Investigation of the lipid lowering mechanism and active ingredients of Danhe granule on hyperlipidemia based on systems pharmacology

**Supplementary Material**

**Preparation of Danhe Granule (DHG)**

Danhe Granule (DHG), a TCM formula, was composed of six herbs, as follows: *Salvia miltiorrhiza* Bunge (Danshen), *Reynoutria japonica* Houtt. (Huzhang), *Crataegus pinnatifida* Bunge (Shanzha), *Citrus × aurantium* L. (Chenpi), *Coix lacryma-jobi var. ma-yuen* (Rom.Caill.) Stapf (Yiyiren), and *Nelumbo nucifera* Gaertn. (Heye). *Saliae Miltiorrhizae Radix et Rhizoma* (Lot No.80270602) [the root and rhizome of *Salvia miltiorrhiza* Bunge, a perennial herbal plant of the *Salvia* L. genus, the Labiatae family], *Polygoni Cuspidati Rhizoma et Radix* (Lot No.80510801) [the root and rhizome of *Reynoutria japonica* Houtt., genus Reynoutria Houtt., family Polygonaceae], *Crataegi Fructus* (Lot No.82230701) [the fruit of *Crataegus pinnatifida* Bunge, the short arbor plants of the *Crataegus* L. genus, the Rosaceae family], *Citri Reticulatae Pericarpium* (Lot No. 81450601) [the peel of *Citrus × aurantium* L., genus *Citrus* L., family Rutaceae], *Coicis Semen* (Lot No.70511101) [the mature kernel of *Coix lacryma-jobi var. ma-yuen* (Rom.Caill.) Stapf, a perennial herbal plant of the *Coix* L. genus, the Poaceae family] and *Nelumbinis Folium* (Lot No. 83720701) [the leaf of *Nelumbo nucifera* Gaertn., genus *Nelumbo* Adans., family Nelumbonaceae] were obtained from Beijing San He Co., Ltd. (Beijing, China) and identified by Professor Xueyong Wang (School of Chinese Materia Medica, Beijing University of Chinese Medicine). Danhe Granule (DHG) [production batch No. D1905007] was provided by the hospital preparation room, China-Japan Friendship Hospital (Beijing, China), which was used for pharmacological research in the study. The preparation processes follows: The total herbs including *Saliae Miltiorrhizae Radix et Rhizoma* (1000g), *Polygoni Cuspidati Rhizoma et Radix* (1000g), *Crataegi Fructus* (1000g), *Citri Reticulatae Pericarpium* (1000g), *Coicis Semen* (3000g) and *Nelumbinis Folium* (1500g) were mixed and extracted 3 times by refluxing with 12-fold of water (volume/weight) for 1 h each time. The liquid extract was filtered and concentrated to a density of 1.08 (60 °C). 95% ethanol was added into the concentrated liquid to 60% ethanol concentration, and then it was precipitated at room temperature for 24 hours. The supernatant was condensed and dried to yield 1060 g powder using decompression drying method at 60 °C.

**Quality control of Danhe Granule (DHG) by high performance liquid chromatography (HPLC)**

1. Chemicals and materials
HPLC-grade acetonitrile, methanol and formic acid were supplied by Merck (Darmstadt, Germany). HPLC-grade phosphoric acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was obtained using a Milli-Q water purification system from Millipore (Bedford, MA, USA). Standards (purity > 98%) of gallic acid (batch No. M-017-161223), polydatin (batch No. M-017-161223), isoquercetin (batch No. Y-076-161216), hesperidin (batch No. 110721-201818), salvianolic acid B (batch No. D-012-170417) and emodin 8-O-β-D-glucoside (batch No. D-018-160928) were purchased from Chengdu Herbpurify Co., Ltd. (China).

Saliae Mil Tiorrhizae Radix et Rhizoma (Lot No. 80271101; Lot No. 20190223; Lot No. 1808094; Lot No. 1808095), Polygoni Cuspidati Rhizoma et Radix (Lot No. 80511101; Lot No. 20181201; Lot No. 1808094), Crataegi Fructus (Lot No. 82231001; Lot No. 20181220; Lot No. 1810120), Citri Reticulatae Pericarpium (Lot No. 81451002; Lot No. 20190122; Lot No. 1811036), Coicis Semen (Lot No. 82511001; Lot No. 20190214; Lot No. 1809075) and Nelumbinis Folium (Lot No. 83720701; Lot No. 20181227; Lot No. 1809012) were purchased from Beijing San He Co., Ltd. (Beijing, China), Beijing Bencao Fangyuan Pharmaceutical Co., Ltd. (Beijing, China) and Beijing Taiyang Pharmaceutical Industry Co., Ltd. (Beijing, China). After random combination of different batches of herbs, we prepared 10 batches of Danhe granules in the laboratory, as follows: XS190312-01, XS190312-02, XS190318-01, XS190326-01, XS190409-01, XS190409-02, XS190412-01, XS190415-01, XS190420-01 and XS190420-02.

Batch numbers of herbs in ten batches of Danhe granules

| DHG Batchs | Saliae Mil Tiorrhizae Radix et Rhizoma | Polygoni Cuspidati Rhizoma et Radix | Crataegi Fructus | Citri Reticulatae Pericarpium | Coicis Semen | Nelumbinis Folium |
|------------|--------------------------------------|------------------------------------|-----------------|------------------------------|-------------|------------------|
| XS190312-01 | 80271101                             | 1808094                            | 20181220        | 81451002                     | 20190214    | 20181227         |
| XS190312-02 | 80271101                             | 20181201                           | 82231001        | 20190122                     | 1809075     | 20181227         |
| XS190318-01 | 80271101                             | 20181201                           | 1810120         | 20190122                     | 1809075     | 20181227         |
| XS190326-01 | 20190223                             | 1808094                            | 1810120         | 20190122                     | 20190214    | 83720701         |
| XS190409-01 | 20190223                             | 20181201                           | 1810120         | 20190122                     | 1809075     | 83720701         |
| XS190409-02 | 20190223                             | 20181201                           | 1810120         | 81451002                     | 82511001    | 1809012          |
| XS190412-01 | 1812062                              | 20181201                           | 20181220        | 1811036                      | 20190214    | 83720701         |
| XS190415-01 | 1812062                              | 20181201                           | 20181220        | 20190122                     | 82511001    | 20181227         |
| XS190420-01 | 1812062                              | 20181201                           | 20181220        | 20190122                     | 82511001    | 20181227         |
| XS190420-02 | 1812062                              | 80511101                           | 82231001        | 20190122                     | 20190214    | 1809012          |

2. Instrumentation and chromatographic conditions

Chromatographic analysis was performed on an Agilent 1260 Infinity high-performance liquid chromatograph system (Agilent Technologies, Waldbronn, Germany). The chromatographic data were processed with Agilent Chem Station. Samples separation was based on a ZORBAX Eclipse XDB-C18 column (4.6×250
mm, 5 μm) with column temperature at 30 ℃. The mobile phase was composed of solvent A (water-0.1% phosphoric acid) and solvent B (acetonitrile), and the flow rate was kept at 1.0 mL/min. The injection volume was 10 μL and the UV detection wavelength was set at 286 nm. The gradient elution was as follows: 0–55 min, 5–30% B; 55–60 min, 30–60% B; 60–70 min, 60–95% B; 70–75 min, maintained at 95% B.

3. Preparation of standard solutions and DHG samples
   Stock solutions of six reference standards were prepared at a concentration of 1.0 mg/mL in methanol, respectively. Serial mixed standard working solutions with different concentrations were prepared through blends and dilutions of the stock solutions with methanol. The stock solutions were filtered through 0.45μm nylon membranes before injection. The DHG sample was weighed 0.20 g and extracted with methanol 50 mL at room temperature by ultrasonic extraction for 30 min, then filtered through 0.45μm nylon membranes before injection.

4. Precision, stability and repeatability validation
   Precision validation: The sample solution (batch No: XS190318-01) was injected for 6 times continuously. Salvianolic acid B was used as the correction peak. The results showed that the RSD of the relative retention time of the common peaks was less than 1.20% and the relative peak area RSD was less than 1.52%. Repeatability validation: The sample (batch No: XS190318-01) was used to made six independently sample solutions. Salvianolic acid B was used as the correction peak. After analyzing the six sample solutions, results showed that the RSD of the relative retention time of the common peaks was less than 0.93% and the relative peak area RSD was less than 1.90%. Stability validation: The sample solution (batch No: XS190318-01) was analyzed at 0, 2, 4, 6, 12 and 24 h. Salvianolic acid B was taken as the correction peak. The results showed that the RSD of the relative retention time of the common peaks was less than 2.83% and the relative peak area RSD was less than 2.61%. The above results indicated that the method was with good accuracy, reliable and reproducible for the similarity and quantification analysis of DHG samples.

5. Establishment of chromatographic fingerprint
   Based on the Similarity Evaluation System for Chromatographic Fingerprint of TCM (2012 edition), the similarities of the 11 batches of DHG samples (batch XS190312-01 as S1, batch XS190312-02 as S2, batch XS190318-01 as S3, batch XS190326-01 as S4, batch XS190409-01 as S5, batch XS190409-02 as S6, batch XS190412-01 as S7, batch XS190415-01 as S8, batch XS190420-01 as S9 and batch XS190420-02 as S10 and batch D1905007 as S11) was evaluated. After peak-picking, template-matching process, the peaks in the spectra were matched automatically (Shown in Figure S1). The reference template was set finally for spectra peak difference and entire similarity evaluation. The similarities of repeatability were greater than 0.94. The results showed that the preparation process of DHG was reasonable and feasible.

Comparability results of reproducibility of DHG samples
6. Quantitative analysis
Under the above chromatographic conditions, based on the comparison of the standard references, six main HPLC chromatographic peaks in DHG sample were identified. In addition, the quantitative analysis of the six compounds in the DHG sample (batch D1905007) which was used in the pharmacological study was carried out. The results are as follows: the contents of gallic acid, polydatin, isoquercetin, hesperidin, salvianolic acid B and emodin-8-O-β-D-glucoside were 7.09 mg/g, 4.37 mg/g, 5.39 mg/g, 9.62 mg/g, 13.09 mg/g, and 2.15 mg/g, respectively. The chromatograms of mixed standards and DHG sample are listed in Supplementary Figure S1.
Figure S1. The chromatograms of DHG and standards by HPLC. Chromatogram profiles of DHG (A); Chromatogram profiles of standards (B); Chemical structures of the main compounds in DHG samples (C). The main components of DHG in the chromatograms are as follows: 1-gallic acid, 2-polydatin, 3-isoquercetin, 4-hesperidin, 5-salvianolic acid B and 6-emetin-8-O-β-D-glucoside.
Figure S2. The extracted ion chromatograms (EICs) of 16 ingredients identified in hamster serum; DHG group (A) and blank group (B) in positive ion mode; DHG group (C) and blank group (D) in negative ion mode (The figures A and C were magnified to show those peaks with smaller area). Chemical structures of the main compounds absorbed into blood after DHG taken orally (E).
Figure S3. The total ion chromatograms of DHG sample
Figure S4. GO enrichment analysis of the targets. The nodes represent GO terms with significant enrichment. The node pie charts represent the molecular function and biological process analysis of these targets. (A) Representative-enriched molecular function relative to the targets. (B) Representative-enriched biological processes relative to the targets.
Figure S5. T-P network. The T-P network was constructed by linking targets and their related pathways classified into 8 groups based on pathway analysis. The nodes represent targets (rhombus), pathways (v-shaped) and different groups (hexagon).
The detailed process of identification of serum ingredients

Ten compounds including polydatin, hyperin, hesperidin, salvianolic acid b, nuciferine, quercetin, emodin 8-O-β-D-glucoside, nobiletin, emodin and tanshinone II-A were identified by comparing with reference standards. In addition, other six compounds were identified based on consulting with literatures and fragment ions analysis. Compound 2 and compound 7 are isomers, exhibiting the [M+H]$^+$ ion at m/z 282.1495 (C$_{18}$H$_{19}$NO$_2$). The differences between them are that compound 7 would produce obvious fragment ion at m/z 265 and compound 7 exhibited a longer retention time on a chromatographic column than compound 2. Then, compound 2 and compound 7 were identified as n-nornuciferine and o-nornuciferine, respectively, by consulting with literatures (Deng et al., 2016; Luo et al., 2005). Compound 4, the isomer of hyperin (compound 3), showed the [M-H]$^-$ ion at m/z 463.0874 and similar fragment ions at m/z 300.0282, m/z 271.0255, m/z 255.0305, m/z 243.0307, m/z 227.0355 and m/z 151.0044. But compound 4 exhibited a longer retention time on a chromatographic column than hyperin and was identified by consulting with literature (Qiao et al., 2014). Compound 5 showed the [M-H]$^-$ ion at m/z 579.1719 and fragment ions at m/z 271.0619 [M-H-Glu-Rha$^-$], m/z 227.0722 [M-H-Glu-Rha-CO$_2$]$^-$, m/z 151.0044 [M-H-Glu-Rha-C$_8$H$_8$O]$^-$, m/z 119.0510 [M-H-Glu-Rha-CO$_2$-C$_7$H$_4$O$_4$]$^-$ and m/z 107.0148 [M-H-Glu-Rha-CO$_2$-C$_7$H$_4$O$_4$-C$^-$]. Therefore, compound 5 was identified as naringin (Ye et al., 2014). Compound 12 showed the [M-H]$^-$ ion at m/z 271.0616 and fragment ions at m/z 151.0042 [M-H-C$_8$H$_8$O]$^-$, m/z 119.0508 [M-H-C$_7$H$_4$O$_4$]$^-$ and m/z 107.0144 [M-H-C$_7$H$_4$O$_4$-C$^-$]. Then, the compound 12 was deduced to naringenin (Sun et al., 2010). Compound 13 exhibited the [M-H]$^-$ ion at m/z 245.0824 and fragment ions at m/z 230.0594 [M-H-CH$_3$]$^-$, m/z 215.0362 [M-H-CH$_3$-CH$_3$]$^-$, m/z 187.0396 [M-H-CH$_3$-CH$_3$-CO]$^-$, and m/z 159.0458 [M-H-CH$_3$-C$_3$H$_6$O$_2$]$^-$]. Therefore, compound 13 was identified as torachryson (Pan et al., 2015). The information of the proposed fragment behaviors of the 16 compounds was also summarized in the following figures.
Onornuciferine

Plasma after DEG taken orally

-ESI

Counts (%) vs. 

m/z 282.1493
m/z 265.1245
m/z 250.1006

H₂O

m/z 235.0772
m/z 191.0862
m/z 219.0815

Reference standard

Plasma after DHO taken orally

-ESI

Counts (%) vs. 

m/z 296.1642
m/z 265.1246
m/z 250.1008

H₂O

m/z 235.0773
m/z 191.0869
m/z 219.0819
Nobiletin

Torachryson

Plasma after DHG taken orally

Reference standard

Plasma after DHG taken orally

-ESI
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