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The diterpenoid esters from *Euphorbia lathyris* L. induce intestinal inflammation via LXRα/ABCA1-regulated lipid rafts and TLR4-mediated pathway

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Author contributions

Wang HN and Wang XY designed this project, performed the experiments, and wrote this article. Wang PH, Jiang MR, Ma SY, Zhu HT and Zhang JL revised the article. Zhang XN, Chen WJ, Mao YF and Xu H analyzed the data. Wang YZ guided the experiment and provided funding for the research. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Peer review information

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Abbreviations

EL, *Euphorbia lathyris* L.; EFL_1, Euphorbia factors L_1; EFL_2, Euphorbia factors L_2; EFL_3, Euphorbia factors L_3; PEL, processed *Euphorbia lathyris* L.; TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa-B; NLRP3, NOD-like receptor family Pyrin domain containing 3; LXRα, Liver-X receptor Alpha; ABCA1, ATP-binding cassette transporter A1; TCM, traditional Chinese medicine; TNF-α, tumor necrosis factor-α IL-1β, interleukin-1β.

Citation

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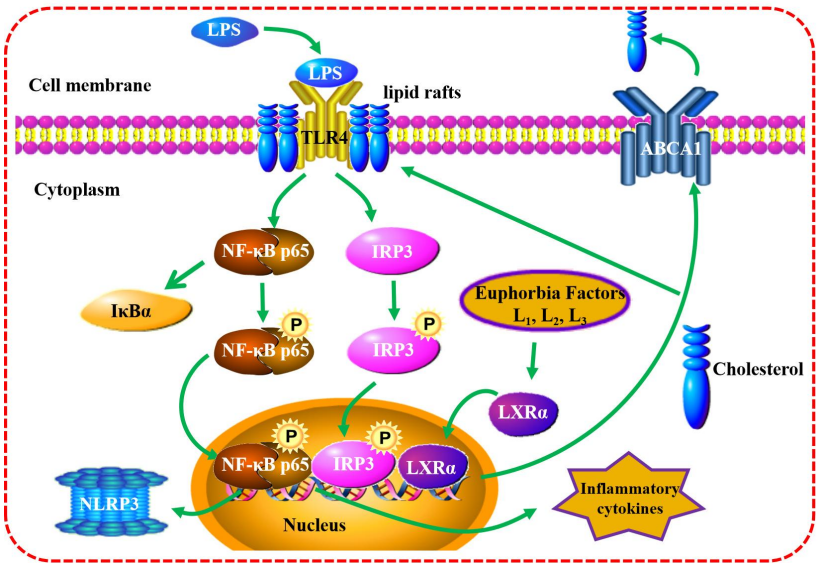
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Abstract

Background: Diterpenoid esters are considered to be the main toxic components and bioactive constituents of *Euphorbia lathyris* L. (EL). Euphorbia factors L_1 (EF_1), L_2 (EF_2), and L_3 (EF_3), the main diterpenoid esters of EL, have been found to cause intestinal diarrhea and induce intestinal inflammation in mice. This research aimed to explore the effects of major diterpenoid esters from EL on intestinal inflammation, as well as to clarify their possible targets and molecular mechanisms in vivo and vitro. **Methods:** Caco-2 cells and BALB/c mice were intervened with EFL_1, EFL_2, and EFL_3, respectively. The expressions of TLR4, NLRP3, NF-κB p65, LXRα, ABCA1, TNF-α and IL-1β were measured by Real-time PCR and ELISA. Cholesterol efflux levels were examined using cholesterol efflux kit. Flow cytometry was applied to detect lipid rafts abundance. Confocal microscopy was applied to investigate co-localization of lipid rafts and TLR4. **Results:** Our results revealed that EFL_1, EFL_2, and EFL_3 inhibited LXRα, ABCA1 expression, and cholesterol efflux, promoted colocalization of TLR4 and lipid rafts, and up-regulated TLR4, NLRP3, NF-κB p65, TNF-α and IL-1β expressions. **Conclusion:** These findings reveal that the mechanisms by which EFL_1, EFL_2, and EFL_3 induce intestinal inflammation may be associated with LXRα/ABCA1-regulated lipid rafts and TLR4-mediated pathways.

Keywords: diterpenoid esters; *Euphorbia lathyris* L.; LXRα/ABCA1; lipid rafts; TLR4



Introduction

The application of toxic traditional Chinese medicine (TCM) is a major feature of the clinical treatment of diseases in TCM. Long-term clinical practice of TCM has proved that toxic TCM have a positive significance to the treatment of difficult and serious diseases and have obvious curative effects. Processing (paozhi) of TCM is an important link to ensure the quality and clinical efficacy of toxic TCM, and toxic TCM can reduce toxicity, increase efficacy or preserve efficacy through rational processing. However, the multi-component, multi-target, and multi-pathway characteristics of TCM determine the complexity of its processing mechanism, and the clinical application of toxic TCM is still facing great challenges. Therefore, the integrated analysis of the material basis and molecular mechanism of toxic TCM processing from multiple perspectives is an important prerequisite to ensure the rational application of toxic TCM in clinical practice.

Euphorbia lathyris L. (EL) has been applied in China and the rest of Asian to treat dropsy, ascites, anuresis, constipation and amenorrhea [1–4]. Modern research display that EL mainly contains diterpenoid esters, fatty oils, proteins, flavonoids, coumarins and volatile oils. Diterpenoid esters are considered to the main toxic components and bioactive constituents of EL, and more than 30 diterpenoid ester compounds have been isolated and identified from EL [5, 6]. Euphorbia factors L_1 (EFL_1), L_2 (EFL_2), and L_3 (EFL_3) are the three diterpenoid esters with the highest content and both have significant bioactivity (The structure of the three components is shown in Figure 1) [7].

Modern pharmacological studies have revealed that EFL_1, EFL_2, and EFL_3 caused intestinal diarrhea and induced inflammation in mice through regulating AQP2, AQP4 and AQP8 expression [8, 9]. ITRAQ proteomics results revealed that these three diterpenoid esters might activate nuclear factor Kappa-B (NF-κB) pathway to induce intestinal inflammatory injury in vivo [3]. The molecular mechanism of the intestinal inflammation induced by EFL_1, EFL_2, and EFL_3 deserved in-depth study.

Liver-X receptor Alpha (LXRα) are key regulators of lipid metabolism and inflammatory signaling, which not only participate in cholesterol efflux related lipid metabolism, but also exert a central role in inflammation [10]. LXRα can promote ATP binding cassette transporterA1 (ABCA1) expression, thereby promoting cholesterol efflux and affecting lipid rafts abundance [11, 12]. Lipid rafts mainly composed of cholesterol, sphingomyelin and lipid raft-linked signal proteins, which occupy an important position in material transport and intracellular signal transduction [13, 14]. Cholesterol efflux is the main way to regulate lipid rafts, and affect the activation of toll-like receptors-mediated signaling pathways and cytokines release in lipid rafts, thus interfering with inflammation [15]. Binding of Toll-like receptor 4 (TLR4) to ligand induces downstream NF-κB into the nucleus, which then causes NOD-like receptor family Pyrin domain containing 3 (NLRP3) activation [16, 17].

Based on this, this study aimed to investigate whether EFL_1, EFL_2, and EFL_3 interfered with cholesterol efflux and lipid rafts abundance through LXRα/ABCA1 pathway, and affected the TLR4-mediated pathway. To explore the mechanism of whether the regulation of three diterpenoid esters from EL to induce the intestinal inflammation, so as to lay an important reference for the safe application of EL in clinic.

Materials and methods

Reagents

EL was purchased from Anhui Bozhou TCM plant, and processed *Euphorbia lathyris* L. (PEL) was prepared using the previously reported method [3, 9]. EFL_1, EFL_2 and EFL_3 were bought from Chengdu Desite Biotechnology Co., Ltd. (Chengdu, China). Cholesterol efflux assay kits were bought from Abcam (Cambridge, UK). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) kits were obtained from Shenzhen xinbosheng Biotechnology Co., Ltd.

Preparation of extracts from EL and PEL

EL and PEL extracts were prepared using the previously reported methods [9]. EL extract contained (3.81 ± 0.03) mg/g EFL_1, (1.49 ± 0.09) mg/g EFL_2, and (3.62 ± 0.07) mg/g EFL_3, and PEL extract contained (2.03 ± 0.01) mg/g EFL_1, (0.86 ± 0.03) mg/g EFL_2, and (1.74 ± 0.03) mg/g EFL_3. Finally, extracts of EL and PEL were dissolved in 0.5% CMC-Na to obtain a solution of 3.17 g/mL.

Cell culture

Caco-2 cell was bought from BNCC (Beijing, China), and was incubated with EL (800 μg/mL), PEL (800 μg/mL), EFL_1 (50 mM), EFL_2 (50 mM) and EFL_3 (50 mM) for 48 h. Control cells were treated with equal volumes of DMEM medium. Cells precipitation and supernatant were collected for follow-up experiments.

Animals and treatment

Male SPF-grade BALB/c mice (4–6 weeks old, 20 ± 2 g) were bought from Beijing SPF Biotechnology Co. Ltd. (License No. SCXK (Beijing) 2019-0010). The mice were acclimated for 5 days in typical laboratory conditions for housing, feeding, lighting and humidity. All experiments were authorized by Animal Care and Use Committee of Beijing University of Chinese Medicine (No. BUCM-4-2022092702-3104).

Thirty-six mice were divided into six groups (n = 6): control group, EL group (145.25 g/kg), PEL group (145.25 g/kg), EFL_1 group (0.5 g/kg), EFL_2 group (0.5 g/kg) and EFL_3 group (0.5 g/kg). At 6 h after administration, the mice were euthanized by inhalation of carbon dioxide, serum and colon were stored at −80 °C.

Real-time PCR assay

Total RNA was extracted with TRIzol (Invitrogen, St. Louis, MO, USA), and then reversely transcribed to cDNA. PCR reactions were performed with SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Beijing, China) kit. Primers used in this research were consistent with our previous study [18]. The relative mRNA expressions of candidate genes were standardized to ACTIN and calculated by $2^{-\Delta\Delta Ct}$ method.

Cholesterol efflux assay

Caco-2 cells were treated with labeling reagent and equilibration buffer. Drug group cells were intervened with HDL and drugs, control group cells were intervened with HDL. Cell supernatant was centrifuged and collected after 24 h. The same amount of cell lysate was added to each well to lysis cells, and the fluorescence value was detected with fluorescence enzyme labeler to calculate cholesterol efflux rate.

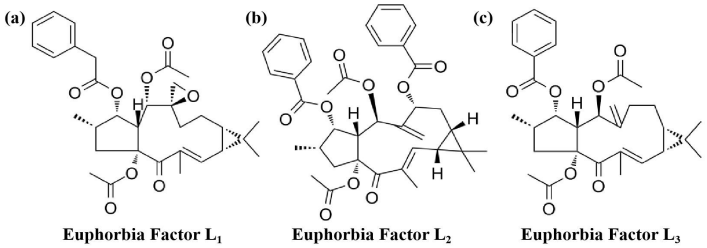


Figure 1 The structure of EFL_1, EFL_2, and EFL_3

Flow cytometer assay

Caco-2 cells were digested and rapidly stained with AlexaFluor488 labeled cholera toxin-B at a concentration of 1 μg/mL for 10 min. Fluorescence intensity was then measured by flow cytometry (Thermo Scientific, Waltham, MA, USA).

Confocal microscopy

Caco-2 cells were immunostained with AlexaFluor488 and fixed with 4% paraformaldehyde. Cells were then blocked with 5% BSA, and stained with TLR4 antibody (1:200) and AlexaFluor594-labeled secondary antibody (1:1,000). Finally, they were observed by confocal microscopy (Leica, Wetzlar, Germany) at 488 nm and 594 nm.

Enzyme linked immunosorbent assay (ELISA)

The supernatant of Caco-2 cells and mouse serum was collected and centrifuged at 3000 rpm for 25 min, and then cell supernatants were aspirated. TNF-α and IL-1β content were then evaluated by ELISA kits (Shenzhen, China), and OD values at 450 nm were detected by the automatic microplate reader (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

The data were analyzed with SAS software (edition 8.2, USA) and expressed as mean ± SD. Differences between multiple groups were analyzed by ANVOA followed by LSD test. Non-parametric data was analyzed by Mann-Wilcoxon test. $P < 0.05$ was considered statistically significant.

Results

EFL_1, EFL_2 and EFL_3 decrease the LXRα and ABCA1 expression

LXRα, the ligand-dependent nuclear receptor, regulates the transcription of ABCA1 to participate cholesterol metabolism and inflammation. According to Figure 2, EFL_2 and EFL_3 decreased LXRα mRNA expressions in Caco-2 cells ($P < 0.01$). Moreover, ABCA1 mRNA levels in Caco-2 cells was down-regulated by EFL_1 and EFL_2 ($P < 0.01$).

EFL_1, EFL_2 and EFL_3 inhibit cholesterol efflux

LXRα-mediated ABCA1 pathway regulate the transmembrane transport of cholesterol, thereby affecting the content of cholesterol and lipid rafts in cells. Figure 3 revealed that EFL_1 and EFL_3 markedly inhibited cholesterol efflux ($P < 0.01$).

EFL_1, EFL_2 and EFL_3 decrease lipid rafts abundance

Cholesterol, the main component of lipid rafts, possess a key position in lipid rafts formation, and cholesterol content will markedly affect lipid rafts abundance. Our studies showed that lipid rafts abundance in Caco-2 cells was decreased under EFL_1, EFL_2 and EFL_3 intervention ($P < 0.01$) (Figure 4).

EFL_1, EFL_2 and EFL_3 promote recruitments of TLR4 into lipid rafts

Lipid rafts are special domains on cell membranes that play a key role in TLR4-mediated pathway transduction and cytokines expression. The results in Figure 5 displayed that EFL_2 and EFL_3 increased the localization of TLR4 to lipid rafts ($P < 0.01$).

EFL_1, EFL_2 and EFL_3 induce the TLR4-mediated pathway transduction

Activation of TLR4 triggers downstream key target proteins expression, leading to NF-κB translocation into the nucleus, which in turn promotes NLRP3 assembly and activation. EFL_1 and EFL_2 significantly increased TLR4 and NLRP3 mRNA levels in colon of mice ($P < 0.05$). And treated with EFL_3 markedly increased NLRP3 and NF-κB p65 mRNA expressions ($P < 0.01$) (Figure 6).

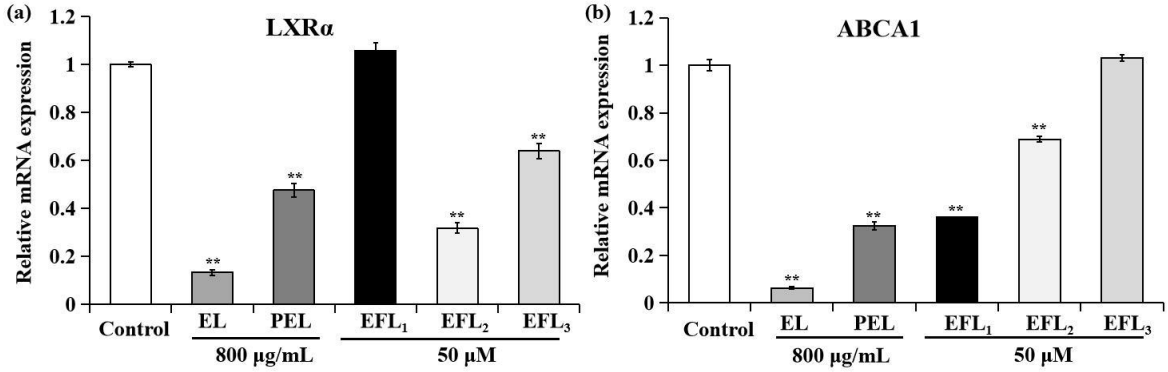


Figure 2 EFL_1, EFL_2 and EFL_3 decrease LXRα (a) and ABCA1 (b) expression (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* control group. EL, *Euphorbia lathyris* L.; EFL_1, Euphorbia factors L_1; EFL_2, Euphorbia factors L_2; EFL_3, Euphorbia factors L_3; PEL, processed *Euphorbia lathyris* L.; LXRα, Liver-X receptor Alpha; ABCA1, ATP-binding cassette transporter A1.

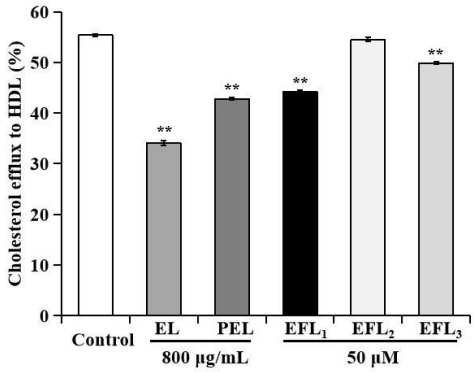


Figure 3 EFL_1, EFL_2 and EFL_3 inhibit cholesterol efflux (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* control group. EL, *Euphorbia lathyris* L.; PEL, processed *Euphorbia lathyris* L.; EFL_1, Euphorbia factors L_1; EFL_2, Euphorbia factors L_2; EFL_3, Euphorbia factors L_3.

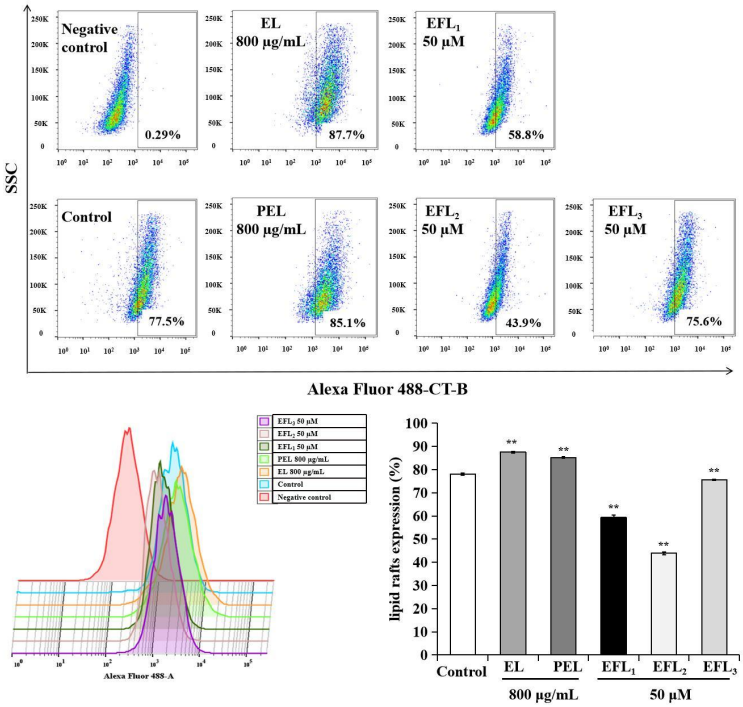


Figure 4 EFL_1, EFL_2 and EFL_3 decrease the lipid rafts abundance (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* control group. EL, *Euphorbia lathyris* L.; EFL_1, Euphorbia factors L_1; EFL_2, Euphorbia factors L_2; EFL_3, Euphorbia factors L_3; PEL, processed *Euphorbia lathyris* L.

EFL_1, EFL_2 and EFL_3 increase the levels of pro-inflammatory cytokines

IL-1β and TNF-α, the critical pro-inflammatory factors in early inflammatory response, activate a variety of cytokines and exert an irreplaceable role in inflammation. Our research revealed that the contents of IL-1β in Caco-2 cells were increased by EFL_1, EFL_2 and EFL_3 ($P < 0.01$) (Figure 7a, 7b). The levels of TNF-α and IL-1β were increased by EFL_1, EFL_2 and EFL_3 in mouse serum ($P < 0.01$) (Figure 7c, 7d).

Discussion

ABCA1 is not only an important transporter for LXRα to regulate cholesterol metabolism, but also a key mediator to exert anti-inflammatory activity [19]. LXRα interfere with membrane cholesterol efflux by regulating the expression of ABCA1, thus affecting lipid raft structure and its mediated TLR4 pathway conduction [11, 20]. Our results showed that EFL_1, EFL_2 and EFL_3 markedly inhibited LXRα and ABCA1 expression. However, ABCA1, ABCG1, ABCG4, and ABCG5/8 both are the downstream receptor proteins of LXR that regulate cholesterol efflux and mediate reverse cholesterol transport [21]. Other nuclear receptors, such as peroxisome proliferation-activated receptors, Farnesoid X receptor can also form heterodimers with retinol X receptor to directly modulate ABCA1 [21–23]. It is hypothesized that LXRα-ABCA1 axis is not the only way by which these three components affect cholesterol efflux, there are multiple other targets that were also regulated by EFL_1, EFL_2 and EFL_3.

LXRα and ABCA1, the key proteins in cholesterol transmembrane transport, participate in the regulation of intracellular cholesterol content and further regulate the function of lipid rafts [24, 25]. Our results displayed that EFL_1, EFL_2 and EFL_3 affected cholesterol efflux and lipid rafts abundance in Caco-2 cells, which revealed that the regulation of inflammation by EFL_1, EFL_2 and EFL_3 may be associated with cholesterol efflux and lipid rafts abundance.

Lipid rafts are sites for recruitment of signaling proteins, and exert an irreplaceable position in TLR4-mediated signal transduction [26]. TLR4 activation is recruited to lipid rafts, contributing to conduction of downstream pathways and release of proinflammatory cytokines. In order to further elucidate the underlying mechanism by which three diterpenoid esters from EL induce the intestinal inflammation, we investigated the regulation of EFL_1, EFL_2 and EFL_3 on co-localization of lipid rafts and TLR4. The results showed that these three components promoted recruitment of TLR4 to lipid rafts through cell membrane microregion lipid rafts, thus inducing intestinal inflammatory response.

According to literature, TLR4 activation induces NF-κB translocation to nucleus, which activates NLRP3 and promotes the maturation and release of pro-inflammatory factors [27]. In this research, we found that EFL_1, EFL_2 and EFL_3 increased TLR4, NLRP3, NF-κB p65, TNF-α and IL-1β expression. According to literature, the cytoplasmic tail of TLR4 and other members of Toll-like receptor family contains a TIR domain, which can recruit various of TIR-containing receptor proteins upon activation [28, 29]. The activation of these critical intracellular signaling pathways can further activate IKK, NF-κB p65, MAPKs, ERK, JNK, PI3K, AKT, IRF3 protein, and ultimately induce the release of cytokines [30, 31]. Therefore, we speculate that EFL_1, EFL_2 and EFL_3 may also induce the intestinal inflammation by interfering with other inflammatory signaling pathways and inflammatory cytokines, and acting in combination with multiple signaling pathways.

Based on the above, the present study revealed that the molecular mechanism of EFL_1, EFL_2 and EFL_3 in regulating intestinal inflammation is related to LXR/ABCA1-regulated lipid raft and TLR4-mediated pathway, but there also exist some shortcomings in this study. Firstly, we only validated three main active components in EL, and did not investigate the synergistic effects of these three components, and other active components and metabolites in EL. Secondly, PXR, ABCG1, MAPK, PI3K, and other pathways that are important in inflammation still need to be more comprehensively investigated. Furthermore, it has been reported that diterpenoid esters from EL show significant anti-tumor activities. Increasing evidence showed that cholesterol-rich cellular microregion lipid raft domains may be potential innovative targets for cancer therapy, and regulation of lipid rafts in tumor cells by cholesterol may be an ideal way to inhibit tumor angiogenesis and prevent cancer metastasis [32, 33]. However, whether cholesterol-mediated lipid rafts also potential anti-tumor targets of EFL_1, EFL_2 and EFL_3 is still unclear, and the

relationship between the regulation of cholesterol and lipid rafts by these components and inflammation and tumor is worthy of further study.

Conclusion

Herin, we investigated the mechanisms by which EFL_1, EFL_2 and EFL_3 induce the intestinal inflammation (Figure 8). Our studies demonstrate that these three components inhibited LXRα/ABCA1 pathway and cholesterol efflux, promoting TLR4 recruitment to lipid rafts, and ultimately inducing TLR4-mediated axis conduction contained in lipid rafts. This study preliminarily revealed that the effect of main diterpenoid esters from EL on intestinal inflammation may be associated with LXRα/ABCA1-regulated lipid rafts and TLR4-mediated pathway. It provides novel understanding about the material basis research and clinical safe application of EL.

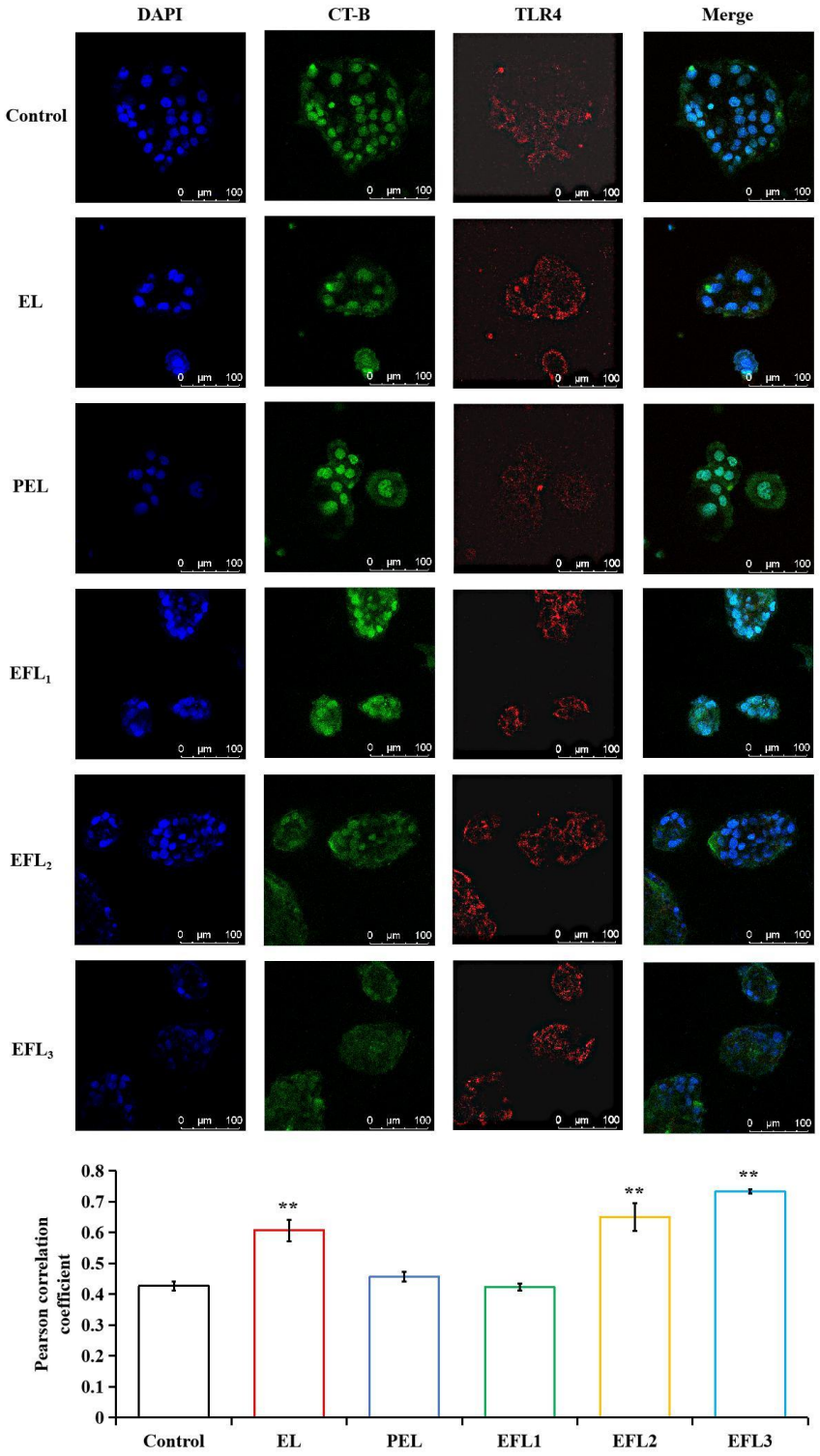


Figure 5 EFL_1, EFL_2 and EFL_3 promote recruitments of TLR4 into lipid rafts (n = 3) (× 100). The lipid rafts were dyed green, TLR4 was red, and nucleus was blue. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* control group. EL, *Euphorbia lathyris* L.; EFL_1, Euphorbia factors L_1; EFL_2, Euphorbia factors L_2; EFL_3, Euphorbia factors L_3; PEL, processed *Euphorbia lathyris* L.; CT-B, cholera toxin-B; TLR4, toll-like receptor 4.

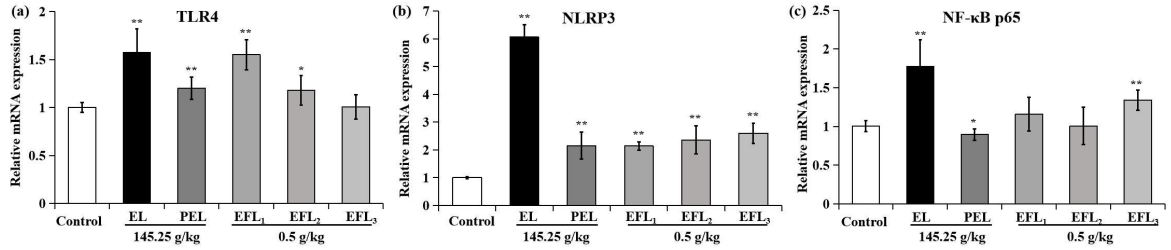


Figure 6 EFL_1, EFL_2 and EFL_3 increase the mRNA expression of TLR4 (a), NLRP3 (b) and NF-κB p65 (c) (n = 6). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* control group. EL, *Euphorbia lathyris* L.; EFL_1, Euphorbia factors L_1; EFL_2, Euphorbia factors L_2; EFL_3, Euphorbia factors L_3; PEL, processed *Euphorbia lathyris* L.; TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa-B; NLRP3, NOD-like receptor family Pyrin domain containing 3.

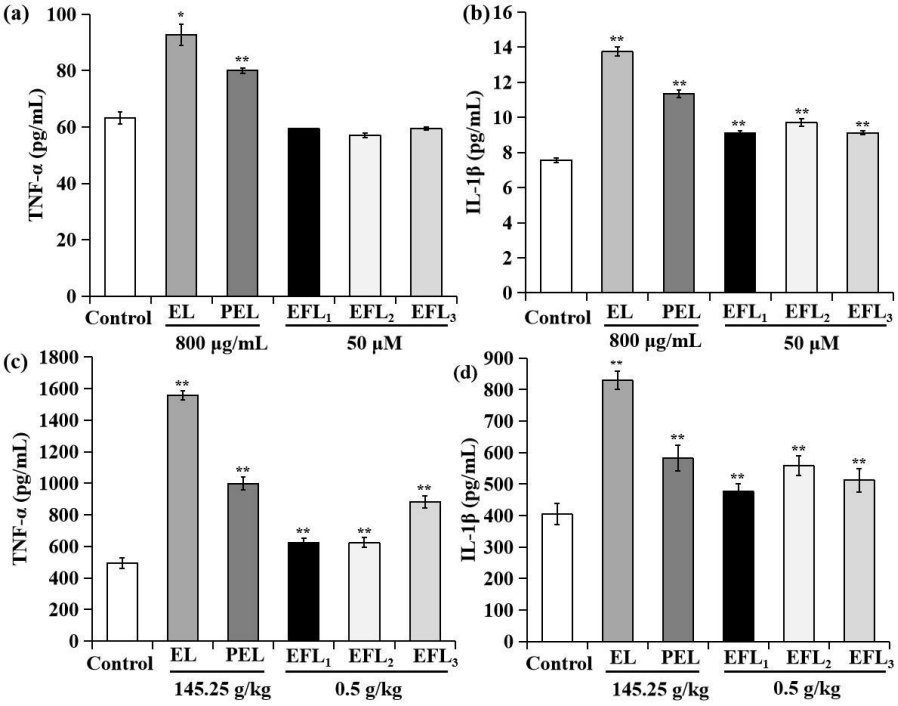


Figure 7 EFL_1, EFL_2 and EFL_3 increase the levels of pro-inflammatory cytokines in Caco-2 cells (a, b) (n = 2) and in mouse serum (c,d) (n = 6). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* control group. EL, *Euphorbia lathyris* L.; EFL_1, Euphorbia factors L_1; EFL_2, Euphorbia factors L_2; EFL_3, Euphorbia factors L_3; PEL, processed *Euphorbia lathyris* L.; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

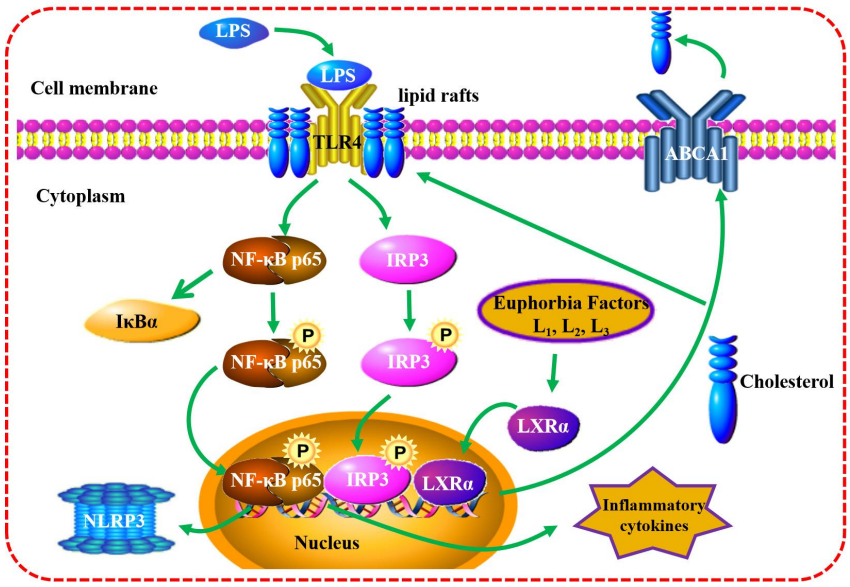


Figure 8 The diterpenoid esters from EL induce the intestinal inflammatory response via LXRα/ABCA1-regulated lipid rafts and TLR4-mediated pathway. TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa-B; NLRP3, NOD-like receptor family Pyrin domain containing 3; LXRα, Liver-X receptor Alpha; ABCA1, ATP-binding cassette transporter A1.

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