Identification of Absorbed Constituents and their Metabolites Related to Estrogen-Like Activity of Total Glycosides of *Cistanche deserticola* in Rat Serum

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**ABSTRACT**

*Cistanche deserticola* Y. C. Ma is a kind of traditional Chinese medicine and food raw material with estrogen-like effect; its active ingredients are glycosides. To explore the absorbed constituents of total glycosides (TGs) *in vivo* and its estrogen-like activity, and reveal its direct acting substances in the body, in this study, 30 female Wistar rats were orally administered with TGs, and serum fingerprints of blood samples collected at 10 different times were established by UPLC-Q-TOF-MS analysis. The estrogen-like activity of TGs was evaluated by determining the proliferation rate of Michigan Cancer Foundation-7 cells. The spectrum-effect relationship was analyzed by serum fingerprint of TGs to characterize the activity *in vivo* and identify the active compounds of TGs. In the results, a total of 75 chemical compounds of TGs were identified in serum, including 11 parent molecules and 64 metabolites. The serum fingerprint of TGs were established, and 36 common peaks were identified. The correlation coefficient and the Grey relational degree between the relative area of the common peaks and the estrogen-like activity of TGs were determined through bivariate analysis and Grey relational analysis, respectively. Total 32 chemical components were identified, including methylated cistanstubuloside B, acetylated tubuloside B, and acteoside, which are the active compounds leading to the estrogen-like activity of TGs *in vivo*. In conclusion, this study is expected to provide a theoretical reference for subsequent in-depth studies on the estrogen-like pharmacodynamic material basis of TGs.

**INTRODUCTION**

*Cistanche deserticola* Y. C. Ma is a medicinal plant growing in arid or semi-arid areas. Its stem, also known as Rou Cong Rong (*Wang* *et al*., 2012), is a precious traditional Chinese medicine and is usually used as a tonic (*Ai et al.*, 2021; *Jiang et al.*, 2009). It can nourish the kidneys, relax the bowels, and protect the liver, exerting anti-oxidant, anti-aging, anti-fatigue, immunoregulation, and estrogen-like effects (*Li et al.*, 2017; *Piwowarczyk et al.*, 2020; *Song et al.*, 2017). It mainly consists of phenylethanol glycosides, iridoids, lignanoids, alkaloids, and polysaccharides (*Fu et al.*, 2020; *Jiang and Tu*, 2009; *Tu et al.*, 2007).

During the perimenopause for women, the estrogen levels *in vivo* will decrease, and usually causes adverse reactions such as bone loss, angina pectoris, hot flashes, night sweats, insomnia, dry eyes, dry and aging skin, increased wrinkles, high cholesterol, and memory loss (*Bittner*, 2009; *Ma and Chen*, 2015; *Zhao et al.*, 2018). Traditional estrogen replacement therapy can treat or alleviate the diseases caused by estrogen deficiency (*Rubinow et al.*, 2015; *Zetterberg*, 2016), however, it is usually accompanied by adverse reactions (*Duenas-Garcia et al.*, 2016), increasing the risk of gynecological cancer (*Decensi et al.*, 2013). In recent years, researches on phytoestrogens have increased, and found that phytoestrogens have good curative effect on related diseases caused by the decline of estrogen levels with little side effects, and is expected to become an alternative treatment method (*Sobenin et al.*, 2016; *Tham et al.*, 1998). Studies have compared the effects of a variety of non-hormone drugs treating on perimenopausal symptoms on cardiovascular diseases including blood pressure, heart rate, stroke, incidence rate and mortality, etc., and showed that phytoestrogen had no adverse effects, moreover, it may also reduce the risk of cardiovascular disease through many ways (*Mareti et al.*, 2019).
Long-term studies on the estrogen-like activity of *C. deserticola* have been performed to determine the components related to its activity. It has been proved that the total glycosides (TGs) of *C. deserticola* were the active components, and an optimal purification process of TGs had been developed (Li *et al*., 2013, 2019). However, there is still a lack of relevant research on the in vivo active compounds of *C. deserticola*, as well as further research on the relationship between the in vivo chemical composition of TGs of *C. deserticola* and their estrogenic activity.

In this study, the serum fingerprint of TGs with MCF-7 (commonly used breast cancer cell line) cell proliferation assays were performed to analyze the spectrum-effect relationship of components in vivo, and to identify the active compounds of TGs in serum. The findings of this study aims to provide support for the future provide experimental basis for revealing the material basis of estrogen-like effect of TGs, use of *C. deserticola* to alleviate estrogen deficiency in females, and have important implications for the development of *C. deserticola* as a natural functional food.

### MATERIALS AND METHODS

**Materials, instruments and the preparation of TGs**

The dry fleshy stems with scale leaves of *C. deserticola* was made into powder. Then, according to the method of Li *et al*., (2019) and Hu *et al*., (2021) in brief, the *C. deserticola* powder was heated and extracted with 75% ethanol, purified with AB-8 macroporous resin, then eluted with 85% ethanol until it was concentrated into extract under reduced pressure to obtain TGs (62.5%, calculated based on verbacoside). Then, TGs were dissolved in distilled water to create a gavage solution containing 75 mg·mL⁻¹ of TGs for subsequent use. The main experimental reagents and instruments were shown in Supplementary Table S1.

**Animal groups and administration**

Total 30 female Wistar rats (weighing 210 ± 20 g) were housed at 22 ± 2°C with a relative humidity of 50 ± 10%, were adaptively fed for 5 days with free access to food and water. The rats were randomly divided into three groups (n=10): drug group (TGs, crude *C. deserticola* dosage at 18.75 g·kg⁻¹·d⁻¹), positive control group (PC; diethylstilbestrol, 0.35 mg kg⁻¹·d⁻¹), and blank control group (BC, same volume of distilled water). All groups were administered intragastrically for four consecutive days, twice daily in the morning and evening. Then the rats were fasted but allowed water for 12 h before blood collection.

**Blood collection and serum samples**

Blood samples (0.5 mL) were collected from the orbital venous plexus for all the groups at 10 different time at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 12 h after the last intragastric administration. After letting stand for 1 h, the serum (supernatant) was obtained by centrifuging at 5000 rpm for 10 min, and stored at −20°C for later use. Serum samples (200 µL) from the TGs and BC groups were collected, thoroughly mixed with 600 µL of methanol respectively, and centrifuged at 12,000 r·min⁻¹ for 10 min. Supernatants were collected, dried with nitrogen, redissolved in 200 µL of 60% methanol, and filtered through a 0.22 µm syringe filter. Thus, 10 serum samples of TGs and BC groups at 10 different time points were obtained for fingerprint and chemical composition analysis, respectively.

**UPLC-Q-TOF-MS analysis**

The time point with the most transitional components were selected to perform the UPLC-Q-TOF-MS analyze; ACQUITY UPLC BEH C₁₈ column (100 mm × 2.1 mm, 1.7 µm) was used, the conditions were according to (Hu *et al*., 2021). Acetonitrile and 0.2% formic acid aqueous solution was used as the solvent system to perform the gradient elution for 20 min. Spectra were recorded in positive and negative ion modes with the scan range of m/z 50-1000. The data were processed and analyzed using Masshunter Qualitative Analysis B.07.00 (Agilent, Santa Clara, CA, USA) (Wu *et al*., 2021).

The in vivo and in vitro component database of TGs of *C. deserticola* was established by consulting the literature, including the name, molecular formula, molecular weight, CAS and other information of reported known compounds. Molecular structure of target compounds was identified by comparing with reference substances and records in the database based on the analysis of elemental composition, accurate molecular ion mass, fragmentation pattern, and retention time.

**Establishment of serum fingerprint of TGs**

UPLC-Q-TOF/MS analysis was performed on the serum samples of 10 different time points, the conditions were the same as above. Then, the collected chromatograms were imported into the Similarity Evaluation System for Chromatographic Fingerprint of TCM (version 2004A) software. The peaks with good stability, reproducibility and stable absorption in the serum fingerprint were selected as common peak, and that with large peak area and moderate retention time as the reference peak.

Moreover, the relative retention time and peak area of each common peak was calculated based on the area (set to unity) and retention time (set to unity) of the reference peak, respectively. A methodological research was
performed on the TGS serum fingerprint, the positive and negative ion mode was assessed, including the precision, stability and reproducibility by calculating the relative standard deviation (RSD) (Dou et al., 2016b).

**MCF-7 cell proliferation assays**

Samples (n=10) from each test group were subjected to MCF-7 cell proliferation experiments. MCF-7 cells in logarithmic growth phase were seeded into a 96-well plate at 1.5×10^4 cells/well, 100 μL per well. After incubation for 24 h, cells were treated with serum of BC, PC and TGS groups (5%), respectively. After 72 h, 100 μL MTT was added and incubated for 4 h. Then the culture medium was discarded and 150 μL dimethyl sulfoxide (DMSO) was added to each well, gently shaken for 10 min. The absorbance was measured at 570 nm using a microplate reader, and the average value and proliferation rate were calculated (Ding et al., 2019).

**Statistical analysis**

SPSS 19.0 (IBM, Armonk, NY, USA) was used for bivariate analysis. The Pearson correlation coefficient between the relative peak area of common peaks of serum fingerprints (as the sub sequence) and the proliferation rate of MCF-7 cells (as the parent sequence) was calculated, the grey relational analysis (GRA) was used to calculate their correlation degree (Sun et al., 2021).

**RESULTS AND DISCUSSION**

**Chemical composition of TGs in rat serum**

To clarify the components and metabolites of TGs in rat serum, UPLC-Q-TOF-MS technology was used to detect the serum samples. In the positive ion mode, total 22 compounds consisting of 4 parent molecules and 18 metabolites were inferred. In the negative ion mode, total 53 compounds (excluding the same chemical components as in the positive ion mode) consisting of 7 parent molecules and 46 metabolites were inferred. In total, 75 compounds consisting of 11 parent molecules and 64 metabolites were inferred (Table I, Supplementary Figs. S2 and S3), indicating that TGS mainly exist as metabolites in vivo.

**Establishing and evaluation of serum fingerprint**

The serum fingerprint of TGS were separately established for the positive and negative ion mode (Fig. 1). In the positive ion mode, a total of 22 components were detected. As some components disappeared or had qualitative changes with time, a total of 16 common peaks are finally determined. In the negative ion mode, total 53 components were detected, and finally 20 common peaks were determined. According to the results in Table I, the specific names of the 36 common peaks can be defined.

The methodological performance in positive and negative ion modes was assessed separately using serum samples collected 1 h after intragastric administration. In the positive and negative ion modes, peak 16 (acetylated mussaenosidic acid) and peak 37 (acetylated eucommin A) were selected as reference peaks. The results showed that the RSD was less than 0.2, indicating satisfactory instrumental precision and methodological reproducibility, as well as sample stability within 24 h.

**Estrogen-like activity of TGs**

In order to clarify the estrogen-like effect of TGS, MCF-7 cell proliferation assays was used to detect the estrogenic activity of serum samples with diethylstilbestrol as the positive drug. In result, the proliferation rate of MCF-7 cells in PC group at all time points was significantly higher than that in BC group (p<0.01, Table II). Compared with BC group, the proliferation rate of MCF-7 cells in TGS group was extremely significant higher at the first 9 time points (0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10 h) (p<0.01), and significantly higher at the last time point (12 h) (p<0.05). In conclusion, serum samples from TGS group at 10 time points could significantly proliferate MCF-7 cells, indicating that it has estrogen-like activity.

**Correlation analysis results**

To clarify the components of TGS with estrogen-like effect in vivo, the analysis methods combining bivariate analysis and grey correlation were used to correlate the common peak of serum fingerprint with the experimental results of estrogen-like activity. The results showed that (Fig. 2), 13 peaks were detected in positive ion mode correlated with the proliferation rate; in which five peaks showed an extremely significant correlation (Peaks 11, 12, 15, 20, and 21). Eight peaks showed a significant correlation (Peaks 2, 5, 7, 8, 9, 13, 14, and 16). In the negative ion mode, 20 peaks were correlated with the proliferation rate, consisting of 15 peaks with extremely
Table I. Identification of compounds of the characteristic peaks in the serum TGs by UPLC-Q-TOF/MS in positive and negative ion mode.

| Ion mode | Peak No. | Retention time (min) | Possible compound | Note | Ion mode | Peak No. | Retention time (min) | Possible compound | Note |
|----------|----------|----------------------|-------------------|------|----------|----------|----------------------|-------------------|------|
| +        | 1        | 5.031                | Acetylated sinapaldehyde glucoside | M     | -        | 17       | 6.932                | Methyleated eucommín A | M    |
| +        | 2        | 5.197                | Acetylated dehydrodiconiferyl alcohol-4-O-β-D-glucoside | M C   | -        | 18       | 7.967                | Geniposide | P    |
| +        | 3        | 5.656                | Methylated acteoside | M     | -        | 19       | 8.438                | Methylated ononin | M    |
| +        | 4        | 6.189                | Sinapaldehyde glucoside sulfated conjugate | M     | -        | 20       | 8.571                | Cistanoside F | P    |
| +        | 5        | 8.590                | Methylated cistantubuloside B | M C   | -        | 21       | 8.695                | Demethylated kankanoside A | M    |
| +        | 6        | 11.118               | Methylated coniferin | M C   | -        | 22       | 9.395                | 3'-Epiloganic acid sulfated conjugate | M    |
| +        | 7        | 11.698               | Dehydrodiconiferyl alcohol-4-O-β-D-glucoside | P     | C        | 23       | 9.565                | Decaffeoyl acteoside | P    |
| +        | 8        | 12.383               | Acetylated tubuloside B | M C   | -        | 24       | 10.155               | Acetylated kankanoside I | M    |
| +        | 9        | 13.776               | Demethylated kankanoside E | M C   | -        | 25       | 10.992               | Kankanoside L sulfated conjugate | M    |
| +        | 10       | 13.836               | Kankanoside J sulfated conjugate | M     | C        | 26       | 11.002               | Cistanoside E glucuronidated conjugate | M    |
| +        | 11       | 13.869               | Methoxylated tubuloside B | M     | C        | 27       | 11.068               | Acetylated daucosterol | M    |
| +        | 12       | 14.459               | Acteoside | P     | C        | 28       | 11.335               | Syringin | P    |
| +        | 13       | 14.462               | Kankanoside L | P     | C        | 29       | 11.674               | Acetylated cistanoside G | M C  |
| +        | 14       | 14.478               | Kankanoside E | P     | C        | 30       | 11.723               | 2'-Acetylaceotside glucuronidated conjugate | M    |
| +        | 15       | 14.479               | Dehydrodiconiferyl alcohol-4-O-β-D-glucoside glucuronidated conjugate | M     | C        | 31       | 11.841               | Hydroxylated cistanoside G | M    |
| +        | 16       | 14.909               | Acetylated mussaenosidic acid | M     | C        | 32       | 11.847               | Methylated tubuloside B | M C  |
| +        | 17       | 15.843               | Hydroxylated sinapaldehyde glucoside | M     | C        | 33       | 12.012               | Dehydroxylated kankanoside L | M    |
| +        | 18       | 15.876               | Acteoside sulfated conjugate | M     | -        | 34       | 12.019               | Ononin | P    |
| +        | 19       | 18.037               | Kankanoside L glucuronidated conjugate | M     | -        | 35       | 12.512               | Dehydroxylated syringin | M    |
| +        | 20       | 18.911               | Methylated mussaenosidic acid | M     | C        | 36       | 12.573               | Kankanoside I glucuronidated conjugate | M    |
| +        | 21       | 20.428               | Methoxylated coniferin | M     | C        | 37       | 12.818               | Acetylated eucommín A | M C  |
| +        | 22       | 20.794               | Tubuloside B glucuronidated conjugate | M     | -        | 38       | 13.118               | Acetylated 2-acetylaceotside | M C  |
| -        | 1        | 1.236                | Cistanoside G glucuronidated conjugate | M     | C        | 39       | 13.144               | Hydroxylated ononin | M C  |
| -        | 2        | 1.269                | 3,4-Dihydroxyphenylethanoid glycoside | M     | C        | 40       | 13.765               | Hydroxylated kankanoside A | M    |

Table continued on next page..............
| Ion mode No. | Retention time (min) | Possible compound | Note | Ion mode No. | Retention time (min) | Possible compound | Note |
|-------------|---------------------|-------------------|------|-------------|---------------------|-------------------|------|
| -3          | 1.886               | Demethylated (2E,6Z)-8-O-β-D-glucopyranosyloxy-2, 6-dimethyl-2, 6-octadienoic acid | M C | -41         | 13.852              | Methoxylated 2-acetylglucomannose | M C |
| -4          | 2.158               | 8-Epiloganic acid | P C | -42         | 14.086              | Dehydroxylated geniposidic acid | M   |
| -5          | 2.765               | Methylated salidrose | M | -43         | 14.982              | Demethylated 8-epidoxypolygolic acid | M   |
| -6          | 3.220               | Syringin sulfated conjugate | M C | -44         | 15.169              | Acetylated geniposidic acid | M  |
| -7          | 3.246               | Syringin glucuronidated conjugate | M C | -45         | 15.403              | Geniposidic acid sulfated conjugate | M   |
| -8          | 3.313               | Syringaresinol-O-β-D-glucopyranoside sulfated conjugate | M | -46         | 15.853              | Methylated cistanoside E | M C |
| -9          | 3.646               | Acetylated cistanoside E | M C | -47         | 15.903              | Methoxylated geniposidic acid | M C |
| -10         | 4.437               | Kankanose | P | -48         | 17.227              | Methoxylated salidrose | M C |
| -11         | 4.787               | Methoxylated arenarioside | M C | -49         | 17.721              | Cistanoside F glucuronidated conjugate | M   |
| -12         | 4.868               | Acetylated decaffeoyl acteoside | M | -50         | 18.321              | Dehydroxylated geniposidic acid | M   |
| -13         | 5.220               | Bartsioside sulfated conjugate | M C | -51         | 19.821              | Methylated bartsioside | M   |
| -14         | 5.865               | Acetylated syringaresinol-O-β-D-glucopyranoside | M C | -52         | 20.443              | Salidroside glucuronidated conjugate | M   |
| -15         | 6.004               | Methylated kankanoside I | M | -53         | 20.872              | Methoxylated decaffeoyl acteoside | M C |
| -16         | 6.788               | Demethylated kankanoside | M | -54         | 21.321              | Methoxylated salidroside | M   |

Table II. Effects of serum TGs collected at different time points on proliferation of MCF-7 cells (n = 10).

| Treatment time | Group | A (PR%) | Treatment time | Group | A (PR%) |
|----------------|-------|---------|----------------|-------|---------|
| 0.25 h         | BC    | 0.255±0.043 | 100.00 | 4 h    | BC    | 0.258±0.034 | 100.00 |
|                | PC    | 0.357±0.060** | 140.00 | PC     | 0.437±0.037** | 169.38 |
|                | TGs   | 0.356±0.035** | 139.61 | TGs    | 0.365±0.071** | 141.47 |
| 0.5 h          | BC    | 0.246±0.051 | 100.00 | 6 h    | BC    | 0.273±0.035 | 139.10 |
|                | PC    | 0.411±0.024** | 167.07 | PC     | 0.346±0.053** | 128.31 |
|                | TGs   | 0.381±0.056** | 154.88 | TGs    | 0.338±0.039** | 123.81 |
| 1 h            | BC    | 0.296±0.054 | 100.00 | 8 h    | BC    | 0.266±0.040 | 120.42 |
|                | PC    | 0.377±0.023** | 127.36 | PC     | 0.431±0.088** | 162.03 |
|                | TGs   | 0.367±0.065** | 123.99 | TGs    | 0.321±0.040** | 120.68 |
| 1.5 h          | BC    | 0.294±0.049 | 100.00 | 10 h   | BC    | 0.215±0.007 | 120.68 |
|                | PC    | 0.403±0.051** | 137.07 | PC     | 0.373±0.034** | 173.49 |
|                | TGs   | 0.377±0.055** | 128.23 | TGs    | 0.333±0.029** | 154.88 |
| 2 h            | BC    | 0.289±0.008 | 100.00 | 12 h   | BC    | 0.279±0.032 | 150.90 |
|                | PC    | 0.402±0.016** | 139.10 | PC     | 0.421±0.064** | 150.90 |
|                | TGs   | 0.348±0.045** | 120.42 | TGs    | 0.332±0.027* | 119.00 |

* p <0.05 and ** p <0.01 in comparison with the blank control group; BC, blank control group; PC, positive control group; TGs, total glycosides of *C. deserticola* administration group; A, absorbance; PR, proliferation rate.
significant correlation (Peaks 1, 4, 6, 7, 9, 11, 14, 29, 32, 37, 38, 39, 46, 48, and 53), and five peaks had a significant correlation (Peaks 2, 3, 13, 41, and 47).

![Fig. 2. Bivariate analysis of estrogen activity and common peaks in serum TGs collected at different time points. (A) positive ion mode; (B) negative ion mode. *significant correlation ($p < 0.05$) when absolute value of correlation coefficient was $>0.3$; ** extremely significant correlation ($p < 0.01$) when absolute value of correlation coefficient was $>0.5$.](image)

Grey relational analysis was performed, and grey relational degrees between the relative area of each common peak in serum fingerprint of TGs and the estrogen-like activity were calculated. The higher the value, the stronger the relation, with the value above 0.5 indicating a certain level of relation between the sub-sequence and the parent sequence. The results showed that (Fig. 3), 15 peaks in positive ion mode and 20 peaks in negative ion mode were highly correlated with cell proliferation rate.

Overall, 32 common components were identified (Supplementary Fig. S1), including methylated cistantubulose B; acetylated tubuloside B; acteoside; acetylated dehydrodiconiferyl alcohol-4-O-β-D-glucoside; dehydrodiconiferyl alcohol-4-O-β-D-glucoside; demethylated kankanoside E; methoxylated tubuloside B; kankanoside E; acetylated mussaenosidic acid; dehydrodiconiferyl alcohol-4-O-β-D-glucoside glucuronidated conjugate; methoxylated muaesenoid acid; methoxylated coniferin; cistanoside G glucuronidated conjugate; 8-epiloganic acid; syringin sulfated conjugate; 3,4-dihydroxyphenylethanoid glycoside; syringin glucuronidated conjugate; acetylated cistanoside E; methoxylated arenarioside; demethylated (2E,6Z)-8-O-β-D-glucopyranosylxylo-2,6-dimethyl-2,6-octadienoic acid; bartioside sulfated conjugate; acetylated syringaresinol-O-β-D-glucopyranoside; acetylated cistanoside G; methylated tubuloside B; acetylated eucommin A; acetylated 2-acetylacteose; hydroxylated ononin; methoxylated 2-acetylacteose; methylated cistanoside E; methoxylated geniposide; methoxylated salidroside; methoxylated decaffeoyl acteoside. These were inferred to be the active compounds of TGs causing estrogen-like effect in vivo.

There have been previous studies on the chemical constituents in C. deserticola (Cui et al., 2016; Li et al., 2015), but they all used C. deserticola or some monomer components in C. deserticola to analyze the chemical components dissolved in blood. At present, few studies were done on the chemical components of TGs of C. deserticola. A relevant study found that C. deserticola medicinal serum could significantly promote the proliferation of breast cancer cells (Song et al., 2019), which is basically consistent with the results of this study. However, no research on correlation analysis between them. The correlation analysis between serum fingerprints and pharmacodynamic experiments is one of the effective methods to reveal the direct acting substances of drugs in vivo. It has been widely used in the material basis research of other traditional Chinese medicine (Dou et al., 2016a; Sun et al., 2021).

**CONCLUSION**

In this experiment, UPLC-Q-TOF-MS was used to characterize the components of TGs of C. deserticola in rat serum samples after intragastric administration, and a total of 75 chemical components were identified. Bivariate correlation analysis and grey correlation analysis were used, 32 direct acting substances that are highly related to estrogen activity in vivo were found in serum in positive and negative ion mode. This study provides a reliable basis for the revelation of TGs estrogen effective substances and their subsequent in-depth development and research.

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Ethical compliance

All experimental procedures with animals were approved by the Animal Ethical Committee of Harbin University of Commerce (Approval No.: HSDYXY-2018001), following all guidelines, regulations, legal, and ethical standards as required for animals.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20210615060610

Statement of conflicts of interest

The authors have declared no conflict of interest.

REFERENCES

Ai, Z.P., Zhang, Y., Li, X.Y., Sun, W.L. and Liu, Y.H., 2021. Widely targeted metabolomics analysis to reveal transformation mechanism of Cistanche desertica active compounds during steaming and drying processes. *Front. Nutr.*, 8: 742511. https://doi.org/10.3389/fnut.2021.742511

Bittner V., 2009. Menopause, age and cardiovascular risk: A complex relationship. *J. Am. Coll. Cardiol.*, 54: 2374-2375. https://doi.org/10.1016/j.jacc.2009.10.008

Cui, Q.L., Pan, Y.N., Bai, X.W., Zhang, W., Chen, L.X. and Liu, X.Q., 2016. Systematic characterization of the metabolites of echinacoside and acteoside from Cistanche tubulosa in rat plasma, bile, urine and feces based on UPLC-ESI-Q-TOF-MS. *Biomed. Chromatogr.*, 30: 1406-1415. https://doi.org/10.1002/bmc.3698

Decensi, A., Bonanni, B., Maisonneuve, P., Serrano, D., Omodei, U., Varriacchio, C., Cazzaniga, M., Lazzeroni, M., Rotmensz, N., Santillo, B., Sideri, M., Cassano, E., Belloni, C., Muraca, M., Segnan, N., Masullo, P., Costa A., Monti, N., Vella, A., Bisanti, L., Aiuto, G.D. and Veronesi, U., 2013. A phase-III prevention trial of low-dose tamoxifen in postmenopausal hormone replacement therapy users: The HOT study. *Annls. Oncol.*, 24: 2753-2760. https://doi.org/10.1093/annonc/mdt244

Ding, J.X., Li, W.L., Hu, Y., Song, H., Sun, X.M. and Ji, Y.B., 2019. Characterization of estrogen active ingredients in *Cuscuta chinensis* Lam. based on spectral characteristics and high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. *Mol. Med. Rep.*, 19: 1238-1247. https://doi.org/10.3892/mmr.2018.9755

Dou, Z.H., Luo, L., Hou, J.Y., Wang, C.P., Meng, P., Gu, W., and Liu, Q.Q., 2016a. Hepatoprotective ingredients of Yinchenhao decoction based on spectrum-effect relationship of serum containing drugs. *Chin. J. Hosp. Pharm.*, 36: 1968-1972.

Dou, Z.H., Xu, B., Liu, Q.Q., Gu, W., Meng, P., and Wang, Z.Y., 2016b. Fingerprint of serum containing drug of Yinchenhao decoction. *Chin. Pharm. J.*, 51: 358-364.

Dueñas-Garcia, O.F., Sullivan, G., Hall, C.D., Flynn, M.K. and O’Dell, K., 2016. Pharmacological agents to decrease new episodes of recurrent lower urinary tract infections in postmenopausal women: A systematic review. *Female Pelvic Med. Res.*, 22: 63-69. https://doi.org/10.1097/SPV.0000000000000244

Fu, Z.F., Han, L.F., Zhang, P., Mao, H.P., Zhang, H., Wang, Y.F., Gao, X.M. and Liu, E.W., 2020. Cistanche polysaccharides enhance echinacoside absorption in vivo and affect the gut microbiota. *Int. J. Biol. Macromol.*, 149: 732-740. https://doi.org/10.1016/j.ijbiomac.2020.01.216

Hu, Y., Ding, J.X., Sun, X.M., Song, H., Xu, B.L., Qi, Z., Wen, J., Liang, W., Li, W.L. and Ding, Z.D., 2021. Chemical compositions and fragmentation pattern of estrogen effective fraction of Cistanche desertica. *Chin. Pharm. J.*, 56: 1149-1159.

Jiang, Y., and Tu, P.F., 2009. Analysis of chemical constituents in Cistanche species. *J. Chromatogr. A*, 1216: 1970-1979. https://doi.org/10.1016/j.chroma.2008.07.031

Jiang, Y., Li, S.P., Wang, Y.T., Chen, X.J. and Tu, P.F., 2009. Differentiation of herb *Cistanches* by fingerprint with high-performance liquid chromatography-diode array detection-mass spectrometry. *J. Chromatogr. A*, 1216: 2156-2162. https://doi.org/10.1016/j.chroma.2008.04.040

Li, W.L., Chen, Q. Yang, B., and Zhang, J.J., 2013. Screening of phytoestrogen effective extracts and dose of Cistanche desertica. *Chin. Herb. Med.*, 5: 292-296. https://doi.org/10.1016/S1674-6384(13)60043-X

Li, W.L., Ding, J.X., Liu, B.M., Zhang, D.L., Song, H., Sun, X.M., Liu, G.Y., Wang, J.Y. and Ji, Y.B., 2019. Phytochemical screening and estrogenic activity of total glycosides of Cistanche desertica. *Open Chem.*, 17: 279-287. https://doi.org/10.1515/chem-2019-0035

Li, W.L., Sun, X.M., Song, H., Ding, J.X., Bai, J. and Chen, Q., 2015. HPLC/Q-TOF-MS-based identification of absorbed constituents and their metabolites in rat serum and urine after oral administration of Cistanche desertica
extract. *J. Fd. Sci.*, **80**: 2079-2087. https://doi.org/10.1111/1750-3841.12975

Li, Y.Q., Chen, Y., Fang, J.Y., Jiang, S.Q., Li, P. and Li, F., 2020. Integrated network pharmacology and zebrafish model to investigate dual-effects components of *Cistanche tubulosa* for treating both osteoporosis and alzheimer’s disease. *J. Ethnopharmacol.*, **254**: 112764. https://doi.org/10.1016/j.jep.2020.112764

Ma, K., and Chen, Y.X., 2015. Discussion on strategy of treatment of perimenopausal syndrome with Chinese and Western Medicine. *China J. Chin. Mater. Med.*, **40**: 3899-3906.

Mareti, E., Ampatzi, C., Paschou, S.A., Voziki E. and Goulis, D.G., 2019. Non-hormonal replacement therapy regimens: Do they have an effect on cardiovascular risk? *Curr. Vasc. Pharmacol.*, **17**: 573-578. https://doi.org/10.2174/1570161116666180911104942

Piwowarczyk, R., Ochmian, I., Lachowicz, S., Kapusta, I., Sotek, Z. and Błaszak, M., 2020. Phytochemical parasite-host relations and interactions: A *Cistanche armena* case study. *Sci. Total Environ.*, **716**: 137071. https://doi.org/10.1016/j.scitotenv.2020.137071

Rubinow, D.R., Johnson, S.L., Schmidt, P.J., Girdler, S. and Gaynes, B., 2015. Efficacy of estradiol in perimenopausal depression: so much promise and so few answers. *Depress. Anxiety*, **32**: 539-549. https://doi.org/10.1002/da.22391

Sobenin, I.A., Myasoedova, V.A., and Orekhov, A.N., 2016. Phytoestrogen-rich dietary supplements in anti-atherosclerotic therapy in postmenopausal women. *Curr. Pharm. Design*, **22**: 152-163. https://doi.org/10.2174/1570161116666151112150520

Song, H., Li, W.L., Liu, B.M., Sun, X.M., Ding, J.X., Chen, N., Ji, Y.B., and Xiang, Z., 2017. Study of the estrogenic-like mechanism of glycosides of cistanche using metabolomics. *RSC Adv.*, **7**: 39403-39410. https://doi.org/10.1039/C7RA06930H

Song, H., Li, W.L., Sun, X.M., Hu, Y., Ding, J.X., Ji, Y.B., and Wang, J.Y., 2019. Estrogenic activity of glycosides from *Cistanche deserticola* as an estrogen receptors adjuvant in vitro. *Pharmacogn. Mag.*, **15**: 693-697. https://doi.org/10.4103/pm.pm_402_18

Sun, X.M., Song, H., Zhao, L.Z., Hu, Y., Xin, K.Y., Li, W.L., and Ding, Z.D., 2021. Direct acting substances discovery of estrogen effect of *Cuscuta chinensis* in vivo. *Acta Pharm. Sin.*, **56**: 1826-1831.

Tham, D.M., Gardner, C.D., and Haskell, W.L., 1998. Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. *Aust. J. clin. Endocr. Metab.*, **83**: 2223-2235. https://doi.org/10.1210/jcem.83.7.4752

Tu, P.F., Shi, H.M., Song, Z.H., Jiang, Y., and Zhao, Y.Y., 2007. Chemical constituents of *Cistanche sinensis*. *J. Asian Nat. Prod. Res.*, **9**: 79-84. https://doi.org/10.1080/10286020500384450

Wang, T., Zhang, X.Y., and Xie, W.Y., 2012. *Cistanche deserticola* Y. C. Ma, Desert Ginseng: A review. *Am. J. Chin. Med.*, **40**: 1123-1141. https://doi.org/10.1142/S0192415X12500838

Wu, X.F., Xie, W., Huang, X.L., Wu, H.Q., Huo, Y.P. and Zhou, X., 2021. Rapid analysis compositions of processed *Citrus medica* L. var. *sarcodactylis* Swingle by UPLC-Q-TOF MS. *J. Chin. Mass Spectrom. Soc.*, **42**: 207-217.

Zetterberg, M., 2016. Age-related eye disease and gender. *Maturitas*, **83**: 19-26. https://doi.org/10.1016/j.maturitas.2015.10.005

Zhao, D., Guallar, E., Ouyang, P., Subramanya, V., Vaidya, D., Ndumele, C.E., Lima, J.A., Allison, M.A., Shah, S.J., Berti, A.G., Budoff, M.J., Post, W.S., and Michos, E.D., 2018. Endogenous sex hormones and incident cardiovascular disease in post-menopausal women. *J. Am. Coll. Cardiol.*, **71**: 2555-2566. https://doi.org/10.1016/j.jacc.2018.01.083
Identification of Absorbed Constituents and their Metabolites Related to Estrogen-Like Activity of Total Glycosides of Cistanche deserticola in Rat Serum

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Supplementary Table S1. List of main materials and instruments used in this study.

| Materials and instruments                        | Manufacturers or Sources                                      |
|--------------------------------------------------|---------------------------------------------------------------|
| Diethylstilbestrol (purity: ≥98%)                | Hanpu Pharmaceutical Co., Ltd., Guangzhou, China              |
| Mass spectrometry (MS)-grade formic acid, acetoniitrile, methanol | Fisher Scientific, Fair Lawn, NJ, USA                        |
| Phenol red-free RPMI 1640                        | HyClone, Logan, UT, USA                                       |
| MTT, DMSO                                        | Sigma-Aldrich, St. Louis, MO, USA                             |
| Agilent 1290 HPLC system, Agilent 6530 series quadrupole time-of-flight LC/MS system | Agilent Technologies, Inc., Santa Clara, CA, USA             |
| ACQUITY UPLC BEH C₁₈ column (100 mm × 2.1 mm, 1.7 μm) | Waters Crop., Jakarta Selatan, Indonesia                      |
| Genius N118LA nitrogen generator                | Peak Scientific, Glasgow, UK                                  |
| Microplate reader                               | Bio-Rad, Inc., Hercules, CA, USA                              |
| The electronic analytical balance AR1140       | Ohaus Corporation, Parsippany, NJ, USA                        |
| XW-80A vortex mixer                             | Yugong Machinery Technology Co., Ltd., Shanghai, China       |
| Raw material of dry Cistanche deserticola       | purchased from Sankeshu Medicinal materials market, Harbin, China |
| Wistar rats (weighing 210 ± 20 g)               | from Harbin Medical University, Harbin, China                |
| MCF-7 cell line                                 | from Research Center of Pharmaceutical Engineering and Technology, Harbin University of Commerce, Harbin, China |

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Supplementary Fig. S1. Total ion chromatogram in the positive ion mode. A: blank serum; B: TGs-containing serum.

Supplementary Fig. S2. Total ion chromatogram in the negative ion mode. A: blank serum; B: TGs-containing serum.
Absorbed Constituents of TGs of *C. deserticola* and their Estrogen-Like Activity

- methylated cistantubuloside B
- acetylated tubuloside B
- acteoside
- acetylated dehydrodiconiferyl alcohol-4-O-β-D-glucoside
- dehydrodiconiferyl alcohol-4-O-β-D-glucoside
- demethylated kankanoside E
- methoxylated tubuloside B
- kankanoside E
- acetylated mussaenosidic acid
- dehydrodiconiferyl alcohol-4-O-β-D-glucoside glucuronidated conjugate
- methylated mussaenosidic acid
- methoxylated coniferin
Absorbed Constituents of TGs of *C. deserticola* and their Estrogen-Like Activity

Supplementary Fig. S3. *In vivo* active compounds leading to the estrogen-like activity of TGs.