chromanyl)-7-hydroxy-8-prenyl-4-chromanone (11), sophorane (12) and sophoranochromene (13). The area ratio of these peaks in TFST and its metabolites (Table 1) indicated that the flavonoid glycosides were decreased, the flavonoid aglycones were increased by human intestinal bacteria, while the isoprenylated flavonoids were not changed (Fig. 1, Table 2).

3.3. The content changes of ingredients in TFST by human intestinal bacteria

Content changes of the seven flavonoid glycosides and five flavonoid aglycones in TFST and its metabolites were determined semi quantitatively by peak area of each incubation time point (0, 12 and 24 h) to 0 h (100 %) (Table S1). Regression equations, correlation coefficients, precision and LOD of reference standards were also evaluated (Table S2). All the contents of daidzein-4′-glucoside-rhamnoside (1), sophorabioside (2), rutin (3), isoquercitrin (4), quercitin (5), ononin (6), trifolirhizin (7) declined as time went on, which were exactly opposite to the content changes of daidzein (1′), genistein (2′), quercitin (3′), formononetin (4′), maackiain (5′) (Fig. 2). Based on the content changes as well as the chemical structures of the twelve constituents of TFST, we inferred that seven flavon glycosides were converted to their aglycones accordingly by human intestinal bacteria.

3.4. Metabolism of flavonoids by human intestinal bacteria in vitro

The UPLC chromatograms of reference standards showed the metabolic results in Fig. S4, which confirmed the transformation of the glycosides to their respective aglycones by intestinal bacteria. Based on the above results, the metabolic pathways of flavonoids with human intestinal bacteria were shown in Fig. 3. It was noteworthy that rutin, isoquercitrin, and quercitin were all metabolized to quercetin. And since there was no commercially available reference standard of daidzein-4′-glucoside-rhamnoside (1), it was hard to verify metabolite directly through the conversion reference product with human intestinal flora. According to chemical structure, fragmentation patterns and the metabolic result of sophorabioside, we speculated that daidzein-4′-glucoside-rhamnoside was metabolized to daidzein by human intestinal bacteria. The results suggested that 0-glucoside flavonoids of TFST were easily converted into their corresponding aglycones with intestinal flora.

3.5. Anticomplement activities through the classical pathway and the alternative pathway

In the transformation of S. tonkinensis flavonoids, there were seven components have changed. Among these components, rutin, isoquercitrin, quercitin, ononin, trifolirhizin were metabolized into quercetin, formononetin and maackiain, with the anticomplement activities were remarkable improved. Particularly, formononetin (CH₅O: 0.082 ± 0.029 mg/mL, AP₅₀: 0.217 ± 0.068 mg/mL), the metabolite of ononin (CH₅O: 3.275 ± 1.352 mg/mL, AP₅₀: NE) showed significant activity comparable to the positive inhibitor. However, neither glycosides nor aglycones of the other isoflavones (sophorabioside, daidzein and...
genistein) showed anticomplement activity (Table 3). Although rutin, isoquercitrin and quercitrin demonstrated anticomplement activities through the classical pathway or alternative pathway, their common metabolite quercetin (CH50: 0.178 ± 0.102 mg/mL, AP50: 0.205 ± 0.139 mg/mL) was quite more potent. Besides, the isoprenylated flavonoids were all inactive.

3.6. Anti-inflammatory activities

In vitro anti-inflammatory assays (Fig. S5) showed that compared with cell + LPS group, rutin, quercitrin, quercetin and formononetin treated cells exhibited significant decreased NO production (P < 0.05). On the other hand, the results were consistent with their anticomplement activities. Quercetin and formononetin showed better activities than their glycosides (rutin, isoquercitrin and ononin). Meanwhile, there was no statistical difference between quercitrin and quercetin. However, both maackiain and trifolirhizin, with very weak anticomplement activity, had no anti-inflammatory activity.

The above results revealed that the metabolic activation of TFST by human intestinal flora was attributed to the content increasing of the more potent flavonol (quercetin) and isoflavone (formononetin) aglycones. This kind of transformation had also occurred in the intestinal bacteria metabolism of the inactive flavone glycosides of Scutellaria baicalensis roots to their anticomplement glycones both in vitro and in vivo [27]. These aglycones, like quercetin and formononetin, might be the potential effective substances of S. tonkinensis to perform the inhibiting inflammation action in vivo.

Our previous study showed that bacteroidetes, proteobacteria and firmicutes accounted for more than 90 % of human intestinal bacteria [27]. Ononin and quercitrin were further metabolized by specific human intestinal bacteria from these three groups, including Bacteroides, Escherichia coli, Lactobacillus and Enterococcus faecalis. The results showed that ononin could be transformed by all the four bacteria into formononetin (Fig. S6). However, the Bacteroides could convert quercitrin to quercetin (Fig. S7). The reason might be the different glycosidic bonds of the two compounds. The β-glucoside in ononin is more common in nature than the α-rhamnoside in quercitrin and thus could be metabolized more easily by most intestinal bacteria. On the other hand, the four bacteria had quite different efficiencies on metabolizing ononin, in which Enterococcus faecalis transformed almost all the compound into its aglycone.

Table 3

| No. | Compound           | CH50 (mg/mL)       | AP50(mg/mL)   |
|-----|--------------------|-------------------|--------------|
| 2   | Sophorabioside     | NE                |              |
| 3   | Rutin              | 0.247 ± 0.133     | 0.307 ± 0.089|
| 4   | Isoquercitrin      | 0.419 ± 0.187     | 0.296 ± 0.103|
| 5   | Quercitrin         | 0.276 ± 0.172     | 0.305 ± 0.164|
| 6   | Ononin             | 3.275 ± 1.352     | NE           |
| 7   | Trifolirhizin      | 3.517 ± 1.367     | NE           |
| 1’  | Daidzein           | NE                | NE           |
| 2’  | Genistein          | NE                | NE           |
| 3’  | Quercetin          | 0.178 ± 0.102***  | 0.205 ± 0.139*** |
| 6’  | Formononetin       | 0.082 ± 0.029     | 0.217 ± 0.068|
| 7   | Maackiain          | 1.940 ± 0.683***  | 2.522 ± 0.710|
| Positive control | Heparin           | 0.046 ± 0.009     | 0.112 ± 0.019 |

Notes: & P<0.05, * P<0.01, **** P<0.0001, compared with the initial peak area of the compound.

Fig. 2. The Peak area changes of the main constituents in flavonoids of S. tonkinensis when incubated with human intestinal bacteria. Date are presented as mean ± SD, n = 3.

\* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, compared with the initial peak area of the compound.
Besides, the roots of *Sophora flavescens* Ait, another commonly used medicinal plant from *Sophora*, are found to contain similar chemical constituents as *S. tonkinensis*, including isoprenylated flavonoids and quinolizidine alkaloids. However, their clinical applications are completely different [28,29]. *S. tonkinensis* is frequently used internally to treat acute throat infections while *S. flavescens* is mostly used externally for the treatment of skin diseases, such as eczema and dermatitis [30]. And their different flavonoid constituents could serve as the qualitative markers for identification of *S. tonkinensis* and *S. flavescens*. *S. flavescens* roots barely contain flavonoid glycosides but mainly contain isoprenylated flavonoids (Fig. S8), which are inactive on the complement system and unable to be metabolized by intestinal bacteria. As expected, almost no change was detected when the flavonoids of *S. flavescens* were incubated with human intestinal bacteria, except trifoliurhizin (1) metabolized to maackitain (1′) (Fig. S9, Table S3). These results further explain the different effects and administration routes of *S. flavescens* and *S. tonkinensis*.

4. Conclusion

Our current study showed that the presence of intestinal flora played a key role in metabolic activation of TFST after oral administration. The contents of flavonoid aglycones with potent anticomplement activity were enhanced significantly under the hydrolytic action of intestinal flora on the flavonoid glycosides. Among the eighteen flavonoids identified using UPLC-ESI-LTQ/MS method, isoflavone and flavonol aglycones (formononetin and quercetin) and three flavonol glycosides (rutin, isoquercitrin and quercitrin) were the major anticomplement constituents of TFST and should be taken as the quality markers of *S. tonkinensis* roots. The in vivo anti-inflammatory and anticomplement effects of formononetin, the most potent constituent and metabolite of TFST, will be investigated in the near future.

**CRediT authorship contribution statement**

**Xin Jin:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Yan Lu:** Writing – review & editing. **Shaoxin Chen:** Project administration. **Daofeng Chen:** Resources, Supervision.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.jpba.2020.113176.

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