Research Article

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Phytochemical screening and estrogenic activity of total glycosides of *Cistanche deserticola*

<https://doi.org/10.1515/chem-2019-0035>

received April 24, 2018; accepted November 30, 2018.

Abstract: Over the decades, there have been continuous efforts to enhance the quality of human life. Postmenopausal syndrome is a serious concern for the well-being of a woman's health. Hormonal therapy is currently the mainstay of treatment for this condition. However, this therapy could lead to estrogen abuse, leading to adverse reactions and side effects. As a result, hormonal therapy has been unsuccessful in ameliorating postmenopausal syndrome. *Cistanche deserticola* is a classical tonic herb in traditional Chinese medicine. It exhibits significant estrogenic activity. The main active compounds of this herb are glycosides. In a previous experiment, three important factors contributing to the total glycoside yield, acteoside yield, and estrogenic activity were identified, namely, eluent concentration, pH, and eluent volume. In this experiment, an optimal purification process was determined using a central composite design-response surface methodology to obtain glycosides from this herb. An eluent (ethanol) concentration of 85% and volume of 25 BV at a pH of 11 was found to be optimal. Twenty-one active compounds were identified by a high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry assay. This study provides valuable insights for further in-depth research evaluating the estrogenic activities of total glycosides of *Cistanche deserticola*.

Keywords: Central composite design; *Cistanche deserticola* total glycosides; LC/Q-TOF-MS; purification technology; uterine growth test.

1 Introduction

Cistanche deserticola is an edible, classical tonic herb. It was first mentioned in Shen Nong's herbal classic and enlisted in the top grade. It is a warm herb and sweet in taste. It has numerous medicinal properties, such as nourishment of the liver and kidney, strengthening of muscles and bones, and improvement of immune regulation along with anti-aging and anti-tumor activities [1-4]. Some natural compounds have been isolated and identified from the extracts of this herb, the major ones being phenylethanoid glycosides, lignanoids, iridoids, polysaccharides, and alkaloids [5-8].

The drugs obtained from medicinal plants contain various active compounds, which are primarily responsible for their therapeutic actions. The efficacy of the same drug obtained from different plant sources can vary because of the differences in the type and quantity of active compounds present in it. Thus, it is important to identify and quantify all the active compounds present in the drugs obtained from medicinal plants. The same applies to *C. deserticola*. Response surface methodology is an experimental method to investigate the interaction between different factors simultaneously [9-10]. It can be used for optimization of extraction parameters for phytopharmaceuticals and quantitative estimation of active compounds in drugs. Central composite design (CCD) is one of the experimental designs useful in response surface methodology. Compared to orthogonal and uniform designs, CCD has higher precision and better predictability [11].

Postmenopausal syndrome can substantially reduce the quality of life in women. Normally, estrogen is used to treat this condition. However, long-term use of estrogen may lead to abuse, thereby causing various adverse

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reactions and side effects. Therefore, it is imperative to choose an alternative therapy, preferably an herbal drug containing estrogen as an active ingredient for the treatment of postmenopausal syndrome [12-13].

In a preliminary experiment, the structures of various natural compounds obtained from *C. deserticola* were identified using mass spectrometry (MS) [14]. It was confirmed that glycosides are the main active compounds having significant estrogenic activity [14-15]. To develop a safe and effective estrogenic active ingredient in a new drug, an in-depth investigation into TGCD after purification is needed. In this study, CCD was first used to optimize the purification of total glycosides of *C. deserticola* (TGCD). Subsequently, uterus growth test was used to evaluate the estrogenic activities of the same glycoside. High-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (HPLC/Q-TOF-MS) was used for qualitative analysis of the compounds of TGCD after purification. This process was applied to explicitly demonstrate the presence of various active compounds with estrogenic activity in TGCD. This can simultaneously provide the basis for its clinical use in postmenopausal syndrome replacing estrogen.

2 Experimental Procedure

2.1 Instruments

Agilent 1290 HPLC system (Agilent Technologies, Palo Alto, CA, USA), Agilent 6530 series quadrupole time-of-flight LC/MS (Q-TOF) system (Agilent Technologies, Palo Alto, CA, USA), and Chemical HPLC-3D workstations were used as chromatographic instruments for data processing. Milli-Q ultrapure water was used for the entire study. AR1140 electronic analytical balance (Ohaus International Ltd.); 680 microplate reader (Bio-Rad Corporation); and 64R high-speed centrifuge (Beckman Coulter Allegra) were used for sample preparation.

2.2 Drugs and chemicals

C. deserticola was purchased from the drug market and identified by Prof. Zhang Delian (Harbin University of Commerce, China). Standard diethylstilbestrol (99% pure, lot no. 60518) was purchased from Dr. Ehrenstorfer (Germany). Other standards acteoside (111530-200505) and echinacoside (111670-200503) were obtained from the National Institute for the Control of Pharmaceutical

and Biological Products, Beijing, China. The purity of each standard was > 98%. Acetonitrile (ACN), methanol, and formic acid (MS grade) were purchased from Thermo Scientific Pierce (Rockford, IL, USA). Ultrapure water was obtained from Hangzhou Wahaha Group Co., Ltd. (Hangzhou, China). All the commercially available reagents were of analytical grade.

2.3 Preparation of total glycosides of *C. deserticola* purification solution

After immersing in 75% ethanol for 12 h, the crude powder of *C. deserticola* (100 g) was extracted with 800 mL of 75% (v/v) ethanol at 80 °C for 150 min under reflux. It was then filtered through a double-deck filter and subsequently extracted with 800 mL of 75% ethanol twice for an additional 150 min. Subsequently, the filtrates were combined and concentrated in vacuum at 45 °C. The extract was obtained by removing the solvent. A certain amount of distilled water was added in the extract to obtain a concentration of 0.5 g/mL, which was used to screen purification process.

For adsorption using AB-8 macroporous resin, the pH of the test sample solution was adjusted to 11. First, 2 BV distilled water was used to wash away the impurities. Then, the eluent at a concentration of 25 BV 85% ethanol was eluted and collected. Finally, the collected purified eluent was merged. A certain amount of distilled water was added in the extract to obtain a concentration of 1.5 g/mL, which was used for intragastric administration. For positive control, diethylstilbestrol solution (20 µg/mL) was prepared with diethylstilbestrol powder.

According to the ratio of purification (0.6), a certain amount of the extract (equivalent to 1 g of *C. deserticola*) was transferred to a 10 mL volumetric flask, dissolved in 50% (v/v) methanol solution in an ultrasonic bath for 5 min, and diluted to 10 mL. The medicinal solution was obtained after filtration of the supernatant through a 0.45 µm filter membrane. Acteoside and echinacoside (1 mg each) were mixed and dissolved completely in a 10 mL 50% (v/v) methanol solution. Finally, the standard solution was filtered through a 0.45 µm Millipore filter prior to analysis.

2.4 LC-MS conditions

Chromatography separation was carried out in an HPLC system (Agilent 1290), equipped with a quaternary solvent delivery system, vacuum degasifier, and photodiode array

detector. MS/MS analysis was performed in an instrument Agilent-1290 HPLC/6530 Q-TOF-MS system, equipped with an electrospray ionization source in both positive and negative ion modes. A Waters Symmetry shield RP C18 column (4.6 × 250 mm, 5 µm) (Waters Corporation, Milford, MA, USA) was used for separation. The mobile phase comprised of 0.2% formic acid aqueous solution (v/v) (A) and ACN (B), and it was pumped at a flow rate of 0.5 mL/min. The injection volume of each sample was 10 µL. The gradient elution program was as follows: 5–23% B for 0–35 min, 23–25% B for 35–65 min, and 25–5% B for 65–70 min. The column temperature was maintained at 30 °C. The chromatograms were monitored and recorded at 330 nm. The atomization gas pressure was set at 30 Psi, and the capillary voltage was 3.5 kV. The flow rate of dry gas was 8 L/min at a temperature of 30 °C. The temperature of sheath gas was set at 400 °C at a flow rate of 12 L/min. The collision energy was set at 10–20 eV for low-energy scans, and 30–50 eV for high-energy scans. The mass spectra data were recorded within the scan range of 50–1000 Da in positive and negative ion scanning modes. In this study, a fast and efficient comparison between TGCD and the standards was carried out under the same LC-MS condition.

2.5 Uterus growth test

This was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental procedures were reviewed and approved by the Animal Ethical Committee of Harbin University of Commerce, China.

Immature female Kunming mice (about 21 d of birth, weaned) weighing 12 ± 2 g, were purchased from Changchun National Biological Industry Base Laboratory Animal Center (Changchun, China). The mice were housed in a temperature-regulated room ($22 \pm 2^\circ\text{C}$) with food and water *ad libitum*. Animal experimentation was initiated after five days of acclimatization. The mice were fasted overnight with water *ad libitum* before intragastric administration of the test solution.

The mice were randomly divided into 22 groups, with 10 animals in each group. They were administered experimental drugs of the same volume for twice a day (morning and evening) for four days as follows:

Group I: Intragastric total glycosides of *C. deserticola* purification solution (20 mL/kg), (solution volume/mouse weight),

Group II: Intragastric distilled water (negative control group), and

Group III: Intragastric diethylstilbestrol (20 µg/mL) (positive control group).

On the fifth day, all the mice were sacrificed. The uteruses were immediately removed and weighed and the uterus coefficients were calculated.

2.6 Statistical analysis

A two-tailed paired-sample *t*-test was used to identify statistically significant differences in the various parameters in the different experimental groups. The analysis was performed using SPSS statistical software (SPSS for Windows v21.0, SPSS Inc., USA). The differences were considered statistically significant at 95% confidence level ($p < 0.05$).

3 Results and discussion

3.1 Linearity and correlation of acteoside and total glycosides yields

The linear regression equation of the acteoside yield was $y = 12831x - 14.75$ (where x is the concentration of acteoside, and y is its corresponding peak area) with a correlation coefficient of $r = 1$ in the concentration range of 0.12–0.72 mg/mL. This indicated a linear calibration curve. The linear regression equation of the total glycosides yield was $y = 26.074x + 0.0866$ (where x is the concentration of total glycosides, and y is its corresponding peak area) with a correlation coefficient of $r = 0.9982$ in the concentration range of 0.013–0.065 mg/mL. This also indicated a linear calibration curve.

3.2 Methodological investigation

The precision, reproducibility, stability, and recovery of the samples were investigated in the methodological investigation. In the precision experiment, the relative standard deviation (RSD) of acteoside and total glycosides was 1.43% and 0.05% respectively. In the reproducibility experiment, the RSD of acteoside and total glycosides was 0.10% and 1.44% respectively. In the 24 h stability experiment, the RSD of acteoside and total glycosides was 0.14%, and 0.90% respectively. In the recovery experiment, the recovery of acteoside was 100.50% with an RSD of

2.08%, while the recovery of total glycosides was 99.12% with an RSD of 1.65%. All the RSD values were less than 3%. These results demonstrated a good precision and reproducibility. In addition, the sample was stable for 24 h. The results of recovery are also within the permissible range (95–105%). Therefore, this method can be used for the determination of acteoside and total glycosides yield after purification.

3.3 Single factor investigation of TGCD

The purification of TGCD using macroporous resin can be affected by many factors, such as resin type, static adsorption factors (adsorption time, leakage concentration, and pH of the sample solution), and elution conditions (flow velocity, volume, and concentration). Using adsorption capacity and rates of desorption and elution of TGCD as indices, the experimental condition was determined based on the results of single factor experiments. Using AB-8 type macroporous adsorption resin, the following optimum conditions were determined: 0.5 mg/mL sample solution, pH of 10, static adsorption time of 8 h, 2 BV distilled water for washing impurities, 20 BV 80% ethanol as eluent, and a flow velocity of 0.5 BV/min. The specific results are shown in Figure 1–7.

3.4 CCD for optimization of TGCD purification technology

Based on the results of single factor investigation, three factors significantly influencing the purification method were selected as indices, namely, pH of the sample solution (x1), eluent concentration (x2), and eluent volume (x3). According to the principle of CCD, each factor has five levels. The maximum and minimum levels of these various factors were set according to the results of the preliminary experiment. The factor levels are shown in Table 1 and the experimental results are shown in Table 2.

The total glycosides and acteoside yields were determined to optimize the purification method for TGCD. Firstly, the total glycosides and acteoside yields were set to the numeric criteria of desirability (d) between 0–1. Then, the overall desirability (OD) $[OD = (d_1, d_2, d_3, \dots, d_n)^{1/n}]$, where n is the index number] was calculated. SPSS21.0 software and design-expert software were used for multiple linear regression and binomial fitting of independent variables and OD, with $p < 0.05$ was considered a statistically significant standard of the equation. The equation with a larger r value (multiple

Figure 1: Graph showing the desorption rates of different resins.

Figure 2: Adsorption capacity of resin AB-8 resin at different times.

Figure 3: Total glycoside content at different concentrations.

Figure 4: Adsorption capacity at different pH values.

Figure 5: Desorption rate at different flow velocities.

Figure 6: Desorption rate at different eluent concentrations.

Figure 7: Desorption rate at different eluent volumes.

correlation coefficient) was selected as the best fit model. The multivariate linear equation is represented as $y = -1.02 - 0.131x_1 + 0.034x_2 + 0.012x_3$ ($r = 0.55$, $p = 0.004$). The binomial equation is $y = -21.92173 - 0.74079x_1 + 0.62914x_2 + 0.041161x_3 + 0.014972x_1x_2 + 2.06050 \times 10^{-4}x_1x_3 + 1.05698 \times 10^{-3}x_2x_3 - 0.029589x_{12} - 4.78730 \times 10^{-3}x_{22} - 2.89446 \times 10^{-3}x_{32}$ ($r = 0.91$, $p = 0.012$). It can be seen from the above equations that the correlation coefficient of the multivariate linear regression equation is lower. The correlation between the independent and dependent variables is very low, and it was considered unfavorable to use in the linear model.

Table 1: Factors and levels of central composite design.

Level	pH value of sample solution (X1)	Eluent concentration (%) (X2)	Eluent volume (BV) (X3)
-1.682	8.32	71.59	11.59
-1	9	75	15
0	10	80	20
+1	11	85	25
+1.682	11.68	88.41	28.41

Table 2: Experimental results of central composite design.

No.	X1	X2	X3	Yield of total glycosides (%)	Yield of acteoside (%)	OD
1	9	75	15	55.9774	1.5662	0.4205
2	11	75	15	38.3164	1.1686	0.0374
3	9	85	15	65.0978	2.3646	0.8485
4	11	85	15	58.1103	1.6770	0.4951
5	9	75	25	63.5201	1.8943	0.6488
6	11	75	25	31.8751	1.1625	0
7	9	85	25	77.1229	2.1875	0.9127
8	11	85	25	73.0339	2.0953	0.8332
9	8.32	80	20	77.9599	2.0025	0.8367
10	11.68	80	20	65.0900	1.8591	0.6462
11	10	71.59	20	52.8052	1.5593	0.3854
12	10	88.41	20	61.5654	1.8154	0.5879
13	10	80	11.59	55.5667	1.8843	0.5532
14	10	80	28.41	71.4362	1.8292	0.6878
15	10	80	20	71.9576	1.9596	0.7578
16	10	80	20	70.9029	2.0543	0.7931
17	10	80	20	71.9363	2.0327	0.7969
18	10	80	20	71.0521	2.0038	0.7714
19	10	80	20	70.7325	1.9873	0.7613
20	10	80	20	70.9028	2.0125	0.7769

But, the correlation coefficient of the binomial equation was high and it resulted in a good fit. Hence, the binomial model was selected.

Based on a comprehensive analysis of the surface figure and contour map combined with the experimental data (OD value near 0.6), the optimum range of purification

Figure 8: Response surface figure and contour map of sample solution pH value (A) and eluent concentration (B). The range of A is from 9 to 11, and the range of B is from 75 to 80%.

Figure 9: Response surface figure and contour map of sample solution pH value (A) and eluent volume (C). The range of A is from 9 to 11, and the range of C is from 15 to 25BV.

method was obtained. From Figure 8, it can be seen that the maximum OD value was generated when the pH of the sample solution (A) was in the range of 9–10, and the eluent concentration (B) was in the range of 79–85%. Figure 9 shows that the maximum OD value was obtained when the pH value of sample solution (A) was in the range of 9–10, and the eluent volume (C) was in the range of 20–25 BV. Figure 10 shows that the maximum OD value was obtained when the eluent concentration (B) was in the range of 80–85%, and the eluent volume (C) was in the range of 20–25 BV. From a comprehensive analysis of these data, the pH of the sample solution, eluent concentration, and eluent volume was determined to be in the range of 9–10, 80–85%, and 20–25 BV respectively. Based on the multivariate binomial equation for variable derivative results and optimal scheme, the best TGCD purification method was found to be at an eluent (ethanol) concentration of 85% and volume of 25 BV at a pH of 11. The corresponding OD value was 0.8332, and the total

glycosides yield was 73.0339%. The visual impression from the figures 8–10 identifies the best method to be the one in which interactions between the two factors were considered, although the best method deduced from the formula recognizes the one in which interactions among the three factors were included. The two results differed and the optimal purification method was considered to be with an eluent (ethanol) concentration of 85% and volume of 25 BV at a pH of 11.

3.5 Uterus growth measurement

The uterus coefficient of each group is shown in Table 3. Compared to the negative control group, the results of the other groups were significantly different. It was found that TGCD obtained from 20 different purification methods all exerted estrogenic actions.

Figure 10: Response surface figure and contour map of eluent concentration (B) and eluent volume (C). The range of B is from 75 to 80%, and the range of C is from 15 to 25 BV.

Table 3: Effects of total glycosides of *Cistanche deserticola* from different purification technologies on the uterine indices of immature mice.

Groups	Uterine indices %
Negative control group	0.061±0.004
Positive control group	0.788±0.304**
1	0.184±0.067**
2	0.160±0.027**
3	0.188±0.037**
4	0.200±0.037**
5	0.127±0.033**
6	0.176±0.037**
7	0.188±0.026**
8	0.288±0.071**
9	0.181±0.076**
10	0.215±0.068**
11	0.214±0.025**
12	0.203±0.038**
13	0.190±0.067**
14	0.165±0.073*
15-20	0.212±0.069**

Compared with the negative control group *p < 0.05, **p < 0.01

3.6 Confirmatory experiment

The comprehensive results of CCD and uterus growth test showed that the optimal purification method was considered to be with an eluent (ethanol) concentration of 85% and volume of 25 BV at a pH of 11. During the

validation process, the average yield of total glycosides was 70.9150%. The average deviation between the predicted and actual values was 2.1180%. Therefore, it can be suggested that the predictability and experimental credibility of this model are good.

3.7 Identification of TGCD after purification

Based on retention time and MS data, 21 natural constituents were speculated, including campneoside 1, 2'-acetylacteoside, cistanoside A, cistanoside B, syringalide A 3'-α- L-rhamnopyranoside, tubuloside A, tubuloside B, salidroside, cistanoside G, geniposidic acid, decaffeoylacteoside, 8-epiloganic acid, echinacoside, cistanoside F, cistantubuloside B1, isoacteoside, acteoside, cis-acteoside, kankanoside E, osmanthuside B, and cistanoside C. The retention time, MS and MS/MS information, formula, and speculated compounds are shown in Table 4.

Q-TOF-MS is particularly suitable for the structural identification of complex molecular components of drugs and food because it can provide possible elemental compositions through exact molecular mass and the structural characteristics of the fragment ions. To establish a systematic structural characterization, Q-TOF-MS, MS data, database seeking, and published reference literature were also used for identification. The molecular formula of each target component was deduced from the parent ion, and it was matched with the known compounds. This formula could be further determined from its related fragment ions. For example, peak 5 showed a predominant deprotonated ion at *m/z* 654 (C₃₀H₃₈O₁₆), which was identical to the elemental composition of campneoside 1. The loss of caffeoyl was formed from a fragment ion

Table 4: Tentative assignments of LC peaks in positive and negative ion modes.

No.	tR (min) ^a	mode	[M+/-H] ⁺ (m/z) ^b	MS ² (m/z) ^c	Formula	Compound presumed
1	6.273	-	299.7058	119.8404	C ₁₄ H ₂₀ O ₇	salidroside
2	10.382	-	445.1028	299.3017; 268.0447	C ₂₀ H ₃₀ O ₁₁	cistanoside G
3	12.352	-	373.0961	211.0607; 123.0448	C ₁₆ H ₂₂ O ₁₀	geniposidic acid
4	18.026	-	461.1810	315.1085	C ₂₀ H ₃₀ O ₁₂	decaffeoylacteoside
5	18.224	+	655.2194	493.3568; 347.1077	C ₃₀ H ₃₈ O ₁₆	campneoside 1
6	18.312	-	375.1105	213.0783	C ₁₆ H ₂₄ O ₁₀	8-epiloganic acid
7	24.690	+	667.2417	625.1981; 505.2354; 463.1662	C ₃₁ H ₃₈ O ₁₆	2'-acetylacteoside
8	29.202	+	801.2636	639.2139; 493.3527; 475.0325	C ₃₆ H ₄₈ O ₂₀	cistanoside A
9	34.001	+	815.2818	653.1939; 180.0305	C ₃₇ H ₅₀ O ₂₀	cistanoside B
10	36.274	+	609.3098	447.0720	C ₂₉ H ₃₆ O ₁₄	syringalide A3'-α-L-rhamnopyranoside
11	38.046	-	785.1080	623.2037; 477.0374; 461.5242	C ₃₅ H ₄₆ O ₂₀	echinacoside
12	38.669	-	487.2797	341.1091; 179.0340	C ₂₁ H ₂₈ O ₁₃	cistanoside F
13	46.717	-	769.2373	623.2046; 487.1453	C ₃₅ H ₄₆ O ₁₉	cistantubuloside B1
14	47.947	-	623.2085	461.1675; 315.1013	C ₂₉ H ₃₆ O ₁₅	isoacteoside
15	48.048	-	623.2084	461.1615; 315.0203	C ₂₉ H ₃₆ O ₁₅	acteoside
16	48.144	+	829.3175	683.2035	C ₃₇ H ₄₈ O ₂₁	tubuloside A
17	48.300	-	623.1655	461.1632; 315.1315	C ₂₉ H ₃₆ O ₁₅	cis-acteoside
18	49.024	-	347.1525	179.0551; 169.1224	C ₁₆ H ₂₈ O ₈	kankanoside E
19	62.410	-	591.1724	445.0762; 145.0290	C ₂₉ H ₃₆ O ₁₃	osmanthuside B
20	64.431	-	637.1748	491.2733	C ₃₀ H ₃₈ O ₁₅	cistanoside C
21	66.868	+	667.4635	625.1659; 505.1346; 463.1226	C ₃₁ H ₃₈ O ₁₆	tubuloside B

^a retention time^b quasi-molecular ion^c MS fragment ions

at m/z 493, and the loss of rha moiety was formed from a fragment ion at m/z 347.

4 Conclusion

Using an LC-Q-TOF-MS technology, a simple and robust qualitative analysis method for TGCD has been developed and fully validated. The validation data for screening and identification of natural compounds from TGCD were satisfactory. Twenty-one bioactive compounds from TGCD were speculated as follows: salidroside, cistanoside G, geniposidic acid, decaffeoylacteoside, campneoside 1, 8-epiloganic acid, 2'-acetylacteoside, cistanoside A, cistanoside B, syringalide A3'-α-L-rhamnopyranoside, echinacoside, cistanoside F, cistantubuloside B1,

isoacteoside, acteoside, tubuloside A, cis-acteoside, kankanoside E, osmanthuside B, cistanoside C, and tubuloside B. The structural characterization of these compounds can provide an experimental foundation for their quality control and further clinical application because of their estrogenic activity. This can offer a novel and improved therapeutic option for treatment of postmenopausal syndrome, thereby avoiding the side effects and adverse reactions of estrogen therapy.

Abbreviations

TGCD *Cistanche deserticola* total glycosides
 LC/Q-TOF-MS liquid chromatography/quadrupole time-of-flight mass spectrometry

RSM response surface methodology
 CCD central composite design
 MS mass spectrometry
 HPLC/Q-TOF-MS High-performance liquid chromatography/
 quadrupole time-of-flight mass spectrometry
 ACN Acetonitrile
 BV bed volume
 OD overall desirability
 LC-MS liquid chromatography- mass spectrometry
 Q-TOF-MS quadrupole time-of-flight mass spectrometry
 TCM traditional Chinese medicines
 RSD relative standard deviation

Acknowledgements: This project was supported by National Natural Science Foundation of China (No. 81073015), Nature Scientific Foundation of Heilongjiang Province (ZD2017014), Young innovative talent training plan of College in Heilongjiang Province (UNPYSCT-2017209). The authors declare that there is no conflict of interest regarding the publication of this paper.

Conflict of interest: Authors state no conflict of interest.

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