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Study on the regulatory effect of liver X receptor in HEK293 cells by six main diterpene esters in *Semen Euphorbiae*

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Competing interests

The authors declare no conflicts of interest.

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Abbreviations

LXR, liver X receptor; RLU, relative luciferase activity.

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Abstract

Background: To study the effects of the main diterpene esters in *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 on the transcriptional activity and protein expression of liver X receptor (LXR). **Methods:** The effect of the main diterpene ester components in *Semen Euphorbiae* on the viability of HEK293 cells were studied by MTT assay. The LXR-Luc plasmid vector was transfected into HEK293 cells and treated with *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 for 24 h. The effect of the main diterpene ester components of *Semen Euphorbiae* on LXR-Luc luciferase activity was investigated by dual luciferase reporter gene system, and the expression of LXRα protein was detected by Western Blot. **Results:** *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 could significantly reduce the relative luciferase activity (RLU) of LXRα, and the expression level of LXRα protein was significantly down-regulated. **Conclusion:** *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 can inhibit the expression of LXR protein level, which may be achieved by inhibiting the transcriptional activity of LXR.

Keywords: *Semen Euphorbiae*; diterpene esters; HEK293; LXR; dual luciferase reporter gene system

Introduction

Semen Euphorbiae is the dried and ripe seeds of *Euphorbia lathyris* L. of family Euphorbiaceae. *Semen Euphorbiae* is "pungent in taste", "warm in nature" and toxic, containing diterpene alcohol esters, fatty oils, coumarins and other chemical components. The main effect are diarrhea and expel water, breaking the blood and eliminating symptoms. Modern research has shown that *Semen Euphorbiae* has diarrhea, anti-tumor, anti-multi-drug resistance, antibacterial, antioxidant and other pharmacological activities [1, 2]. At the same time, it has been proven to exhibit stimulating effect on the gastrointestinal tract and it needs to be used in clinical practice after degrease-frosting [3–6].

Liver X receptor (LXR) is a member of the nuclear receptor superfamily, which can be activated by ligands to regulate transcription. It is a key regulatory factor involved in the transmission of inflammatory signals and lipid metabolism *in vivo*, and plays an important role in the research of traditional Chinese medicine detoxification and toxicity-efficacy basis [7–9]. Previous studies have shown that, the crude extract of *Semen Euphorbiae* can inhibit the transcription and translation of LXR, and the downregulation of LXR transcription and translation levels after frosting is significantly weakened [10]. The toxicity attenuation mechanism of *Semen Euphorbiae* after frosting may be related to the regulation of LXR signals, but the material basis for *Semen Euphorbiae* regulation of LXR is currently unclear. Therefore, based on the six main diterpene esters components of *Semen Euphorbiae*, including *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8, the effects of *Semen Euphorbiae* on LXR transcription activity and protein expression in HEK293 cells were studied to screen the main active components of *Semen Euphorbiae* regulating LXR [11–12].

Instruments and reagents

1550 Enzyme marker (Thermo, USA), MiliQ Biocel pure water machine (Millipore, USA), Cell culture chamber (Thermo, USA), Fuorescence quantitative PCR instrument (Thermo, USA).

Semen Euphorbiae was purchased from Bo Zhou, Anhui Province, China (Lot No. 1203070692). After testing, the oil content of *Semen Euphorbiae* is 48.61%, which meets the requirements of the 2020 edition of the Chinese Pharmacopoeia. *Semen Euphorbiae Pulveratum* is prepared from the same batch of *Semen Euphorbiae* by removing oil and making frost. HEK293 cells were obtained from BeNa Culture Collection.

Euphorbia factor L_1 (Lot No.DSTDQ007001), *Euphorbia* factor L_2 (Lot No.DSTDD013401), *Euphorbia* factor L_3 (Lot No.DSTDD006201), *Euphorbia* factor L_{7a} (Lot No.DSTDQ021901), *Euphorbia* factor L_{7b} (Lot No.DSTDQ022001) and *Euphorbia* factor L_8 (Lot No.DSTDQ022101) have the purity of 99.91%, 99.69%, 99.42%, 98.33%, 99.45% and 100.00%, respectively, which were obtained from Chengdu Desite Biotechnology Company Co., Ltd; DMSO (cell grade) was purchased from Sigma-Aldrich, USA; DMEM high glucose medium, fetal bovine serum, PBS buffer and trypsin were purchased from Gibco, USA; LXR-Luc luciferase reporter gene plasmid (1 μg), pRL-TK plasmid, RG027 Dual-Luciferase Reporter Gene Detection Kit, EZ trans transfection reagent, T0901317 LXR agonist (10 mg) were purchased from Beijing BioDee Biotechnology Co., Ltd; Anti-LXR alpha antibody was purchased from Abcam, UK.

Experimental methods

Preparation of extracts of *Semen Euphorbiae* and *Semen Euphorbiae Pulveratum*

A certain amount of *Semen Euphorbiae* was taken to prepare *Semen Euphorbiae Pulveratum* according to the frosting method in the general rule 0213 of the 2020 edition of the Chinese Pharmacopoeia. The oil content was 19.40%, which was in line with the requirements of the Pharmacopoeia.

Separately weigh a certain amount of *Semen Euphorbiae* and *Semen Euphorbiae Pulveratum*, and heat reflux extracted by adding 6 times of 95% ethanol for 3 times. Combined extracts, and the solvent was recovered under reduced pressure to no alcohol taste. Isocratic petroleum ether extraction, recovery under reduced pressure, obtained the extracts [13].

Cell culture

Inoculate HEK293 cells into culture flasks with DMEM medium, then place in a CO_2 thermostat incubator with 5% CO_2, 37 ˚C, and saturated humidity for static culture [14].

The effect of diterpene esters of *Semen Euphorbiae* on the survival rate of HEK293 cells

HEK293 cells in logarithmic growth phase were seeded in 96-well cell culture plates. The blank group, *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 (6.25, 12.5, 25, 50, 100 μmol/L) groups were set up. After the cells were adherent, add 100 μL corresponding drugs to each group, and the blank group was added to the medium without drugs for 48 h. Add 20 μL MTT (5 mg/mL) solution, incubate at 37 ˚C for 4 h, discard the supernatant, add 150 μL of DMSO to each well, and calculate the cell survival rate by detecting the absorbance (A) value at 490 nm with an enzyme marker.

Cell transfection and determination of relative luciferase activity of LXRα

When the cells grew well, seed in 24-well cell culture plates the day before transfection. The blank group, T0901317 positive drug group, *Semen Euphorbiae* and *Semen Euphorbiae Pulveratum* extract group (calculated as 800 μg/mL based on the amount of raw drug volume), *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 group (50 μmol/L) were set up, with 3 repeat holes in each group. Culture for about 24 h, ready for transfection.

The LXR-Luc luciferase reporter gene plasmid was used to detect the level of LXRα transcriptional activity, and the pRL-TK plasmid was used as an internal reference. The diluted EZ Trans transfection reagent was added into the plasmid solution, mixed, and incubated at room temperature for 15 min. After each well was added with mixed reagents, the culture medium containing EZ Trans-DNA complex was removed at 37 ˚C, 5% CO_2 incubator for 12 h. Replace with fresh culture medium and leave overnight. Each group was treated with corresponding drugs for 24 h. 200 μL lysate was added. Supernatant 20 μL was added to each well in an opaque 96-well microplate, and 100 μL of pre-mixed LAR II was added. After 2 s, the data were measured immediately to detect the luciferase reaction intensity. After the determination of firefly luciferase was completed, 100 μL of pre-mixed Stop & Glo was added to each well, the 96-well plate was gently shaken, and the data were immediately measured after 2 s of rest. The internal reference renilla luciferase reaction intensity was detected and the readings were recorded.

Relative luciferase activity (RLU) = experimental group luciferase activity/blank group luciferase activity, each group luciferase activity = firefly luciferase activity / renilla luciferase activity.

Detection of LXRα protein expression levels by western blot

Cells were seeded in 6-well plates at a density of 2×10^5 cells per well and allowed to adhere. Subsequently, the cells were allocated into three groups: control, *Semen Euphorbiae* extract, and *Semen Euphorbiae Pulveratum* extract (at a concentration equivalent to 800 μg/mL of raw drug volume), as well as seven *Euphorbia* factors (L_1, L_2, L_3, L_{7a}, L_{7b} and L_8) at a concentration of 50 μmol/L each. After 48 hours of drug treatment, the cells were harvested, washed with PBS, and lysed. The cell lysates were then denatured by boiling and subjected to SDS-PAGE electrophoresis, followed by membrane transfer. The membrane was incubated overnight at 4 ˚C with primary antibodies, washed with TBST, and then incubated with secondary antibodies. Finally, ECL luminescent solution was applied, and the protein bands were analyzed using Image J. The entire process adhered to standard Western blotting procedures.

Results

Effects of *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 on the survival rate of HEK293 cells

Compared with the blank group, drug-dosing groups significantly reduced the viability of HEK293 cells in a concentration-dependent manner over the concentration range of 12.5–100 μmol/L ($P < 0.01$). The results are shown in Table 1.

Effects of *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 on the relative luciferase activity of LXRα in cells

The activity of LXR-Luc luciferase reporter gene was analyzed for the main diterpene ester components in *Semen Euphorbiae*, The results are shown in Figure 1. Compared with the blank control group, T0901317 positive drug could significantly increase the RLU of LXRα ($P < 0.001$), indicating that the luciferase reporter gene system responded normally. The RLU of LXRα in HEK293 cells was significantly decreased by 800 μg/mL extract of *Semen Euphorbiae* and *Semen Euphorbiae Pulveratum* ($P < 0.001$). The activity of LXRα in 50 μmol/L *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 groups was (0.60 ± 0.04) times ($P < 0.001$), (0.87 ± 0.02) times ($P < 0.01$), (0.81 ± 0.01) times ($P < 0.001$), (0.88 ± 0.05) times ($P < 0.01$), (0.90 ± 0.05) times ($P < 0.01$) and (0.91 ± 0.04) times ($P < 0.05$) of that in the blank group, respectively. All of them can reduce the RLU of LXRα, and have a significant inhibitory effect, which is basically consistent with the trend of the extract. The results suggest that *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 may be the main components of the regulation of LXRα by *Semen Euphorbiae*.

The effects of *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 on the expression of LXRα protein in cells

Western Blot was used to detect the effect of main diterpene ester components on the expression of LXRα protein in HEK293 cells. The results are shown in Figure 2. Compared with the control group, 800 μg/mL extract of *Semen Euphorbiae* and *Semen Euphorbiae Pulveratum* and 50 μmol/L *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 could significantly down-regulate the expression level of LXRα protein ($P < 0.001$), and *Euphorbia* factor L_3 could down-regulate the expression level of LXRα protein ($P < 0.05$). The results showed that the six main diterpene esters in *Semen Euphorbiae* could affect the expression level of LXRα protein in HEK293 cells, suggesting that *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 may be related to the regulation of LXRα protein expression level by *Semen Euphorbiae*.

Table 1 The effects of different concentrations of *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8 on the survival rate of HEK293 cells

Concentration (μmol/L)	Cell viability (%)					
	Euphorbia factor L_1	*Euphorbia* factor L_2	*Euphorbia* factor L_3	*Euphorbia* factor L_{7a}	*Euphorbia* factor L_{7b}	*Euphorbia* factor L_8
0	100.00 ± 1.41	100.00 ± 1.41	100.00 ± 1.41	100.00 ± 1.41	100.00 ± 1.41	100.00 ± 1.41
6.25	97.30 ± 3.39	100.54 ± 2.04	92.32 ± 6.44**	93.90 ± 4.12**	81.94 ± 5.65**	91.43 ± 6.01**
12.5	89.24 ± 3.18**	89.90 ± 2.52**	72.38 ± 5.51**	73.21 ± 4.86**	66.83 ± 5.05**	79.05 ± 5.49**
25	65.59 ± 4.56**	79.62 ± 2.28**	60.95 ± 3.99**	61.71 ± 4.87**	49.24 ± 3.27**	62.89 ± 6.09**
50	45.24 ± 4.28**	68.19 ± 5.68**	43.21 ± 1.77**	38.70 ± 1.15**	25.40 ± 3.67**	51.65 ± 3.71**
100	28.19 ± 4.21**	61.52 ± 4.50**	27.59 ± 3.75**	34.95 ± 3.44**	20.44 ± 3.11**	43.56 ± 2.51**

Note: Compared to the blank group, *$P < 0.05$, **$P < 0.01$.

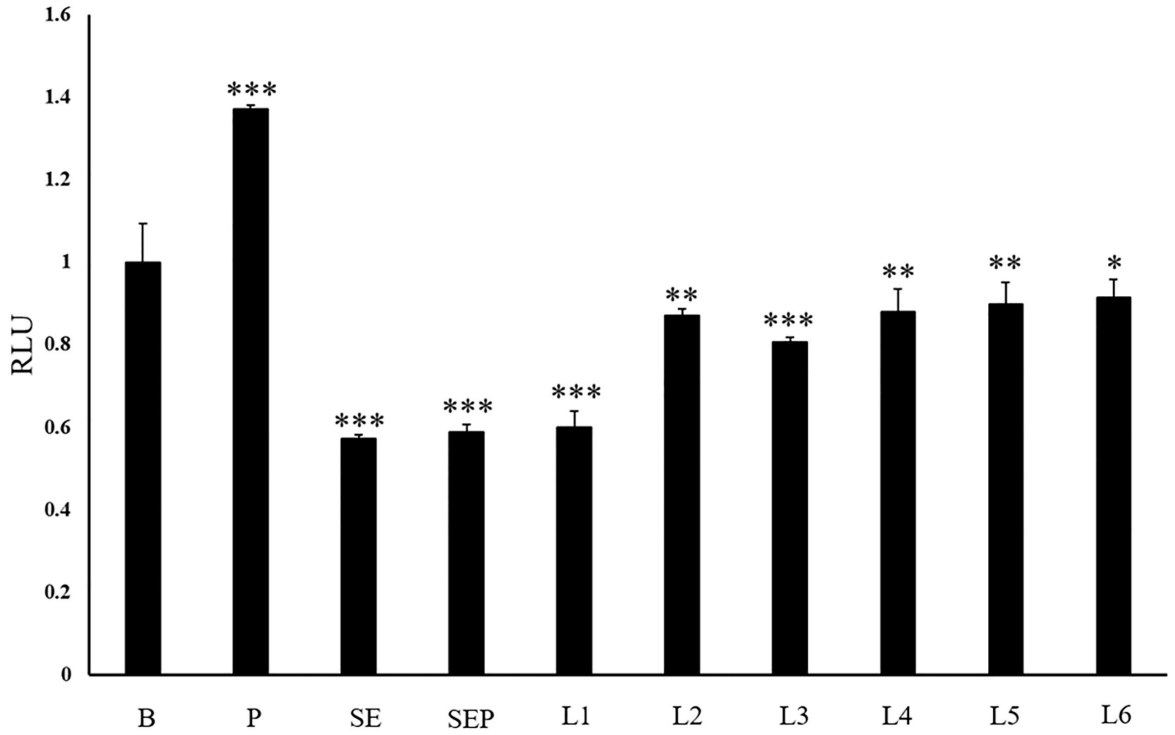

Figure 1 The effects of *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b}, L_8 and extract on the relative fluorescence intensity of LXRα. B: Blank Control group; P: Positive Drug group; SE: *Semen Euphorbiae* group; SEP: *Semen Euphorbiae Pulveratum* group; Compared with the blank control group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

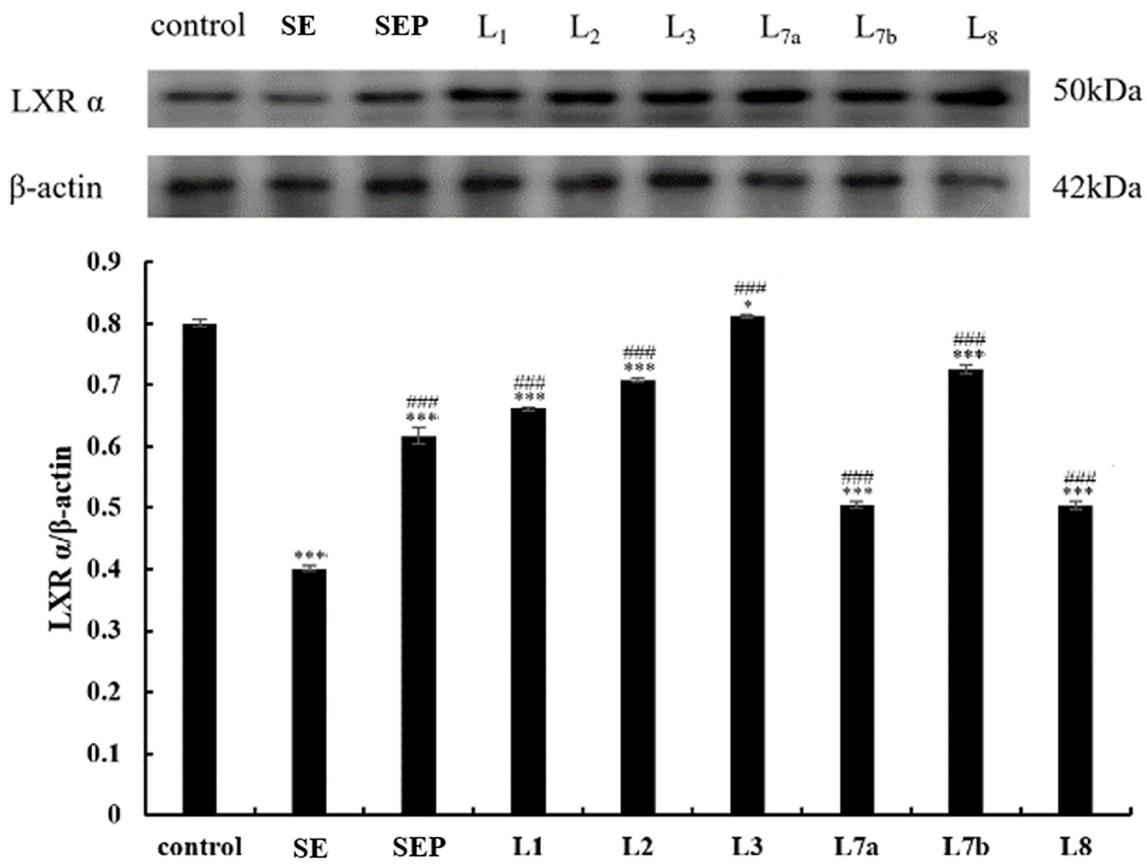

Figure 2 The effect of *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b}, L_8 on the expression of LXRα protein. SE: *Semen Euphorbiae* group; SEP: *Semen Euphorbiae Pulveratum* group; Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Compared with the SE group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

Discussion

Dual luciferase reporter gene system is a reporter system for detecting firefly luciferase and marine coelomic luciferase activities using luciferin as a substrate.The biological fluorescence released by the reaction of luciferase and substrate during oxidation is detected by chemiluminescence. It can detect gene expression sensitively, quickly and efficiently. It is an important means to detect the activity of transcription factors [15–18]. Western Blot is a hybridisation technique that combines high-resolution gel electrophoresis with immunochemical analysis, which is widely used for quantitative and qualitative analysis of proteins, and has the advantages of high analytical capacity, high sensitivity and high specificity [19–21]. LXR is involved in the regulation of lipid metabolism, which can induce gene expression involved in reverse cholesterol transport, hepatic glycogen metabolism and fatty acid synthesis. It is a key substance to regulate lipid balance [7–8, 22]. It also has the effect of inhibiting the expression of inflammatory genes and thus controlling immunity and inflammation [23, 24]. LXRα, as an isoform of LXR, is mainly expressed in the liver, intestine, and kidney, preferentially regulating the expression of genes related to the regulation of lipid metabolism, apoptosis, and inflammation.Preliminary studies have found that Chrysanthemum officinale extracts can modulate LXRα and improve the level of intracellular cholesterol [10, 25]. However, the active components and mechanism of action are still unclear. Therefore, in this study, we selected the diterpene esters represented by *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b}, L_8, and examined the transcriptional and translation levels of the six diterpene ester components of *Semen Euphorbiae* that regulate LXRα, based on the LXR-Luc Dual-Luciferase Reporter Gene System and Western Blot method. The results showed that the regulation of *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b}, L_8 on the luciferase activity of LXRα in HEK293 cells was consistent with that of the extract of *Semen Euphorbiae* and *Semen Euphorbiae Pulveratum*, which significantly inhibited the transcriptional activation of LXRα and the expression level of LXRα protein. It was speculated that *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b}, L_8 could down-regulate the expression of LXRα protein by inhibiting the transcriptional activity of LXRα.

Conclusion

In this study, we investigated the effects of the major diterpene ester components, *Euphorbia* factor L_1, L_2, L_3, L_{7a}, L_{7b} and L_8, on LXR in the extracts of *Semen Euphorbiae*, by using the dual luciferase reporter gene system and Western Blot method, and the results indicated that the major diterpene ester components in *Semen Euphorbiae* could regulate the LXR in renal HEK293 cells, and might be the main active components of *Semen Euphorbiae* in the modulation of LXR, which provided experimental basis and reference for the subsequent in-depth discussion of theoretical research, pharmacological effects and mechanism of action of *Semen Euphorbiae*.

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