Screening of Phytoestrogenic Effective Extracts and Dose of *Cistanche deserticola*

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Abstract: **Objective** To screen the phytoestrogenic effective extracts and dose of *Cistanche deserticola* including estrogenic and anti-estrogenic activities. **Methods** The effect of phytoestrogen was determined through uterus growth test in low and high estrogen female model mice. Then MTT assay of the estrogen-dependent breast cancer cells MCF-7 was conducted with the medicated serum of mice. **Results** After ig administration with 95% ethanol extract of *C. deserticola* [EECD, 30 g/(kg·d)], the uterus coefficient of low estrogen model mice increased. The medicated serum of 30 g/(kg·d) EECD significantly promoted the proliferation of MCF-7 cells. 40 g/(kg·d) EECD + diethylstilbestrol significantly inhibited the growth of the uterus in high estrogen model mice and the proliferation of MCF-7 cells as well. **Conclusion** With the dose of 30 g/(kg·d), EECD could exert quasi estrogen effect, and with the dose of 40 g/(kg·d), EECD could exert the estrogen antagonistic action. The method established is accurate and reliable, which could be used for the follow-up studies on the phytoestrogen material basis of *C. deserticola*.

Key words: *Cistanche deserticola*; effective extracts and dose; MTT; phytoestrogens; uterus growth test
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Introduction

Phytoestrogens are natural compounds in some plants, which could bind to estrogen receptors *in vivo* and demonstrate estrogenic and anti-estrogenic activities (Usui, 2006). Phytoestrogens act as estrogen when the concentration is low in the body, while act as anti-estrogen when the concentration is higher than a certain threshold (Zhu *et al*, 2012). Thus, they are also known as "selective estrogen receptor modulator" which could regulate estrogen-related diseases such as menopausal syndrome (Tu *et al*, 2011). However, if the effective parts or dose is misemployed, there may be counterproductive effects. Therefore, it is necessary to screen the effective extract parts and dose of the Chinese materia medica with phytoestrogenic effect. *Cistanche deserticola* Y. C. Ma (Orobanchaceae) (Pharmacopoeia Committee of P. R. China, 2010) was initially recorded in *Shennong's Herbal Classic* and was clinically used in male impotence, female infertility, insufficiency of essence and blood, weakness of spine and knee caused by kidney-*Yang* deficiency. As a classic tonic, *C. deserticola* and its compound preparation are widely used for the treatment of estrogen disorders (Gao, 2011).

In this study, we extracted *C. deserticola* with water and 95% ethanol, respectively. Low estrogen model (sexually immature mice) and high estrogen model female mice (induced by diethylstilbestrol) were ig administered with different extract parts and dosages, respectively. Classical uterus growth test (He *et al*, 2012) was performed to investigate the uterine coefficients, and after that, MTT assay (Zhao *et al*, 2007) was used to observe the influences on estrogen- dependent breast cancer cells MCF-7 by medicated serum. The active extract parts and dosage of phytoestrogens from *C. deserticola* were obtained by the comprehensive analyses of *in vitro* and *in vivo* results. This result could provide not only a rational use of *C. deserticola* in the

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treatment of menopausal related diseases, but also a basis on the research of phytoestrogens substance of the medicine.

Apparatus and materials

Apparatus

AR1140 Electronic Analytical Balance (Ohaus International Ltd., USA); 680 Microplate Reader (Bio-Rad Corporation, USA); IX70 Inverted Microscope (Olympus Corporation Olympus, Japan); CO_2 Incubator (NBS Corporation, USA); Clean Bench (Beijing East Hal Instrument Manufacturing Co., Ltd., China); Standard PB-10 pH Meter (Sartorius Company, Germany); TL-2000MM-III Micro Oscillator (Jiangyan Tianli of Medical Devices, Co., Ltd., China).

Medicinal materials

Cistanche deserticola Y. C. Ma was purchased from Natural Herbs Medicine Distribution Market, and identified by Prof. ZHANG De-lian (Harbin University of Commerce, China).

Reagents

Diethylstilbestrol standard (99%, Lot 60518) was purchased from Dr. Ehrenstorfer (Germany). RPMI 1640 and RPMI1640 without phenol red were purchased from HyClone Company (USA). Fetal bovine serum (FBS) without mycoplasma and chlamydia was purchased from Hangzhou Evergreen Biological Engineering Company (Hangzhou, China). Trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich Co. LLC (St Louis, USA). Human breast cancer MCF-7 cell line was provided by the Research Center on Life Sciences and Environmental Sciences, Harbin University of Commerce, China.

Animals

Sexually 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 [SCXK-(Kyrgyzstan) 2003-0004].

Methods

Preparation of *C. deserticola* solution

C. deserticola crude drug (600 g) was crushed, divided into two parts equally, then marked I and II. I and II were extracted by hot efflux extraction with distilled water and 95% ethanol, respectively. The amount of solvent and the extracting time were 1:10 (material-solution ratio) and 150 min, 1:6 and 75 min, respectively. Subsequently, the extracting solution was blended in the rotary evaporator at about 50 ℃. After completing the evaporation of the solvent, the blend was scraped and dried, then kept in the refrigerator at −20 ℃ for further use. The extracts were dissolved in distilled water before administered to mice. The diethylstilbestrol standard was dissolved in distilled water to suspension of 20 μg/mL.

Animal grouping and administration

According to the principle of weight balance and randomization, the mice were divided into three groups estrogen-like (A), anti-estrogen (B), and control (C) groups. Each group continued to be divided into sub-groups on the following basis.

Group A: Kunming mice (100) were divided into 10 groups (10 in each group). The mice in five groups were given water extract of *C. deserticola* and the mice in other five groups were given 95% ethanol extract of *C. deserticola*, and the doses were 5, 10, 20, 30, and 40 g/(kg·d) (crude drug given), respectively.

Group B: Kunming mice (100) were divided into 10 groups (10 in each group). The mice in five groups were given water extract of *C. deserticola* with diethylstilbestrol and the mice in other five groups were given 95% ethanol extract of *C. deserticola* with diethylstilbestrol, and the doses were 5, 10, 20, 30, and 40 g/(kg·d) (crude drug given) + 0.35 mg/(kg·d), respectively.

Group C: The mice were divided into two groups (10 in each group), blank control and positive control groups, respectively. The mice in positive control group were treated with 0.35 mg/(kg·d) diethylstilbestrol and the mice in blank control group were given an equal volume of distilled water.

Uterus growth test in mice

The mice in groups A, B, and C were ig administered twice daily for consecutive 4 d. After administration in the evening of the day 4, the mice were fasted for 12 h. On the next morning, blood was collected from the mice eye socket, then stored at 4 ℃ overnight. Whole blood was centrifuged at 3000 r/min for 10 min. Then the serum was separated carefully,

inactivated complement by water bath (56 ℃, 30 min), and filtered through 0.22 μm membrane. The treated serum was stored in the refrigerator at −20 ℃. After drawing blood, the mice uterus were removed and weighed immediately to get the uterine wet weight. The mouse uterus coefficient was calculated (i.e. uterine wet weight / body weight × 100%).

MTT assay

Cell culture MCF-7 cells (estrogen-dependent cells) were cultured in RPMI1640 supplemented with 10% FBS in a CO_2 incubator (37 ℃, 5% CO_2). The culture medium was replaced each 3 d. Four days before the beginning of the test, the cells were washed with PBS for three times, and the culture medium was changed to RPMI1640 without phenol red (containing 5% CDT-FBS) for running out of the intracellular estrogen residues (Wang *et al*, 2013).

MTT assay by medicated serum After treated with RPMI1640 without phenol red (containing 5% CDT-FBS), the MCF-7 cells at logarithmic growth phase were used for the following experiments. The cells were washed for three times with 3 mL PBS, dispersed with 0.25% trypsin, then added with (−)phenol red RPMI1640. Cells were seeded in 96-well culture plate at a density of 2×10^3 cells/well uniformly. The total volume of culture medium per well was 100 μL. After 24 h observation, the medium was changed to RPMI 1640 with 10% medicated serum for the following culture. The serum of mice in each group was mixed into six wells equally. At 72 h, 20 μL MTT (5 mg/mL in PBS) was added to each well for 4 h incubation. After the culture medium was removed, DMSO (150 μL) was added for coloration. The plates were shaken with protecting from light to dissolve the formazan crystals completely. Setting zero by DMSO, the absorbance (*A*) of each well was measured at 570 nm by ELISA detector. The average value of *A* and proliferation rate (PR) were calculated as the formula: PR = average value of *A* in the experimental group / average value of *A* in blank control group.

Statistical analysis

All data were expressed as $\bar{x} \pm s$, and the statistical analysis was performed with software SPSS15.0. The data of multiple groups were compared by One-way ANOVA. $P < 0.05$ was taken as significance, and $P < 0.01$ was taken as higher significance. The phyto-estrogen active extract parts and the optimal dose of *C. deserticola* could be drawn.

Results

Effect of different extracts and doses on mouse uterus

In group A, 95% ethanol extract of *C. deserticola* [40 g/(kg·d)] could increase the uterine coefficient of immature mice ($P < 0.05$). Moreover, the effect of 30 g/(kg·d) was more significant ($P < 0.01$), compared with diethylstilbestrol, which promotes a lesser extent. In group B, with diethylstilbestrol as control, 95% ethanol extract of *C. deserticola* [40 g/(kg·d)] + diethylstilbestrol significantly inhibited the growth of uterus, compared with diethylstilbestrol group, with statistical significance ($P < 0.01$). The results were shown in Table 1.

Table 1 Uterine coefficients of *C. deserticola* in mice

Groups	Extraction solvent	Dosages / ($g \cdot kg^{-1} \cdot d^{-1}$)	Uterine coefficients / %
A	water	5	0.071 ± 0.003
		10	0.069 ± 0.002
		20	0.072 ± 0.011
		30	0.067 ± 0.003
		40	0.069 ± 0.016
	95% ethanol	5	0.072 ± 0.007
		10	0.068 ± 0.002
		20	0.079 ± 0.005
		30	0.143 ± 0.015**
		40	0.116 ± 0.018*
B	water	5	0.867 ± 0.008
		10	0.869 ± 0.004
		20	0.873 ± 0.008
		30	0.864 ± 0.006
		40	0.851 ± 0.087
	95% ethanol	5	0.871 ± 0.001
		10	0.860 ± 0.003
		20	0.853 ± 0.007
		30	0.851 ± 0.007
		40	0.823 ± 0.005**
C	blank control	—	0.064 ± 0.008
	diethylstilbestrol	0.35×10^{-3}	0.864 ± 0.036

*$P < 0.05$ **$P < 0.01$ *vs* group C; same as below

Cell proliferation by MTT assay

In group A, 95% ethanol extracts of *C. deserticola* [30 g/(kg·d)] significantly promoted the proliferation of MCF-7 cells ($P < 0.01$), compared with diethyl-stilbestrol, which promoted a lesser extent. In group B, with diethylstilbestrol as control, diethylstilbestrol

group could significantly promote the proliferation of MCF-7 cells. However, each medicated serum of the two extracts could inhibit the cell proliferation significantly ($P < 0.01$). The results were shown in Table 2.

Table 2 Proliferative rate of *C. deserticola* in MCF-7 cells

Groups	Extraction solvent	Dosages / ($g \cdot kg^{-1} \cdot d^{-1}$)	*A*	PR / %
A	water	5	0.683 ± 0.012	98.84
		10	0.692 ± 0.005	100.14
		20	0.694 ± 0.016	100.43
		30	0.684 ± 0.002	98.99
		40	0.689 ± 0.015	99.71
	95% ethanol	5	0.699 ± 0.004	101.15
		10	0.683 ± 0.021	98.84
		20	0.690 ± 0.005	99.86
		30	0.785 ± 0.016**	113.60**
		40	0.698 ± 0.004	101.01
B	water	5	0.838 ± 0.023	121.27**
		10	0.810 ± 0.019	117.22**
		20	0.676 ± 0.022	97.83**
		30	0.693 ± 0.017	100.29**
		40	0.676 ± 0.033	97.83**
	95% ethanol	5	0.808 ± 0.013	116.93**
		10	0.798 ± 0.015	115.48**
		20	0.640 ± 0.020	92.62**
		30	0.659 ± 0.016	95.37**
		40	0.642 ± 0.008	92.91**
C	blank control		0.691 ± 0.006	100
	diethylstilbestrol		0.889 ± 0.017	136.77

The whole animal experiments and MTT assay were integrated, and the result indicated that 95% ethanol extracts of *C. deserticola* were effective, dose of 30 g/(kg·d) exerted estrogenic effect, 40 g/(kg·d) exerted anti-estrogenic action.

Discussion

The phytoestrogenic effect of *C. deserticola* has been reported. *C. deserticola* could increase the estrogen level in serum when used alone (Zhao, 2007). However, there is no in-depth research on the method of its application. Based on the uterine growth test, "a gold standard" for the detection of estrogen, and in combination with MTT assay *in vitro*, 95% ethanol extracts of *C. deserticola* were investigated. The dose of 30 g/(kg·d) could exert quasi estrogen activity and 40 g/(kg·d) exert estrogen antagonist effect. In the uterus growth test, after given diethylstilbestrol, the uterus affinity for water in mice was increased (Smith *et al*, 2002). In the test, the uterus had high water content when the mice were dissected. Meanwhile, as the volume of uterus became larger, the uterine wall became thinner and more fragile. Nevertheless, in group A (the estrogen-like group) when the mice were administered with *C. deserticola* alone, the uterus weight increased but still had a good elasticity. This phenomenon suggested that the phytoestrogen of *C. deserticola* could reduce the risks of diseases such as fibroids and endometrium abnormality caused by synthetic hormone (Chen *et al*, 2011). In the high estrogen model group, the mice were co-administered with *C. deserticola* and diethylstilbestrol, the water content of uterus tended to decrease obviously, compared with the positive control, which suggested that the herb also had the effect of unopposed estrogens. MCF-7 cells in the following MTT assay are estrogen-dependent, sensitive to the changes of the amount of estrogen. MTT assay is a classical method to reflect the serum hormone levels indirectly and it will provide theory reference for the clinical medication on breast cancer.

This study combines the advantages of *in vivo* and *in vitro* experiments fully, investigates the phyto-estrogenic activity of *C. deserticola* comprehensively. The results may provide a basis for the following research on the phytoestrogenic activity of *C. deserticola*. It could offer the possibility of *C. deserticola* to be a substitute source of synthetic estrogen drug, thereby it could reduce the risks of synthetic hormones.

References

Chen HG, Zhu L, Cui QC, Lang JH, Li B, 2011. Preparation rat animal model of uterine fibroids with estrogen. *Acta Acad Med Sin* 33(4): 408-411.

Gao ZY, 2011. Application of Chinese medicine Jia Rong and estrogen in the treatment of climacteric syndrome of department of gynaecology. *J Clin Med Pract* 15(24): 117-118.

He QF, Wu HQ, You LL, Jin XM, 2012. Research of dose-effect on β-cypermethrin promoting MCF-7 proliferation and uterine weight of immature female mice. *J Toxicol* 26(3): 175-177.

Pharmacopoeia Committee of P. R. China, 2010. *Pharmacopoeia of the People's Republic of China*, Vol. I. China Medical Science and Technology Press: Beijing.

Smith P, Rhodes NP, Ke Y, Foster CS, 2002. Upregulation of estrogen and androgen receptors modulate expression of FGF-2 and FGF-7 in human, cultured, prostatic stromal cells exposed to high concentrations of estradiol. *Prostate Cancer Prostatic Dis* 5(2):

105-110.

Tu PF, Jiang Y, Guo YH, Li XB, Tian YZ, 2011. Review on the research progress and industry development of *Cistanche deserticola*. *Chin Pharm J* 46(12): 882-887.

Usui T, 2006. Pharmaceutical prospects of phytoestrogens. *Endocr J* 53(1): 7-20.

Wang YZ, Gao YT, Hu J, Gao HS, Chen H, 2013. Effect of resveratrol on proliferation of different estrogen receptor subtypes of MCF-7 cells. *J Chin People's Armed Police Forces* 24(3): 229-231.

Zhao PW, 2007. *Research on the Phytoestrogenic Effects and Their Mechanisms of Ten Kinds of Chinese Medicine*. Beijing University of Chinese Medicine: Beijing.

Zhao PW, Wang DW, Niu JZ, Wang JF, Wang LQ, 2007. Evaluation on phytoestrogen effects of ten kinds of Chinese medicine including *Flos Carthami*. *China J Chin Mater Med* 32(5): 436-439.

Zhu DN, Wang L, Wang ST, Zhang WS, 2012. Advances in studies on phytoestrogens. *Chin Tradit Herb Drugs* 43(7): 1422-1428.

Introduction of Cover Picture

Bupleurum scorzonerifolium Willd., Plants 30—60 cm, perennial. Taproot stout, dark reddish-brown, branched. Stems 1—3, flexuose, greatly dichotomously branched, base clothed with fibrous remnant sheaths. Basal leaves linear, 6—16 × 0.2—0.7 cm, thick-papery, rigid, nerves 3—5, prominent abaxially, margin white cartilaginous, base slightly narrowed and clasping. Upper leaves small. Umbels numerous, 1.2—4 cm across; bracts 1—3, subulate, 0.5—4 × 0.2—0.6 mm, unequal, deciduous; rays (3–)4—6(–8), 1—2 cm, very slender, spreading; bracteoles 5, lanceolate, 2.5—4 × 0.5—1 mm, equaling or slightly exceeding umbellules; umbellules 2—5 mm across, (6–)9—11(–15)-flowered; pedicels 0.2—1 mm. Petals yellow. Stylopodium low-conic, dark yellow. Fruit ellipsoid, dark brown, 2.5—3 × 1.5—2 mm; ribs pale, prominent; vittae 5—6 in each furrow, 4—6 on commissure.

This *Bupleurum* is one of two primary species the roots of which are used for the major traditional Chinese medicine "*Chaihu*" (*B. chinense*).

Picture provided by ZHOU You
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