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# *Liquidambaris fructus* inhibits osteosarcoma through PTGS2/TGFB1 and regulates efferocytosis.

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**Abstract**: **Background**: Liquidambaris Fructus can play an anti-tumor role by triggering cell cycle blockade and apoptosis and inhibiting cancer cell proliferation. **Objectives**: To evaluate the effects and potential mechanism of Liquidambaris Fructus on 143B cells and MNNG/HOS CI #5 cells. **Methods**: Cell viability of 143B cells and MNNG/HOS CI #5 cells was evaluated using the CCK-8 assay. A transwell cell migration assay was performed to detect cell migration and invasion. Cell apoptosis was assayed by flow cytometry. Network pharmacology method was used to predict the potential targets and pathway of Liquidambaris Fructus against osteosarcoma. Protein levels were determined using Western blot, while mRNA levels were detected using RT-qPCR analysis. In vitro efferocytosis assay of apop143B-MHSW and apopMNNG-HMDMs was detected by flow cytometry. **Results**: Liquidambaris Fructus can effectively increase 143B and MNNG/HOS CI #5 cell apoptosis levels and inhibit 143B and MNNG/HOS CI #5 cell viability, migration and invasion. Network pharmacology showed that PTGS2 and TGFB1 were two targets related to the enriched efferocytosis pathway. Liquidambaris Fructus inhibited proteins and mRNA of PTGS2 and TGFB1 expression levels in 143B and MNNG/HOS CI #5 cells. Liquidambaris Fructus inhibited PTGS2 and TGFB1 levels in the co-culture of osteosarcoma cells and macrophages. Liquidambaris Fructus inhibited efferocytosis. **Conclusion**: Liquidambaris Fructus can target PTGS2 and TGFB1 to inhibit osteosarcoma cell growth and metastasis, as well as to inhibit efferocytosis, thus alleviating osteosarcoma.

**Keywords**: Efferocytosis; *Liquidambaris fructus*; Mechanism; Network pharmacology; Osteosarcoma

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## INTRODUCTION

Osteosarcoma (OS) is a primary malignant tumor caused by osteogenic mesenchymal cells (Li, *et al*. 2020), and presents a high prevalence in children and adolescents. The disease is characterized by strong local aggressiveness and early distant metastasis (Lin, *et al*. 2022). Distant terminal metastases have occurred in approximately 20% of patients when diagnosed (Ritter and Bielack 2010). Worldwide, the incidence of osteosarcoma is about 1 to 3 cases per million people per year (Zhao, *et al*. 2019). The therapeutic effect and the prognosis of osteosarcoma have improved considerably after neoadjuvant chemotherapy combined with surgical treatment (Cersosimo, *et al*. 2020). With the combination therapy regimen, patients can achieve a 5-year survival rate of 78%, but for the metastasis and recurrence patients, the 5-year survival rate is only 20% (Gaspar, *et al*. 2018; Kansara, *et al*. 2014). In the process of treating osteosarcoma, patients may develop resistance to commonly used chemotherapies, which have a serious impact on a patient's life (Chen, *et al*. 2021; Hansen, *et al*. 2006). Therefore, it is particularly important to develop safe and effective novel drugs to treat osteosarcoma.

*Liquidambaris fructus* (LF), also called Lu Lu Tong, is the dry infructescences of *Liquidambar formosana Hance* (Li, *et al*. 2021; Qian, *et al*. 2020). They are mainly distributed in East China, South China, Southwest China, etc. It was first recorded in Compendium of Materia Medica. Phytochemical studies on the resin of *Liquidambar formosana* were carried out and five undescribed pentacyclic triterpenes were isolated and identified. (Zhu, *et al*. 2021). Previous studies have shown LF has anti-tumor, hepatoprotective and anti-inflammatory properties. In addition, LF has been demonstrated to inhibit a variety of tumor cells (Wang, *et al*. 2022b). Meanwhile, reports have demonstrated that LF can play an anti-tumor role by triggering cell cycle blockade and inhibiting cancer cell proliferation (Li, *et al*. 2021). Pharmacologic studies of LF have shown that treatment with LF can effectively reduce levels of the inflammatory factors IL-6, IL-1β, and TNF-α (Lin, *et al*. 2022). Inhibition of Prostaglandin-endoperoxide synthase 2 (PTGS2) and transforming growth factor-β (TGF-β) is critical for their anti-inflammatory efficacy. However, the lack of evidence on the mechanism of drug action limits the use of LF in modern clinical practice. Our study aims to investigate the effect and mechanism of LF on cell viability, apoptosis, migration and invasion of 143B cells and MNNG/HOS CI #5 cells. Network pharmacology was applied to predict the potential targets of LF on osteosarcoma and the major pathways likely to be involved. The effects of LF on efferocytosis were explored with *in-vitro* experiments.

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## MATERIALS AND METHODS

### *Cell culture*
Murine alveolar macrophage cell line MHS (SUNNCELL) and human monocyte-derived macrophages HMDMs (SUNNCELL). Both cells were incubated in RPMI 1640 medium (Yuanye, Shanghai, China) supplemented with 10% fetal bovine serum (Gibco, Grand Island, U.S.A.) and 1% penicillin/streptomycin (Gibco, Grand Island, U.S.A.). The incubation conditions were 37°C and 5% $CO_2$. 143B cells and MNNG/HOS CI #5 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (Yuchun, Shanghai, China). The incubation conditions were 37°C, and 5% $CO_2$.

### *Preparation of Liquidambaris Fructus extracts*
*Liquidambaris fructus* was purchased from Jiangzhong Pharmaceutical Co., Ltd. (Nanchang, China). The quality of *Liquidambaris fructus* was identified using chromatographic fingerprint(GC-8860, Agilent). *Liquidambaris fructus* (800 g) was ground. LF powder was extracted with 6 times 95% ethanol (w/v) reflux 3 times. Ethyl acetate and petroleum ether were used to concentrate the extract. Finally, the three concentrated extracts obtained were dried and mixed well (Li, *et al*. 2021).

### *Animal selection*
Thirty rats were randomly divided into five groups (n=6). The extract was dissolved in distilled water. Three groups of rats were given 75 mg/kg/day, 100 mg/kg/day, and 130 mg/kg/day, respectively (Li, *et al*. 2021). The drug was administered by gavage for 20 consecutive days. Rats were euthanized one hour after the last dose. The venous blood was obtained to prepare serum, assigned as low serum, moderate serum, and high serum, respectively. The control group was given an equal amount of physiological saline solution, assigned as serum-ctl.

### *Cell viability assay*
Logarithmic growth phase 143B cells (Biobw, Beijing, China) and MNNG/HOS CI #5 cells (KALANG, Shanghai, China) were inoculated in 96-well plates. The cells were cultured until cell attachment and then treated with the serum from rats that received different concentrations of LF (75 mg/kg/day, 100 mg/kg/day, and 130 mg/kg/day). After continuing the incubation for 48 h at 37ºC, a dose of CCK-8 reagent was added to each well. After 2 h, the absorbance of cells in each well was measured at 450 nm using an Agilent Synergy H1 multifunction microplate reader (Agilent Technologies, Santa Clara, USA), and cell viability was calculated.

### *Cell apoptosis assay*
Logarithmic growth phase: 143B cells and MNNG/HOS CI #5 cells were inoculated in 96-well plates and cultured for 24 h. The cells were treated with LF serum, centrifuged at 4℃ for 10 min, and resuspended in 500 μL binding buffer. Then, 5 μL Annexin V-FITC and 5 μL PI were added to each sample tube, followed by incubation for 15 min at room temperature away from light (Qi, *et al*. 2024). Finally, we used flow cytometry to detect the cells.

### *Cell migration assay*
The 143B cells and MNNG/HOS CI #5 cells were resuspended and then 100 μL of cell suspension was added to the upper chamber of the Transwell. 500 μL of serum-containing medium was added to the lower chamber of the Transwell. The devices were then incubated overnight at 37°C, 5% $CO_2$ (Wang, *et al*. 2024). After culture, cells that had not migrated into the upper chamber were gently wiped away. The cells were then fixed and stained. The migrated cells were detected at Ex/Em=530/590 nm(Li, *et al*. 2018).

### *Cell invasion assay*
The Matrigel was spread in the upper chamber of the Transwell. Then, the invasion assay was performed as described in the cell migration assay.

### *Network pharmacology analysis*
Pharmacokinetic properties for natural compounds in LF were searched in the TCMSP database, involving oral bioavailability (OB) and drug-likeness (DL). The potential pharmaceutical components of LF were filtered using the drug screening criteria of OB≥20 and DL≥0.18. The relationships between components and targets were captured using TCMSP and HERB databases. The OMIM and GeneCards databases were searched using the keyword "osteosarcoma" to collect targets related to osteosarcoma.

The genes with more than five times the average Relevance score (8.38) were collected (Wang, *et al*. 2022a). The intersection of the two sets of targets was taken using Venny 2.1.0. GO and KEGG enrichment analysis of intersecting targets using R language, to find out the major cellular components (CC), molecular functions (MF), biological processes (BP), and pathways involved in the intersecting targets. Then, the herb-compound-target-all pathways and herb-compound-target-top 10 pathway networks were constructed using Cytoscape software.

### *Western blot analysis*
143B cells and MNNG/HOS CI #5 cells were collected and RIPA lysate was added to each group of cells. Using a BCA kit to test the protein concentration. Using SDS-PAGE to separate proteins and followed by Western blot analysis. The gels were stained with a vinylidene difluoride membrane and then exposed to the corresponding antibodies (PTGS2, TGF-β1). After the membrane was washed, the II antibody was added for 2h at 25ºC. The film was washed and developed. Finally, ImageJ1.8.0 calculated the grayscale value.

### *RT-qPCR analysis*
Total RNA from 143B cells and MNNG/HOS CI #5 cells was extracted using Trizol reagent (ThermoFisher, Beijing,

China), and cDNA was synthesized by reverse transcription. Primer sequences were synthesized by Beijing Qingke Biological Co. PCR amplification was performed using GAPDH as an endogenous reference gene. The relative expression of the target gene was calculated using $2^{-\Delta\Delta Ct}$. Mouse ptgs2 primers: 5'-TTCAACACACTCTATCACTGGC-3' (sense), and 5'-AGAAGCGTTTGCGGTACTCAT-3' (antisense). Mouse tgfb1 primer: 5'-CACAGAGAAGAACTGCTGTG-3' (sense) and 5'-AGGAGCGCACAATCATGTTG-3' (antisense). Mouse β-actin (reference gene) primers: 5'-GGCTGTATTCCCCTCCATCG-3' (sense), and 5'-CCAGTTGGTAACAATGCCATGT-3' (antisense). Human PTGS2 primers 5'-CCTGTGCCTGATGATTGC-3' (sense) and 5'-CTGATGCGTGAAGTGCTG-3' (antisense). Human TGFB1 primers: 5'-GGCTACCATGCCAACTTCT-3' (sense) and 5'-CCGGGTTATGCTGGTTGT-3' (antisense). Human β-actin (reference gene) primers: 5'-AGAGGGAAATCGTGCGTGAC-3' (sense), and 5'-CAATAGTGATGACCTGGCCGT-3' (antisense).

***Detection of PEG2 release***

Using a PGE2 ELISA kit (SINOBESTBIO, Shanghai, China), the levels of PGE2 in the culture medium were detected. The experimental procedure was carried out in accordance with the manufacturer's requirements.

***Measurement of extracellular TGF-β1***

A TGF-β1 ELISA kit (Yiyan Bio-technology Co., Shanghai, China) was used to detect the levels of TGF-β1 in the cell supernatants. The experimental procedure was performed according to the manufacturer's requirements, and calculated as pg/mL.

***In-vitro efferocytosis assay***

Apoptotic 143B cells (apop143B) and MNNG/HOS CI #5 (apopMNNG) were induced by 400 μW/cm$^2$ UV for 2 h. Using an annexin V Apoptosis Detection Kit with PI (YaJiBiological) to test the apoptosis and exposure of phosphatidylserine on the cell surface. Apoptotic 143B cells and MNNG/HOS CI #5 cells were labeled with IncuCyte pHrodo red labeling reagent (BIOCREATIVE TECHNOLOGY CO) according to the kit instructions. After apoptotic 143B cells or MNNG/HOS CI #5 cells were added to the coincubation for 1 h, unengulfed cells were washed away. The MHS or HMDMs that captured apoptotic cells (CFSE positive cells)-henceforth 143Bpo-MHS or MNNGpo-HMDMs- were purified by sorting and quantified.

***Statistical analysis***

The data were expressed as (Mean±SD). Data analysis was performed using Graphpad Prism 9.0. Unpaired t-test, one-way, or two-way analysis of variance was performed. $p < 0.05$ was statistically significant.

## RESULTS

***Effects of Liquidambaris fructus on osteosarcoma cells***

CCK-8 results showed that cells were exposed to LF with different drug regimens for 48 h. The cell viability of 143B cells and MNNG/HOS CI #5 cells progressively decreased with increasing LF drug concentration compared to the serum-ctl group ($p < 0.001$, Fig. 1A). The apoptosis results showed that the apoptosis rate was significantly higher in experimental groups after LF treatment compared to the serum-ctl group; the effect of elevated apoptosis rate was most significant in the high serum group ($p < 0.001$, Fig. 1B). Meanwhile, the effect of LF on 143B cells and MNNG/HOS CI #5 cells were explored using cell migration and invasion assays. The results of the cell migration assay showed that the cell migration rate was significantly decreased in all groups after treatment with LF compared to the serum-ctl group, and the reduction effect was most significant in the high serum group ($p < 0.001$, Fig. 1C). The results of cell invasion experiments showed that the cell invasion rates of all groups after treatment with LF were significantly reduced, and the reduction effect was most significant in the high serum group ($p < 0.001$, Fig. 1D). Therefore, LF may be effectively suppress the viability, migration and invasion of osteosarcoma cells and increase the apoptosis rate of osteosarcoma cells.

***Prediction of potential targets of Liquidambaris fructus on osteosarcoma***

A total of seven potential pharmaceutical components were collected in LF using the TCMSP database (Table 1). These potential pharmaceutical components targeted 63 genes and 91 pathways (Fig. 2). Using Venny 2.1.0., we obtained 5 shared key targets between LF targets and osteosarcoma-associated genes (Fig. 3A). After KEGG pathway enrichment analysis, it was found that these five shared targets were mainly enriched in the Pathways in cancer, Apoptosis-multiple species, efferocytosis, etc (Fig. 3B). GO enrichment analysis showed that the BP mainly involved response to steroid hormone, response to estradiol, and epithelial tube morphogenesis; CC analysis showed that the targets were mainly involved in the organelle outer membrane, outer membrane, and nuclear membrane; MF consisted of protease binding, cytokine receptor binding, and cysteine-type endopeptidase activity involved in apoptotic (Fig. 3C, Fig. S1). After the construction of the herb-components-target-top 10 pathway network, PTGS2, and TGFB1 were found to be involved in the efferocytosis pathway (Fig. 3C, Fig. S2).

***Effect of Liquidambaris fructus on PTGS2 and TGFB1 expression levels in osteosarcoma cells***

To explore the effect of LF on the expression of PTGS2 and TGFB1 in osteosarcoma cells, mRNA and protein levels of PTGS2 and TGF-β1 after LF treatment were detected.

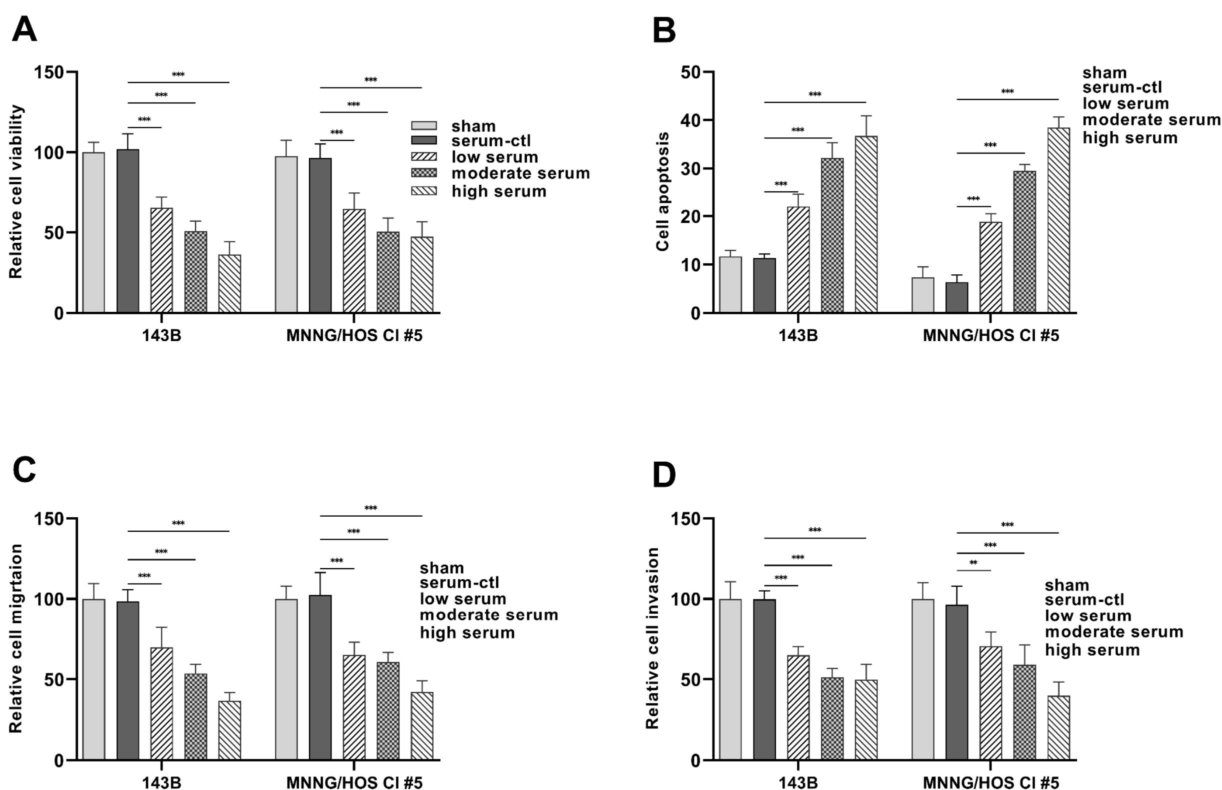


**Fig. 1**: Effects of *Liquidambaris fructus* on osteosarcoma cells.
(A) Cell viability was assayed by the CKK-8 method. (B) Cell apoptosis was assayed using flow cytometry analysis. (C) Cell migration was tested by Transwell assay. (D) Cell invasion was detected by the Transwell assay. ***$p < 0.001$ vs. serum-ctl.

**Table 1**: The potential active chemical substances in *Liquidambaris fructus*.

| Mol ID | Molecule Name | Molecular weight | Oral bioavailability (%) | Drug-likeness |
|---|---|---|---|---|
| MOL000263 | oleanolic acid | 456.78 | 29.02 | 0.75 |
| MOL000357 | sitogluside | 576.95 | 20.63 | 0.62 |
| MOL000358 | beta-sitosterol | 414.79 | 36.91 | 0.75 |
| MOL000516 | bornyl cinnamate | 284.43 | 21.52 | 0.21 |
| MOL000517 | isostyracin epoxide | 280.34 | 92.52 | 0.22 |
| MOL000519 | coniferin | 314.41 | 31.1 | 0.32 |
| MOL000095 | delta 7-stigmastenol | 416.81 | 25.32 | 0.74 |

Western blot showed that the treatment of LF inhibited the mRNA and protein levels of PTGS2 ($p < 0.001$, Figs. 4A and 4B). Similarly, Western blot results showed that LF treatment inhibited the protein expression level of TGFB1 mRNA and TGF-β1 protein ($p < 0.001$, Fig. 4C and 4D).

***Effect of Liquidambaris fructus on efferocytosis in-vitro***

After co-culturing apoptotic 143B cells (human) with MHS (rats) cells, the primers for detecting PTGS2 and TGFB1 were designed based on mouse mRNA sequences. Ptgs2 and Tgfb1 mRNA come from macrophages rather than engulfed apoptotic cells. The decrease of Ptgs2 and Tgfb1 mRNAs in MHS macrophages was examined ($p < 0.001$, fig. 5A and 5B). In addition, LF treatment effectively reduced *in-vitro* efferocytosis of MHS ($p < 0.001$, Fig. 5C). In addition, when apoptotic MNNG/HOS CI #5 cells co-cultured with HMDMs cells, the release of PEG2 and TGF-β1 was reduced by LF ($p < 0.001$, Figs. 5D and 5E). In addition, LF decreased the *in-vitro* efferocytosis of HMDMs that co-cultured with apoptotic MNNG/HOS CI #5 cells ($p < 0.001$, Fig. 5F).

## DISCUSSION

The main cause of the recurrence of osteosarcoma is the malignant proliferation, migration, and invasion of osteosarcoma cells (Kansara, *et al*. 2014; Wolf-Dennen, *et al*. 2020).

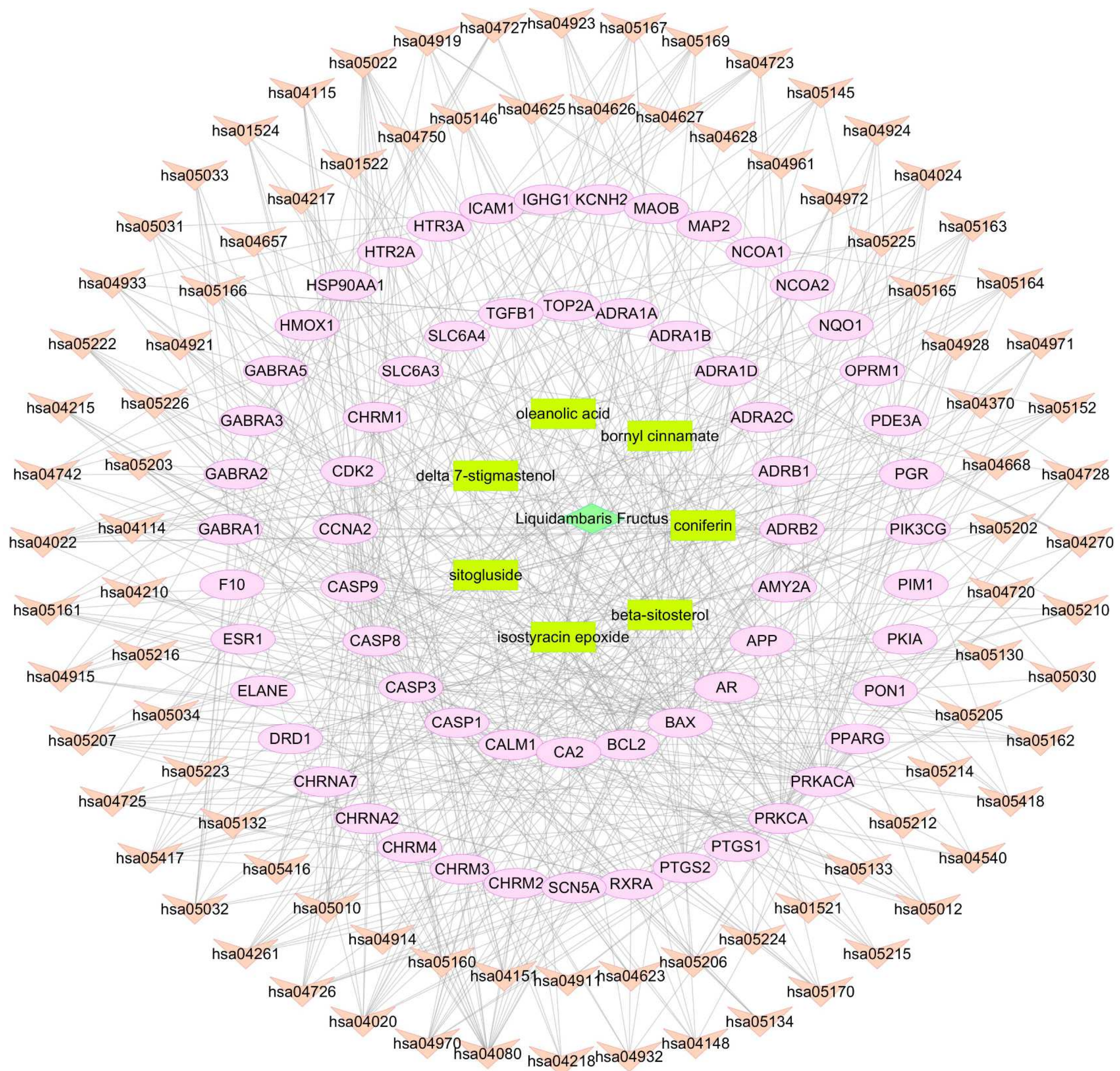


**Fig. 2**: Prediction of potential targets of *Liquidambaris fructus* on osteosarcoma.
Herb-compound-all target-pathway. The pathway with p.adjust less than 0.05 was included.

At present, first-line chemotherapeutic agents, azithromycin and cisplatin, mainly inhibit the growth of osteosarcoma cells through promoting apoptosis and inhibition of cell migration and invasion (Goel, *et al*. 2018; Shoaib, *et al*. 2022). LF is not only used to treat various inflammatory conditions but also plays an important role in the treatment of certain cancers (Ahmed, *et al*. 2020; Qian, *et al*. 2020). However, little research has been reported on LF for the treatment of osteosarcoma. Therefore, our study aimed to elucidate the mechanism of action of LF against osteosarcoma. In our study, it was observed that cell viability, migration and invasion were inhibited, while apoptosis was increased in 143B cells and MNNG/HOS CI #5 cells after treatment with LF-containing serum from rats that received different concentration of LF (75 mg/kg/day, 100 mg/kg/day, and 130 mg/kg/day). A network pharmacology method was used to analyze potential targets and the pathways of LF against osteosarcoma. The potential mechanism of LF in alleviating osteosarcoma was predicted to inhibit efferocytosis (fig. 3D). Osteosarcoma cells were redirected to immunogenic secondary necrosis (Wu, *et al*. 2023). They release danger signals and associated antigens to activate innate and adaptive anti-tumor immune responses to achieve therapeutic effects. Therefore, in this study, our team worked to demonstrate that LF inhibits efferocytosis to prevent immunosilencing apoptotic osteosarcoma cells from being cleared too quickly.

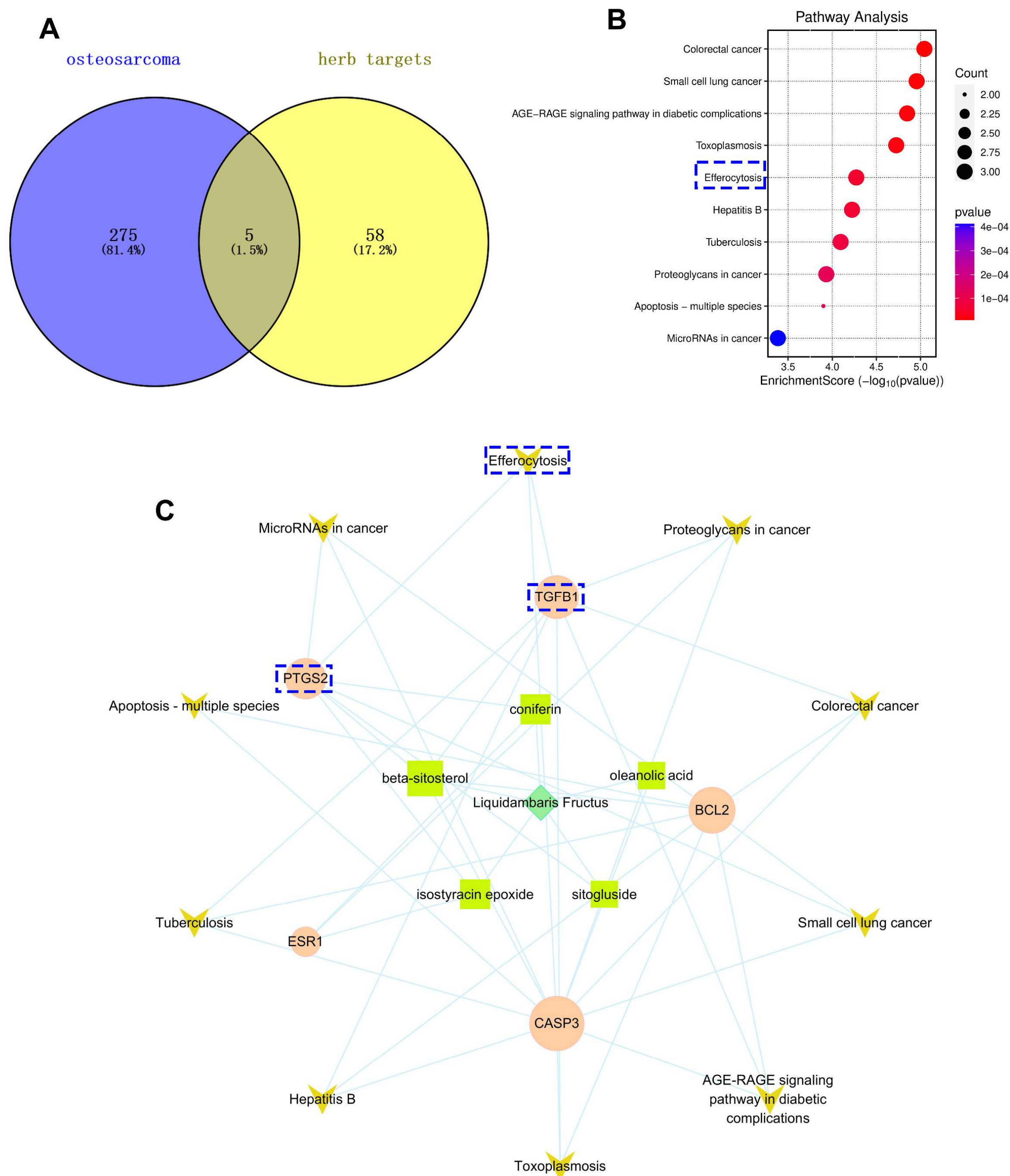


**Fig. 3**: Prediction of potential pathway of *Liquidambaris fructus* on osteosarcoma.
(A) Venn diagram of predicted *Liquidambaris fructus* targets and osteosarcoma genes from TCMSP, HERB and GeneCards databases. (B) The top 10 markedly enriched signaling pathways were analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) clustering analysis. (C) The herb-compound-disease target-top 10 pathway network.

PTGS2 is mainly found in tumor cells, inflammatory cells, and damaged tissues (Geesala, *et al*. 2019; Ohnesorge, *et al*. 2021). TGF-β regulates cell growth and differentiation (Wu, *et al*. 2022; Zhang, *et al*. 2023). TGF-β is found to be detectable in almost all tumors (Chen, *et al*. 2015). It was demonstrated that LF can be effective in relieving osteosarcoma via targeting PTGS2 and TGF-β.

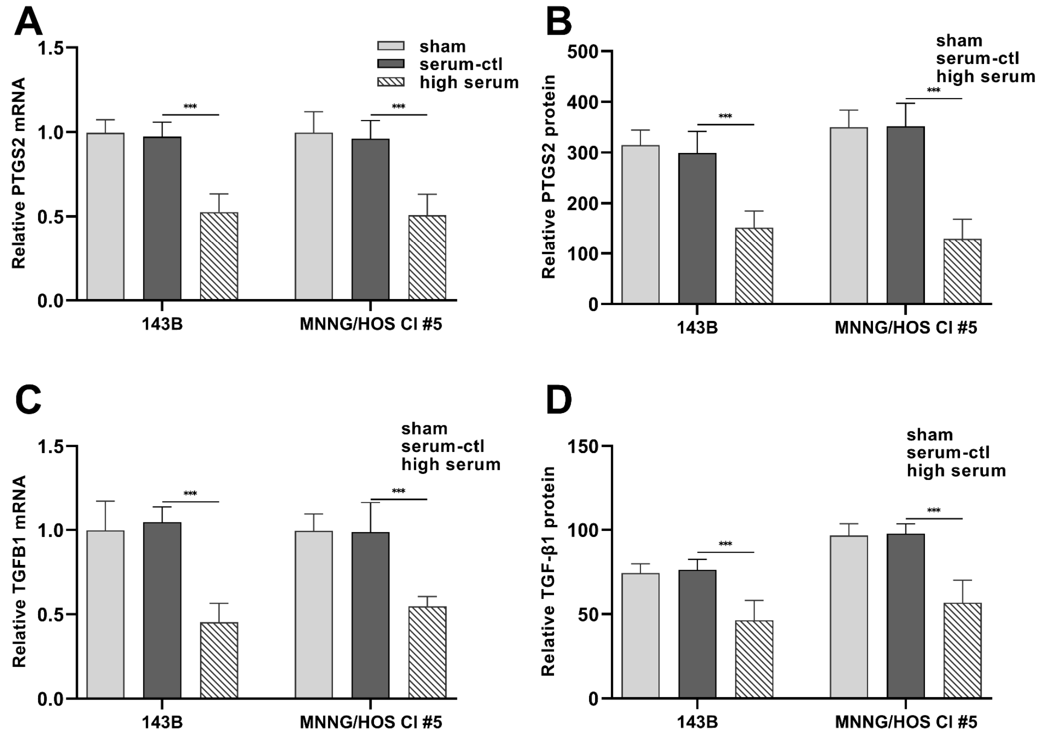


**Fig. 4**: Effect of *Liquidambaris fructus* on 143B cells and MNNG/HOS CI #5 cell protein expression levels.
(A) PTGS2 mRNA expression levels were tested by RT-qPCR. (B) PTGS2 protein levels were tested by Western blot. (B) PEG2 release was determined by the PGE2 ELISA kit. (C) TGFB1 mRNA expression was detected by RT-qPCR. (D) TGF-β1 protein levels were detected by Western blot.

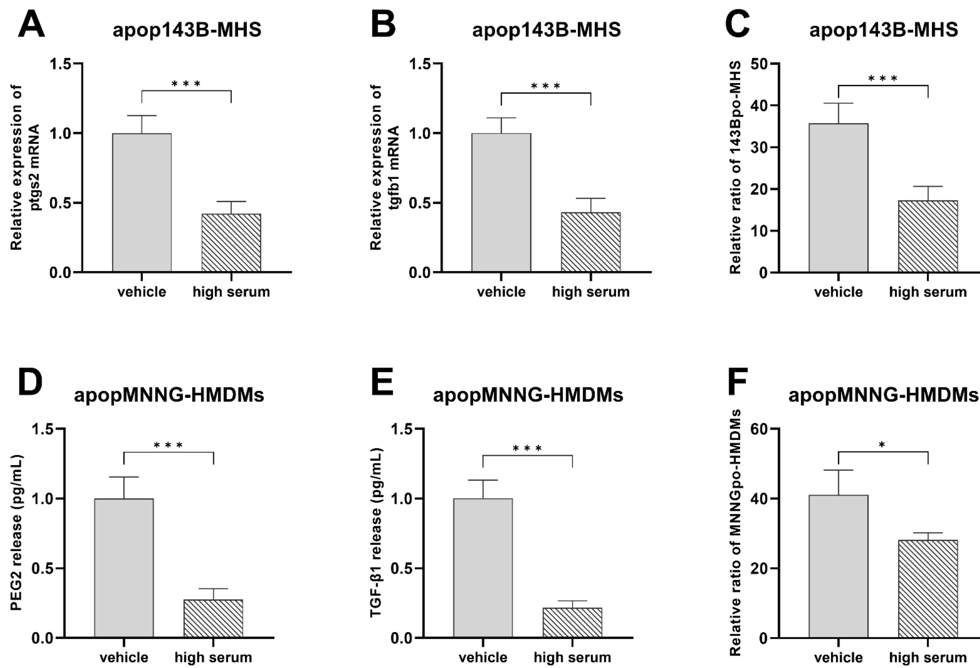


**Fig. 5**: Effect of *Liquidambaris fructus* on the level of efferocytosis *in-vitro*
ptgs2 (A) and tgfb1 (B) mRNA expression levels in apop143B-MHS co-culture were detected by RT-qPCR. (C) *In-vitro* efferocytosis assay of MHS was detected by using an Apoptosis Detection Kit. PTGS2 (D) and TGFβ1 (E) mRNA expression levels in apopMNNG-HMDMs coculture were detected by RT-qPCR. (F) *In-vitro* efferocytosis assay of HMDMs was detected by using an Apoptosis Detection Kit.

Firstly, the expression of PTGS2 and TGF-β1 in 143B and MNNG/HOS CI #5 cells was determined. The results showed that LF could significantly reduce the expression of PTGS2 and TGF-β1 (Fig. 4A-C). Then the release of rate PEG and TGF-β were detected. The results of this experiment showed the same trend of change as the results of the Western blot. Therefore, LF may effectively treat osteosarcoma. Efferocytosis is the process by which cells undergo programmed death and are further engulfed and removed by phagocytes (Boada-Romero, *et al*. 2020; Doran, *et al*. 2020; Mehrotra and Ravichandran 2022). Efferocytosis affects TAM in three main ways: (1) promoting the secretion of anti-inflammatory factors such as IL-10 and TGF, and suppressing the secretion of pro-inflammatory factors such as IL-1 and TNF-α (Cheng, *et al*. 2022). (2) Promoting TAM polarisation to the M2 phenotype. (3) Regulation of the activation and maturation of immune cells (Zhang, *et al*. 2022). Previous studies have shown that efferocytosis promotes inflammation reduction while also producing immunosuppression (Nitahara-Kasahara, *et al*. 2023). Previous studies in colon and breast cancer. Efferocytosis enhances the release of immunosuppressive factors (Huang, *et al*. 2020; Werfel, *et al*. 2019). Therefore, macrophages and osteosarcoma were co-cultured to study *in-vitro* efferocytosis. The results showed that the treatment of LF effectively suppressed the mRNA expression of PTGS2 and TGFβ1 in the apop143B-MHS assay (Fig. 5A-B). Meanwhile, the experimental results of *in-vitro* efferocytosis level showed that the treatment of LF significantly inhibited the efferocytosis level (fig. 5C). Delightedly, the apopMNNG-HMDMs experiments had the same trend of change (Fig. 5D-F). Therefore, LF may effectively treat osteosarcoma by inhibiting efferocytosis.

Although this study has made preliminary findings on the treatment of osteosarcoma with LF through both cell experiments and animal experiments, there are still the following limitations. Firstly, cell experiments are conducted in highly controlled in vitro environments, where specific biological mechanisms can be effectively observed. However, they cannot fully simulate the complex microenvironment within the human body, such as intercellular interactions and systemic physiological regulation. This may affect the applicability of the research conclusions in the human body. Secondly, although animal experiments provide a model that is closer to the overall situation, there are significant differences among species. For instance, metabolic rates and immune responses are different from those in humans, making it difficult to directly generalize the experimental results to clinical practice. Furthermore, the sample size may be limited, especially in the animal group, which will affect the statistical power and the reliability of the conclusions. These limitations suggest that the current findings should be used with caution in human scenarios, and further studies are needed to verify them. Future research will focus on the following directions to further enhance the research outcomes: (1) Validate the mechanism in more diverse animal models, especially in large animals or primate models, to reduce interspecies differences and enhance the translatability of the results. (2) Develop more complex in vitro systems, such as organoids, to integrate the key factors of cell-microenvironment interaction and enhance the physiological relevance of cell experiments. (3) Expand the sample size and collect diverse data through cross-institutional cooperation to reduce selection bias and enhance statistical efficacy.

## CONCLUSION

LF can effectively increase 143B and MNNG/HOS CI #5 cell apoptosis levels and inhibit cell viability, migration and invasion. Experiments with co-cultures of macrophages and osteosarcoma cells have shown that LF may inhibit efferocytosis to prevent immune-silencing apoptotic osteosarcoma cells from being cleared too quickly. However, the action mechanism of LF treatment of osteosarcoma remains to be further elucidated *in vivo*.

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None.

***Authors' contribution***
Wenhui Chen developed the original idea and the protocol, abstracted and analyzed data, wrote the manuscript and is a guarantor; Lan Deng, Mo Jiang, Chen Zhang, Rihui You and Zhiyao Huang contributed to the development of the protocol, abstracted data and prepared the manuscript.

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***Data availability statement***
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

***Ethical approval***
The animal experiment was approved by the Institutional Animal Care and Use Committee of The Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine (No.: 2024-011).

***Conflict of interest***
No conflict of interest.

***Supplementary data***
https://www.pjps.pk/uploads/2026/03/SUP1774332992.pdf

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