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Research on phytoestrogenic effective fraction of cuscuta chinensis

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ABSTRACT

Effective fraction of Cuscuta chinensis were studied using a combination of in vitro and in vivo assays. Estrogen-like activity was determined by uterus growth test in low and high estrogen female model mice. Then MTT assay of the MCF-7 cells was conducted with the medicated serum of the mice. Estrogen-antagonistic effect was also determined by uterus rate of mice. 95% ethanol extracts of Cuscuta chinensis dose of 24 g • kg^{-1} • d^{-1} after intragastric administration increased the uterus rate of immature mice; The medicated serum of 24 g • kg^{-1} • d^{-1} significantly promoted the proliferation of MCF-7 cells. 24 g • kg^{-1} • d^{-1} 95% ethanol extracts + diethylstilbestrol significantly inhibited the growth of the uterus of high estrogen model mice. 95% ethanol extracts were effective fraction of Cuscuta chinensis. The method established is accurate, reliable, which can be used for the further studies on the phytoestrogen material basis of Cuscuta chinensis.

KEYWORDS

Cuscuta chinensis;
Phytoestrogens;
Effective extracts and dose;
Uterus growth test;
MTT.

INTRODUCTION

Cuscuta chinensis is only contained in the "Shen Nong's Herbal Classic", as a top grade. It is the dried ripe seed of Cuscuta australis R.Br. or Cuscuta chinensis Lam, which is being recorded in Pharmacopoeia of People's Republic of China (2010 edition). It proves that the Cuscuta chinensis has multiple benefits to human health. Active Components of Cuscuta chinensis have affinity with estrogen receptor, and have dual-directional regulation on estrogen level[4,5]. But it is still difficult to gain international recognition for lack knowledge of Active Components of Cuscuta chinensis. Therefore, it's necessary to screen the effective extract parts and dose of the Cuscuta chinensis with phytoestrogen effect.

In the study, Cuscuta chinensis were extracted with water and 95% ethanol respectively. Low estrogen model (sexually immature mice) and high estrogen model female mice (induced by Diethylstilbestrol) were intragastric administrated with different extract parts and dosage, respectively. Classical uterus growth test[2,3,7] was performed to investigate the uterine coefficient, after that, MTT assay[1,6] was used to observe the influences on estrogen-dependent breast cancer cells MCF-7 by medicated serum. Phytoestrogens active extract parts of *C.deserticola* and dosage were obtained from the comprehensive

analyses of *in vitro* and *in vivo* results. This result can provide not only a rational use of Cuscuta chinensis in the treatment of menopausal related diseasesÿbut also a basis on the research of phytoestrogens substance of the medicine.

MATERIALS AND METHODS

Apparatus

Centrifuge TDL80-2B (Feige., China); Electronic Analytical Balance AR1140 (Ohaus International Ltd., USA); 680 Microplate Reader (Bio-Rad Corporation, USA); Inverted Microscope IX70 (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 the Medical Devices Co., Ltd., China).

Medicinal materials

Cuscuta chinensis was purchased from Anguo of Hebei, and identified by Prof. Zhang Delian (Harbin University of Commerce).

Reagents

Diethylstilbestrol was purchased from HEFEI JIULIAN PHARMACEUTICAL CO.,LTD, Lot 20100920. RPMI1640 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-diphenyltetrazoliumbromide (MTT), 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 Harbin University of Commerce, Research Center on Life Sciences and Environmental Sciences.

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].

Preparation of sample solution

Cuscuta chinensis (1kg) was crushed, divided into two parts equally, marked I, II. I, II were reflux extraction with 8 times amount of distilled water and 95% ethanol, respectively, and extraction for 3 times, 3h per time. combine the filtrate, concetrated it into extraction. The extracts were dissolved in distilled water before administered to mice.

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 assigned into three groups, A: estrogen-like groups; B: anti-estrogen groups; C: control groups. Each group continued to sub-group on the following basis:

Group A:

A total of 80 Kuming mice were divided into 8 groups (10 in every group). 4 groups were given Cuscuta chinensis water extracts and the other 4 groups given 95% ethanol extracts, and the dose were 3,6,12,24 g • kg^{-1} • d^{-1} (crude drug given), respectively;

Group B

80 Kuming mice were divided into 8 groups (10 in every group). 4 groups were given water extracts and diethylstilbestrol and 4 groups were given 95% ethanol extracts and diethylstilbestrol, and the dose were 3,6,12,24g • kg^{-1} • d^{-1} (crude drug given)+ 0.35 mg • kg^{-1} • d^{-1}, respectively;

Group C

The mice were divided into 2 groups (10 in every group), as blank control group and positive control group, respectively.

Blank control group

The mice were given an equal volume of distilled water.

Positive control group

The mice were treated with diethylstilbestrol (0.35 mg • kg^{-1} • d^{-1}.)

Uterus growth test in mice

The mice in group A, group B, group C were orally

administered twice a day for consecutive 4 d, morning and evening. After administration on the evening of the fourth day, the mice were fasted for 12 h. On the next morning, blood was collected from the mice eye socket then stored at 4 °C overnight. Whole blood was centrifuged at 2000 r • min^{-1} for 10 min. Then the serum was separated carefully, inactivated complement by water bath (56°C 30 min), filtered from 0.22 μm membrane. The treated serum was stored in the refrigerator at -20 °C; After drawing blood, the mice uterus were removed and weighed immediately to get the uterine wet weight. Calculate the mouse uterus coefficient (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 (at 37 °C, 5%CO_2). The culture medium was replaced each three days. 4 d before starting the test, the cells were washed with PBS 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.

MTT assay by medicated serum

After MCF-7 cells treated with (-)phenol red RPMI1640 (containing 5% CDT-FBS), the cells at logarithmic growth phase were used for following experiments. The cells were washed three times with 3 ml PBS each, dispersed with trypsin (0.25%), then added (-)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 when the cells were observed adherent, the medium was changed to 1640 with 10% medicated serum (estrogen-like groups)for the following culture. Each group of the test mice serum was mixed into six wells equally. After 72 h, 20 μL MTT (5 mg/mL in PBS) was added to each well for another 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 completely dissolve the formazan crystals. Setting zero by DMSO, absorbance (A) of each well was measured at 570 nm by ELISA detector. Calculate the average value of A and proliferation rate (PR). PR = (the average value of A in the experimental group/ the average value of A in blank control group) x100%.

Statistical analysis

All data were expressed as x ± s, and performed with software SPSS17.0.t test was used statistically.

RESULTS AND DISCUSSION

Effect of different extracts and dose on mouse uterus

In group A, 95% ethanol extracts of Cuscuta chinensis, dose of 24 g • kg^{-1} • d^{-1} can increase uterine coefficient of immature mouse ($P < 0.05$). In group B, 95% ethanol extracts of Cuscuta chinensis, 24 g • kg^{-1} • d^{-1} + diethylstilbestrol group significantly inhibited the growth of the uterus ($P < 0.05$). The results are shown in TABLE 1:

TABLE 1: Uterine coefficient of cuscuta chinensis in mice

	Dosage (g·kg^{-1}·d^{-1})	Uterus Coefficient (%)
Group A	W3	0.077±0.013
	W6	0.076±0.017
	W12	0.053±0.006
	W24	0.056±0.010
	E3	0.060±0.007
	E6	0.055±0.007
	E12	0.063±0.015
	E24	0.145±0.025*
Group C	Blank Control	0.044±0.008
	Postive Control (diethylstilbestrol)	0.732±0.146
Group B	W3+diethylstilbestrol	0.776±0.191
	W6 +diethylstilbestrol	0.717±0.104
	W12 +diethylstilbestrol	0.779±0.145
	W24 +diethylstilbestrol	0.941±0.269
	E3 + diethylstilbestrol	0.874±0.134
	E6 +diethylstilbestrol	0.760±0.299
	E12 +diethylstilbestrol	0.738±0.153
	E24 +diethylstilbestrol	0.699±0.051*
Group C	Blank Control	0.115±0.018
	Postive Control (diethylstilbestrol)	0.870±0.091

W: water extraction; E: 95% ethanol extraction.

MTT cell proliferation by medicated serum

In group A, 95% ethanol extracts of Cuscuta chinensis, dose of 24 g • kg^{-1} • d^{-1} significantly promoted the proliferation of MCF-7 cells ($P < 0.05$), however, compared with diethylstilbestrol, which promoted a lesser extent. The results are shown in TABLE 2:

TABLE 2 : Proliferation rate of *C.deserticola* in MCF-7 cells

	Dosage (g·kg^{-1}·d^{-1})	A	PR (%)
Group A	W3	0.701±0.04	107.79%
	W6	0.745±0.04	114.61%
	W12	0.625±0.08	96.13%
	W24	0.745±0.04	103.38%
	E3	0.669±0.05	102.95%
	E6	0.630±0.04	96.95%
	E12	0.677±0.053	104.18%
	E24	0.789±0.076*	121.28%*
Group C	Blank Control	0.691±0.006	100%
	Postive Control (diethylstilbestrol)	0.889±0.06**	136.77%**

Integrated the whole animal experiments and MTT assay, the result was that 95% ethanol extracts of Cuscuta chinensis, dose of 24 g • kg^{-1} • d^{-1} exerted estrogenic effect.

CONCLUSION

In the test, we observed that 95% ethanol extracts of Cuscuta chinensis, dose of 24 g • kg^{-1} • d^{-1} displayed estrogen-like activity in group A,meanwhile, displayed estrogen-antagonistic effect in group B. The results showed that: 95% ethanol extracts of Cuscuta chinensis has two-way regulation. Mechanism of dual-directional regulation need further studying.

This study fully combined the advantages of *in vivo* and *in vitro* experiments, investigated phytoestrogen activity of Cuscuta chinensis comprehensively. The result may provide a basis for the following research on the pharmacodynamics basis on the phytoestrogen activity of Cuscuta chinensis. It can offer the possibility of Cuscuta chinensis to be a substitute source of synthetic estrogen drug, thereby it can reduce the risks of synthetic hormones.

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