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ORIGINAL ARTICLE

Estrogenic Activity of Glycosides from *Cistanche deserticola* as an Estrogen Receptors Adjuvant *in vitro*

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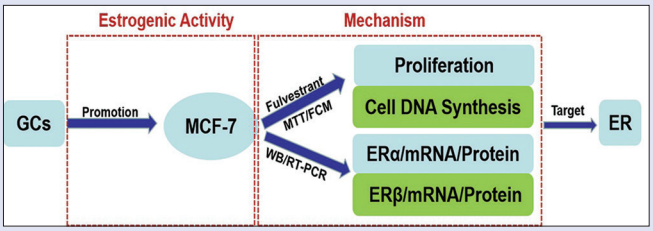
ABSTRACT

Background: *Cistanche deserticola*, a traditional Chinese herb medicine, has been widely used for thousands of years with the activities of hormone regulation, immunomodulatory, antioxidative, neuroprotective, anti-inflammatory, and estrogen. Glycosides of *Cistanches* (GCs) were the main bioactivity components of the herb. **Objective:** The objective of the study is to study estrogenic activity and the mechanism about estrogen receptors (ERs) of GCs. **Materials and Methods:** Cell proliferation was measured using the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide assay for MCF-7 cells. The cell cycle was detected using flow cytometry, and proliferation index was calculated. The mRNA and protein expressions of ERα and ERβ were detected by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis as the reported method with minor modifications. **Results:** GCs group at the concentrations of 1.75, 17.5, and 175 μg/mg could enhance proliferation of the MCF-7 cell lines with a time and dosage-dependent manner. Combined medication group (fulvestrant with estradiol [E_2] or GCs) could lead to the incline of proliferation rate compared with the individual medication group ($P < 0.01$). Flow cytometry analysis indicated that GCs could advance MCF-7 cell lines from G0/G1 phase cells to S and G2/M phase, which could promote cell DNA synthesis. The mechanism of GCs on MCF-7 was similar to that of E_2. RT-PCR and western blot analysis indicated that after treatment with GCs for 48 h, contents of ERα and ERβ mRNA and proteins in MCF-7 increased as a dosage-dependent manner with that of GCs. GCs can play a role of estrogenic activity according upregulated mRNA and proteins of ERα and ERβ. **Conclusion:** This study indicated the estrogenic activity of GCs, and also, ER is the target of GCs. GCs can play a role of estrogenic activity according upregulated mRNA and proteins of ERα and ERβ.

Key words: *Cistanche deserticol,* estrogen receptors, estrogenic activity, glycosides of *Cistanche*, MCF-7

SUMMARY

- GCs (Glycosides of *Cistanches*) were the main bioactivity components of traditional Chinese medicine *Cistanche deserticola.*
- GCs could enhance the proliferation and affect the cell cycle transformation of the MCF-7 cell lines.
- GCs can play a role of estrogenic activity according upregulated mRNA and proteins of ERα and ERβ.



Abbreviations used: GCs: Glycosides of *Cistanches*; ERs: Estrogen receptors; E_2: Estradiol; MTT: 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide; ERα: Estrogen receptors α; ERβ: Estrogen receptors β; RT-PCR: Reverse transcription polymerase chain reaction; PBS: Phosphate buffer saline; PR: Proliferation rate; PI: Proliferation index; FBS: Fetal bovine serum; CDT: Charcoal dextran treated; ATCC: American type culture collection; DMSO: Dimethyl sulfoxide; CMG: Combined medication group.

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INTRODUCTION

Cistanche deserticola ("Rou Cong Rong" in Chinese), a traditional Chinese herb medicine which first recorded in *ShenNongBenCaoJing*, has been used for the treatments of kidney deficiency, impotence, senile constipation, and blood deficiency for thousands of years.[1] It is mainly distributed in desert region of Northwestern China, such as Gansu and Xinjiang.[2] Modern pharmacology research demonstrated that *C. deserticola* can advance broad medicinal functions, such as hormone regulation, immunomodulatory, antioxidative, neuroprotective, anti-inflammatory, and estrogenic activity.[3,4] Glycosides of *Cistanches* (GCs) were considered as one of main active components with various biological effects.[5,6]

Estrogen receptors-α (ERs-α) and ERβ are the subtypes of the ERs which could regulate the physiological functions.[7] These proteins could regulate transcription of target genes by binding to associated DNA regulatory sequences in the cell nucleus. Both of the subtypes are markedly expressed in the cardiovascular and central nervous systems.[8,9] ERα is existed mainly in mammary gland, uterus, ovary, bone, male reproductive organ, and prostate. ERβ is present mainly in the prostate, ovary, and bladder.[10,11] Moreover, there are some common physiological roles for the two subtypes, such as in the development and function of the ovaries and in the protection of the cardiovascular system.[12,13] Previous research in our group revealed the estrogenic-like mechanism of GCs by the method of metabolomic analysis.[14] In a continuous study, we analyzed the mechanism of estrogenic activity of GCs on MCF-7 cell lines related with ERs.

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MATERIALS AND METHODS

Instruments

ECO-170P-230 incubator was from New Brunswick Scientific (China) Co., Ltd., Bio-Rad 680 microplate reader and electrophoresis apparatus were from Bio-Rad Laboratories, Inc., CX21 microscope was from Olympus Co., Ltd., GIS-2019 gel imaging system was from Tanon Science and Technology Co., Ltd., flat bottom plate was from Corning Inc., EPICS-XL flow cytometry was from Beckman-Coulter Inc., and StepOnePlus Real-Time polymerase chain reaction (PCR) system was from Thermo Fisher Scientific.

Chemicals and reagents

GCs were prepared in our laboratory as the method previous reported,[14] and the purity of GCs was determined to be 52% (acteoside was used as a marker to determine the GCs) by ultraviolet spectrophotometry. Estradiol (E_2) was purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS) and RPMI-1640 were from Gibco Co., Ltd., 96-well flat bottom plate was from Corning Inc., and dimethyl sulfoxide (DMSO) and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Corporation. TRIzol reagent, Reverse Transcription (RT) Kit, and TB Green™ RT-PCR Kit were purchased from TaKaRa Co., Ltd., Dalian, China. Anti-ERα Rabbit pAb, anti-ERβ Rabbit pAb, and β-actin antibody were purchased from Wanlei Biotechnology Co., Ltd. All the other common chemicals were purchased from standard commercial suppliers.

Sample preparation

Preparation of glycosides of Cistanches stock solution

GCs extract was dissolved in DMSO to make a stock solution with the concentration of 0.1051 g/ml and stored at 4°C. Phenol red-free RPMI-1640 was used to dilute the stock solution to various concentrations before use.

Preparation of estradiol stock solution

E_2 extract was dissolved in DMSO to make a stock solution with the concentration of 0.27 mg/ml and stored at 4°C. Phenol red-free RPMI-1640 was used to dilute the stock solution to various concentrations before use.

Preparation of charcoal dextran treated-fetal bovine serum

Hundred milliliters of FBS was mixed with 250 mg of activated charcoal powder and 25 mg of dextran. After incubating for 45 min at 55°C, the mixture was centrifuged at 4°C, 1000 rpm for 15 min. The supernatant was filtered using Millipore Express PES membrane to get the charcoal dextran-treated (CDT)-FBS and stored at −20°C.

Cell culture

The human breast cancer MCF-7 cell lines were obtained from the American type culture collection (ATCC). Moreover, the cells were cultured with RPMI-1640 medium containing 10% FBS and 1% penicillin streptomycin at 37°C under a humidified 5% CO_2 atmosphere. Cells were cultured in the phenol red-free RPMI-1640 medium containing 5% CDT-FBS 4 days before MTT assay so that the estrogen in the cells should be cleared.

Cell proliferation analysis of estradiol on MCF-7 cell lines

Cell proliferation was measured using the MTT assay for MCF-7 cells.[15,16] E_2 was dissolved in RPMI-1640 culture media containing 0.1% DMSO at final concentrations of 0.1, 1, 10, 100, and 1000 nM. MCF-7 cell lines were cultured in phenol red-free RPMI-1640 medium for 4 days. After washing by phosphate-buffered saline (PBS) for three times, 100 μL of FBS-free RPMI-1640 medium was added into 96-well plates at 1.5×10^4 per well and incubated at 37°C for 24 h. Subsequently, the cells were treated with various concentrations of the E_2 solution for 72 h. Then, 100 μL of MTT solution (0.5 mg/mL) was added into each well. Cells were incubated for 4 h. Finally, 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm by a microplate reader, and the proliferation rate (PR) was calculated.

Cell proliferation analysis of glycosides of *Cistanches* on MCF-7 cell lines

Cell proliferation was measured using the MTT assay for MCF-7 cells. GCs were dissolved in RPMI-1640 culture media containing 0.1% DMSO at final concentrations of 0.0175, 0.175, 1.75, 17.5, and 175 μg/ml. MCF-7 cell lines were cultured in phenol red-free RPMI-1640 medium containing 5% CDT-FBS for 4 days. After washing by PBS for three times, 100 μL of FBS-free RPMI-1640 medium was added into 96-well plates at 1.5×10^4 per well and incubated at 37°C for 24 h. Subsequently, the cells were treated with various concentrations of the GCs solution for 24, 48, and 72 h, respectively. Then, 100 μL of MTT solution (0.5 mg/mL) was added to each well. Cells were incubated for 4 h. Finally, 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm by a microplate reader, and the PR was calculated. Phenol red-free RPMI-1640 and medium containing E_2 (2.725×10^{-3} μg/ml) were the negative and positive group, respectively.

Cell proliferation analysis of glycosides of *Cistanches* with fulvestrant on MCF-7 cell lines

Cell proliferation was measured using the MTT assay for MCF-7 cells. GCs were dissolved in RPMI-1640 culture media containing 0.1% DMSO at final concentrations of 1.75, 17.5, and 175 μg/ml. MCF-7 cell lines were cultured in phenol red-free RPMI-1640 medium containing 5% CDT-FBS for 4 days. After washing by PBS for three times, 100 μL of FBS-free RPMI-1640 medium was added into 96-well plates at 1.5×10^4 per well and incubated at 37°C for 24 h. Subsequently, the cells were treated with various concentrations of the GCs solution and incubated with fulvestrant (6.06×10^{-5} μg/ml) for 48 h. Then, 100 μL of MTT solution (0.5 mg/mL) was added into each well. Cells were incubated for 4 h. Finally, 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm by a microplate reader, and the PR was calculated. Phenol red-free RPMI-1640 and medium containing E_2 (2.725×10^{-3} μg/ml) were the negative and positive group, respectively.

Cell cycle assay

GCs were dissolved in RPMI-1640 culture media containing 0.1% DMSO at final concentrations of 1.75, 17.5, and 175 μg/ml. MCF-7-cell lines were cultured in phenol red-free RPMI-1640 medium containing 5% CDT-FBS for 4 days. After washing by PBS for three times, 1 mL of FBS-free RPMI-1640 medium was added into 6-well plates at 2.5×10^5 per well and incubated at 37°C for 24 h. Subsequently, the cells were treated with various concentrations of the GCs solution and incubated with fulvestrant (6.06×10^{-5} μg/ml) for 48 h. Subsequently, the cells were collected and washed with PBS for three times, centrifuged at 4°C, 1000 rpm for 10 min, and fixed with 70% ethanol at −20°C, overnight. After washing with PBS for twice, the cells were stained with PI solution (50 mg/mL) containing 1 mg/mL of RNase A and 0.1% Triton X-100 for 30 min in the dark at 4°C. The cell cycle was detected using flow cytometry, and proliferation index (PI) was calculated as follows. Phenol

red-free RPMI-1640 and medium containing E_2 (2.725×10^{-3} μg/ml) were the negative and positive groups, respectively.

$PI = (S + G_2M)/(G_0/G_1 + S + G_2M) \times 100\%$

RNA isolation and real-time quantitative polymerase chain reaction analysis

To measure the level of mRNA, a RT-quantitative PCR (qPCR) assay was used. Total cellular RNA was extracted with TRIzol reagent. For qPCR, RNA was reverse transcribed to cDNA from 4 μg of total RNA using a RT kit. RT-PCR analyses were conducted using TB Green™ RT-PCR Kit. All protocols were performed according to the manufacturer's instructions. The primer pair used for amplification of ERα was 5'-GGGAAGTATGGCTATGGAATCTG-3' (forward) and 5'-TGGCTGGACACATATAGTCGTT-3' (reverse); the primer pair used for amplification of ERβ was 5'-AGTGCCGCTCTTGGAGAGCTG-3' (forward) and 5'-CCTGGGTCGCTGTGACCAGA-3' (reverse). The PCR conditions for ERα and ERβ were 94°C for 3 min followed by 37 and 40 cycles, respectively, of 94°C for 30 s, 58°C for 2 min, and 72°C for 2 min. The primer pair used for amplification of GAPDH was 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse). The PCR conditions for GAPDH were 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 58°C for 2 min, and 72°C for 2 min. Every time RT-qPCR experiment was repeated more than two times. For this, GAPDH was used as internal control. The relative gene expression of ERα/ERβ of transfectants in relation to control cells was calculated: $2^{-(\Delta\Delta Ct)}$.

Western blotting

The expression of ERα and ERβ proteins was detected by western blot analysis as the reported method with minor modifications. Briefly, the MCF-7 cells were grown in 6-well plates at 2.5×10^5 cells per well and treated with GCs at the final concentration of 1.75, 17.5, and 175 μg/ml for 48 h, respectively. After incubation, the whole-cell extracts were prepared using RIPA buffer containing 1 mM PMSF. Proteins in the whole-cell extracts were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membrane was blocked with 5% (w/v) skim milk dissolved in TBST buffer and incubated with primary and secondary antibodies in turn. Bound antibodies were observed.

Statistical analysis

The results were expressed as means and standard deviations, and statistical significance was performed using Student's *t*-test with SPSS 21.0 software (IBM Co., Ltd. America). P <0.05 was considered statistically significant.

RESULTS

After 24 h of E_2 treatment, the proliferation of MCF-7 cells was obviously enhanced [Figure 1a]. 10nM of E_2 solution could stimulate the growth of the cells obviously (123.89%). However, with the increase of E_2 concentration, the cell proliferation ability weakened. Hence, 10nM was the optimal concentration of E_2 in this experiment.

GCs group at the concentrations of 1.75, 17.5, and 175 μg/mg could enhanced proliferation of the MCF-7 cell lines with a time and dosage-dependent manner and exhibited a significant difference with negative group [Figure 1b]. Compared to negative group, E_2 group exhibited obvious proliferation (120.06%).

Combined medication group (CMG) (fulvestrant with E_2 or GCs) in the CDT-FBS medium were incubated with MCF-4 cells. After 72 h of incubation, CMG could lead to the incline of PR compared with the individual medication group (IMG) ($P < 0.01$) [Figure 1c].

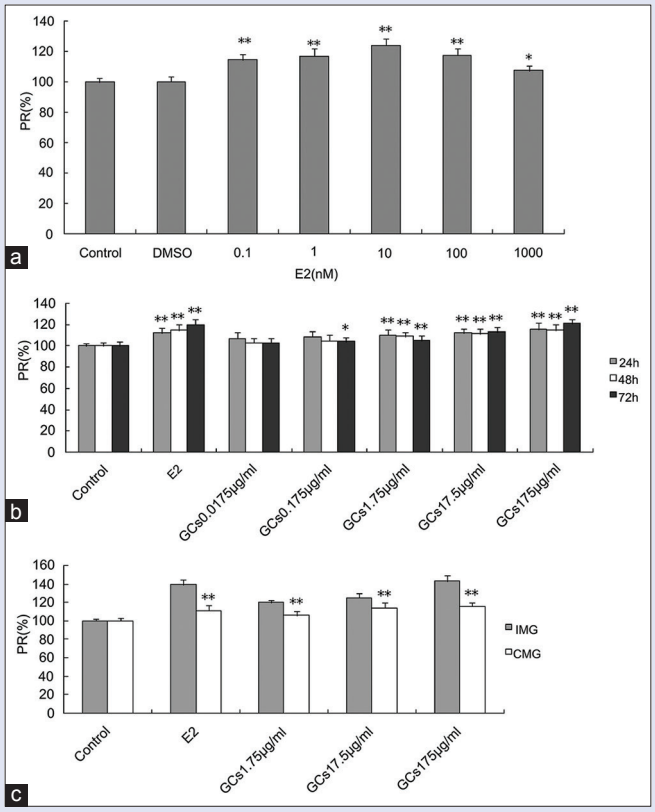

Figure 1: Proliferation function of estradiol, glycosides of *Cistanches* and antagonist intervention of glycosides of *Cistanches* in human breast cancer MCF-7 cells; (a) sensitivity experiment of estradiol in human breast cancer MCF-7 cells; (b) proliferation function of glycosides of *Cistanches* in human breast cancer MCF-7 cells; (c) antagonist intervention of glycosides of *Cistanches* in human breast cancer MCF-7 cells)

Flow cytometry analysis showed that GCs could slightly increase the PI value which suggesting that GCs could advance G0/G1 phase cells to S and G2/M phase [Figure 2 and Table 1] and promote cell DNA synthesis. The result indicating that the mechanism of GCs on MCF-7 was similar to that of E_2. In CMG (fulvestrant with GCs), PI value of cell PI was decline to that of IMG slightly ($P < 0.05$).

RT-PCR analysis indicated that the expressions of ERα and ERβ mRNA were increased compared with control group ($P < 0.01$) in a dose-dependent manner after being treated by different concentrations of GCs for 48 h [Figure 3a and b].

Western blot result indicating that after treatment with various concentrations of GCs for 48 h, contents of ERα and ERβ proteins in MCF-7 increase with the GCs increased as a dosage-dependent manner [Figure 3c-e].

CONCLUSION

In this research, the effect of GCs on the proliferation and cell cycle of MCF-7 was assayed by the method of MTT and flow cytometry, respectively. GCs could increase the proliferation of MCF-7 cells at the concentration of 1.75, 17.5, and 175 μg/ml after incubated for 72 h. However, the increase tend slows down when CCs and fulvestrant were used together. GCs could increase the PI value of MCF-7 cells, decrease the cell of G_1 phase, and increase the cell of S and G_2 phase.

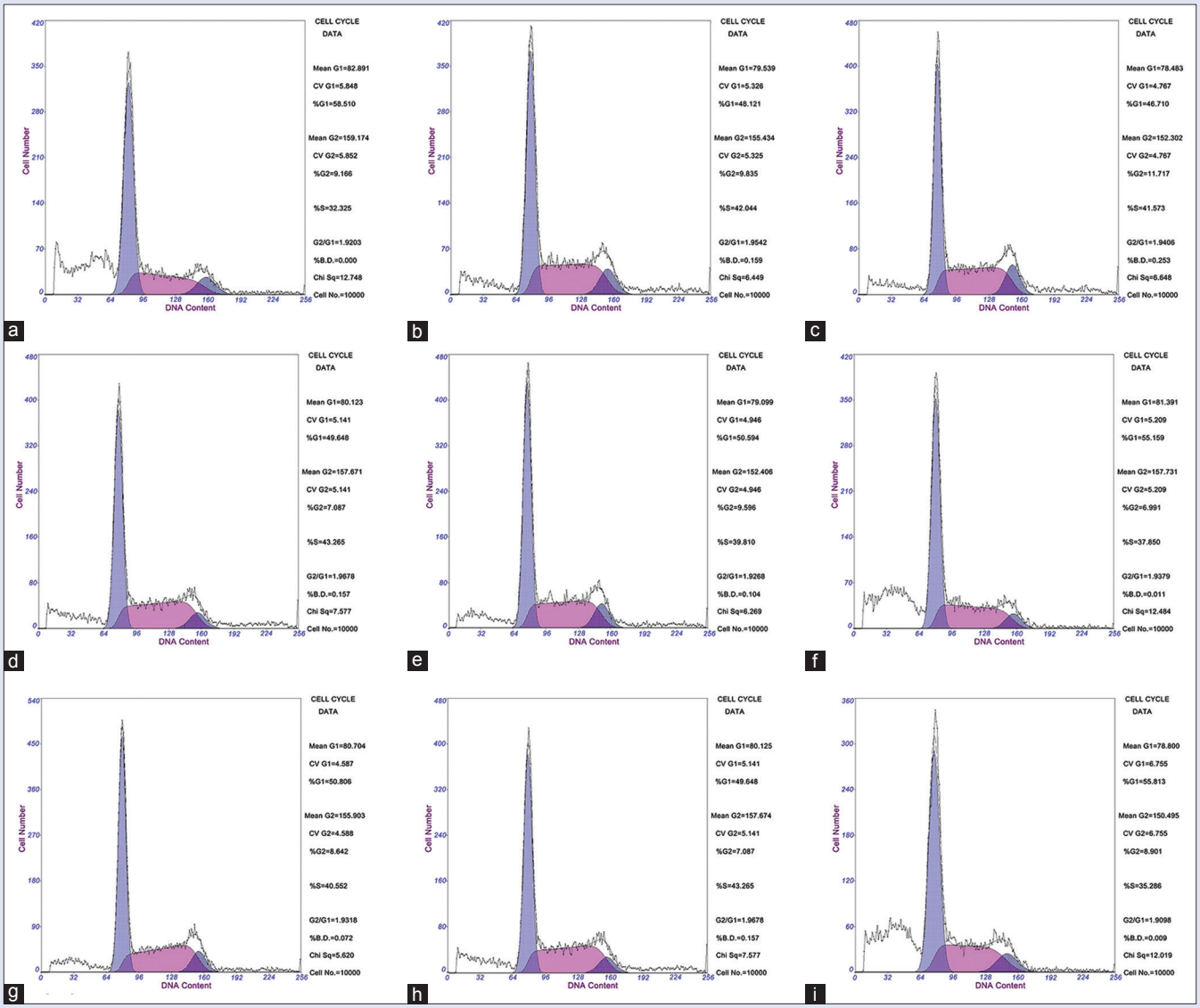

Figure 2: Cell cycle of glycosides of *Cistanches* in human breast cancer MCF-7 cells (a) control; (b) 2.725 × 10-3 μg/ml estradiol; (c) 175 μg/ml glycosides of *Cistanches*; (d) 17.5 μg/ml glycosides of *Cistanches*; (e) 1.75 μg/ml glycosides of *Cistanches*; (f) estradiol + fulvestrant; (g) glycosides of *Cistanches* 175 μg/ml + fulvestrant; (h) glycosides of *Cistanches* 17.5 μg/ml + fulvestrant; (i) glycosides of *Cistanches* 1.75 μg/ml + fulvestrant)

Table 1: Cell cycle of glycosides of *Cistanche* in on MCF-7 cells

Group	Dose(μg/ml)	G_0/G_1(%)	S (%)	G_2/M (%)	PI
Negative		58.29±1.70	33.47±1.03	8.24±1.62	41.71
IMG					
E_2		49.09±2.12**	42.37±1.38**	8.54±1.28	50.91
GCs	175	46.73±1.51**	41.62±2.25**	11.65±0.74**	53.27
	17.5	49.05±1.46**	42.72±2.42**	8.23±1.41	50.95
	1.75	50.29±1.41**	39.38±2.90**	10.33±1.61*	49.71
CMG					
E_2 + fulvestrant		54.98±1.87▲	37.61±2.23▲	7.41±0.51	45.02
GCs + fulvestrant	175	50.62±1.32△	40.14±1.76	9.24±0.66△	49.38
	17.5	51.24±1.75	38.86±1.59△	9.90±0.23	48.76
	1.75	55.67±1.52▲	35.50±1.38△	8.83±0.16	44.33

Comparison between IMG and negative group, *P<0.05, **P<0.01; Comparison between CMG and IMG, △P<0.05, ▲P<0.01. IMG: Individual medication group; GCs: Glycosides of *Cistanches*; CMG: Combined medication group; E_2: Estradiol; PI: Proliferation index; MCF-7: Human breast cancer cells

Fulvestrant is an ER-specific antagonist, which blocks the nuclear localization of ER by impairing receptor dimerization and energy-dependent nuclear transport.[17] In this research, a conclusion has confirmed that fulvestrant can weaken the proliferation of GCs on MCF-7 cells, and it could affect the cell cycle transformation. Hence, this study indicated the estrogenic activity of GCs, and also, ER is the

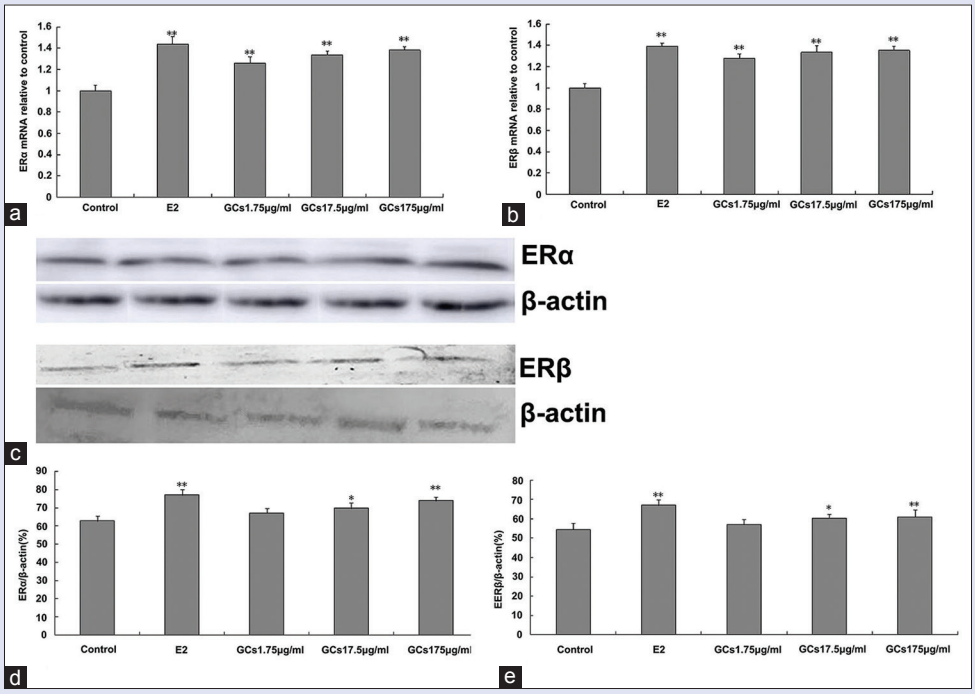

Figure 3: Effect of glycosides of *Cistanches* on estrogen receptors α and estrogen receptors β mRNA and protein expression in MCF-7 cells (a) estrogen receptor α mRNA relative expression; (b) estrogen receptors β mRNA relative expression; (c) estrogen receptors α and estrogen receptors β protein expression in MCF-7; (d) estrogen receptors α protein expression in each group; (e) estrogen receptors β protein expression in each group)

target of GCs. In the RT-PCR and western blot experiment, mRNA and protein expressions of ERα and ERβ in the MCF-7 cells increased with the increase of GCs concentration. Hence, GCs can play a role of estrogenic activity according upregulated mRNA and proteins of ERα and ERβ.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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