

Houttuynia cordata Thunb. Essential Oil Inhibits Diffuse Large B-cell lymphoma Growth

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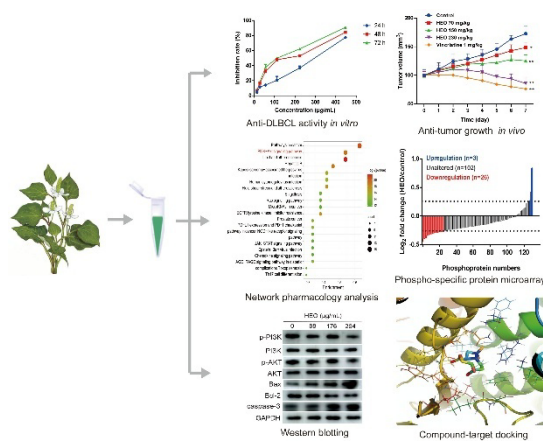
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Abstract: Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL) with high clinical heterogeneity and poor prognosis. Therapeutic drugs that can effectively improve the survival rate of DLBCL patients are lacking. *Houttuynia cordata* Thunb. is a well-known Asian medicinal and edible plant with anti-inflammatory and anti-tumor activities. This study investigated the potential therapeutic benefits of *Houttuynia cordata* Thunb. Essential Oil (HEO) on DLBCL. We found that HEO inhibited the proliferation of SUDHL-4 cells, induced apoptosis and cell cycle arrest at G0/G1 phase in a time and concentration-dependent manner. Moreover, intraperitoneal administration of HEO (70, 150, and 230 mg/kg) dose-dependently inhibited tumor growth of DLBCL in an athymic nude mouse xenograft model, while no significant changes in body weight were observed, indicating no obvious toxicity. Network pharmacology analysis indicated that HEO mainly regulated protein phosphorylation and PI3K/Akt signaling pathway. Phospho-specific protein microarray results also showed that HEO regulated the phosphorylation of PI3K/Akt signaling pathway. Further investigation confirmed that HEO significantly inhibited the protein expression of p-PI3K and p-Akt. In addition, HEO decreased the protein expression of B-cell lymphoma 2 (Bcl-2) and increased the protein expression of Bcl-2-associated X (Bax) and caspase-3. Compound-target docking results displayed that bornyl acetate (the main components of HEO), caryophyllene oxide and terpineol had strong binding interactions with the active sites of PI3K and Akt, indicating they contribute to the therapeutic effect. These results demonstrated that HEO exerts anti-tumor effects in DLBCL *via* suppressing PI3K/Akt signaling pathway, indicating HEO may be a potential inhibitor of PI3K/Akt signaling pathway for the treatment of DLBCL.



Keywords: *Houttuynia cordata* Thunb essential oil, diffuse large B-cell lymphoma, apoptosis, PI3K/Akt, Bcl-2

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1 Introduction

Diffuse large B-cell lymphoma (DLBCL) has been identified as the most common aggressive lymphoma subtype, representing approximately one-third of all cases worldwide^{1, 2}. Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) is used as a standard approach for the treatment of DLBCL and cures ~60 % of DLBCL including in advanced stage. However, ~40 % of intermediate- or high-risk DLBCL patients are not cured with R-CHOP^{3, 4}. In addition, doxorubicin and cyclophosphamide treatment often lead to significant cardiotoxicity and hematologic toxicity^{5, 6}. Hence, it is necessary to develop novel agents with low toxicity that can effectively restrain DLBCL.

Houttuynia cordata Thunb. (*H. cordata*) is well known among the people of diverse cultures across China, Japan, Korea and North-East India for its medicinal properties⁷. Traditionally it is used for the treatment of inflammation, pneumonia, severe acute respiratory syndrome, muscular sprain, stomach ulcer etc^{8, 9}. Recent studies have shown that it possesses antioxidant, anti-cancer, anti-inflammatory, anti-mutagenic, antiviral, as well as antimicrobial properties¹⁰⁻¹³. A wide range of phytochemicals including enriched in flavonoids, alkaloids, sterols, phenolics and amino acids have been identified in *H. cordata*¹⁴. More recent investigations suggested that *H. cordata* exhibits antitumor activities against several cancers, including leukemic, prostate, breast, gastric, lung and melanoma cancers¹⁵⁻²⁰. For example, *H. cordata* suppressed cell proliferation and stimulated apoptosis via inactivation of AKT/ERK/MAPK in both androgen-sensitive prostate cancer and castration-resistant prostate cancer cell lines¹⁹. Also, *H. cordata* induced cell cycle arrest by downregulating cyclinD1 and CDK4 expression and upregulated the expression of caspases and pro-apoptotic Bcl-2 family proteins in breast cancer cells¹⁸. An *in vivo* study has showed *H. cordata* significantly suppressed benzo(a)pyrene-induced lung tumorigenesis by activating the Nrf2-HO-1/NQO-1 signaling pathway¹⁷. In our previous study, *H. cordata* essential oil (HEO) showed significant anti-tumor effects on Burkitt's lymphoma by promoting cell cycle arrest and inducing cell apoptosis²¹. However, the investigation of HEO against DLBCL is still lacking.

The role of constitutive phosphatidylinositol 3-kinase (PI3K) signaling in B cells has been implicated as a central mechanism for relaying cell survival, adhesion, and proliferative signals. PI3K via protein kinase B (Akt) achieves transcriptional, translational, and posttranslational regulation of B-cell lymphoma 2 (Bcl-2) family proteins²². The Bcl-2 family members are key regulators of the intrinsic apoptotic pathway, which are represented by both antiapoptotic and pro-apoptotic members^{23, 24}. Importantly, the ratio between Bcl-2 (antiapoptotic member) and Bcl-2-associated X (Bax, pro-apoptotic member) is considered predictive of the forthcoming cell death²⁵. The pro-apoptotic protein BAX can promote cell death by permeabilizing the outer mitochondrial membrane and subsequent initiation of the caspase cascade^{26, 27}. Recent studies showed constitutive activation of the PI3K/Akt pathway are observed in 25-52 % of DLBCL patients. Moreover, several studies have demonstrated that overexpression of phosphorylated Akt (p-Akt) is significantly correlated with poorer progression-free survival in ~1/4th of DLBCL patients^{28, 29}. Also, Bcl-2 overexpression is presented in about 25 % of DLBCL and is associated with poor prognosis^{30, 31}. The PI3K/Akt pathway and Bcl-2 are thought to be effective therapeutic targets for DLBCL^{32, 33}.

In this study, the therapeutic effect of HEO on DLBCL was evaluated *in vitro* and *in vivo*. We found that HEO treatment significantly inhibited the proliferation and induced apoptosis of DLBCL *in vitro*, and markedly inhibited tumor growth of DLBCL in a mouse xenograft model. Also, network pharmacology showed HEO induced cell apoptosis of DLBCL by modulating the phosphorylation of apoptosis associated protein. Further investigation of phospho-specific protein microarray revealed that HEO suppressed the phosphorylation of PI3K/Akt signaling pathway and increased the ratio of Bax/Bcl-2, resulting in inhibition of proliferation and promotion of apoptosis of DLBCL. In addition, the active molecules of HEO against DLBCL were clarified by molecular docking. These results demonstrated that HEO likely acts as a novel inhibitor of PI3K/Akt signaling pathway and may provide a potential strategy for the treatment of DLBCL.

2 Materials and Methods

2.1 Extraction of *H. cordata* essential oil

H. cordata were obtained from Jiangyou country, Sichuan province, China. The plant was identified and authenticated by the Professor Chengming Dong (Henan University of Chinese Medicine). A voucher specimen has been deposited in Institute of Medical and Pharmaceutical Sciences, Zhengzhou University, with collection NO. ZZU-2020001. HEO was extracted by a distillation extraction method. Briefly, 100 g of *H. cordata* were soaked with 1000 mL distilled water for 10 h and then reflux for 10 h. Anhydrous sodium sulfate was employed to remove moisture from the HEO, followed by storage at -20 °C.

2.2 Gas chromatography tandem mass spectrometry (GC-MS) analysis

The chemical fingerprint analysis of HEO was conducted using GC-MS method. Chromatographic separation was achieved using an Agilent 7890 system with flame ionization detection and an Agilent 7683B automated sampler. A HP-5MS capillary column (30 m × 0.25 mm i.d.), coated with a 0.10 µm film of crosslinked 5 % phenyl methyl silicone was operated with helium carrier gas at 1 mL/min constant flow. The oven temperature program consisted of an initial 5 min isothermal phase at 70°C, followed by sequential ramps: 5°C/min to 100°C, 2°C/min to 123°C (holding for 3 min), 1.5°C/min to 140°C, and finally 15°C/min to 250°C (holding for 13 min). The injector temperature was maintained at 280 °C. The split ratio was set to 20:1.

Detection was performed on an Agilent 5975C mass spectrometer with an electron impact ionization mode. The parameter settings were as follows: ionization voltage, 70 eV; ion source temperature, 230 °C; quadrupole temperature, 150 °C. Mass spectra were recorded from *m/z* 20 to 500. Identification of the constituents was based on comparison of the spectra with the NIST databases using a probability-based matching algorithm.

2.3 Cell Counting Kit-8 (CCK-8) assay

100 µL of SUDHL-4 cells at a density of 4×10^4 cells/ml was seeded in 96-well plate. After incubation for 12 h at 37 °C in a 5 % CO₂, the cells were treated with HEO at six concentrations (14, 28, 56, 112, 224 and 448 µg/mL) and incubated for 24 h, 48 h and 72 h, respectively. 10 µL of CCK-8 (Dojindo, Japan) was then added to cells and incubated for 2 h at 37 °C in a 5 % CO₂ incubator. The absorbance of each well was measured at 450 nm. The concentration of HEO that inhibited cell proliferation by 50 % was defined as the 50 % inhibitory concentration (IC₅₀).

2.4 Cell cycle analysis

The inhibitory effect of HEO on cell cycle arrest was assessed by flow cytometry using Cell Cycle Detection Kit (Key GEN Bio TECH, China). SUDHL-4 cells were cultured in 6 well-plates at an initial density of 4×10^5 cells/well and incubated for 12 h. The cells were treated with vehicle or HEO (88, 176 and 264 µg/mL) for 48 h. Cells were collected, washed with phosphate buffer (PBS), and fixed in 70 % ice-cold ethanol for 30 min at 4 °C. The cells were then suspended in 200 µL PBS containing 20 µg/mL propidium iodide (PI), 0.2 mg/mL RNase, and 0.1 % triton X-100, and incubated at 37 °C for 30 min. The cell cycle distribution was performed using flow cytometry.

2.5 Acridine orange/Ethidium bromide (AO/EB) double fluorescent staining

Morphological study of apoptotic cells induced by HEO using acridine orange /ethidium bromide (AO/EB) double fluorescent staining. Briefly, SUDHL-4 cells were seeded in 96-well plates at an initial density of 5×10^3 cells/well and incubated for 12 h. Then, SUDHL-4 cells were treated with 88, 176 and 264 µg/mL of HEO for 48 h. Subsequently, SUDHL-4 cells were exposed to AO (5 µg/mL) and EB (5 µg/mL) (Beijing Leagene Biotech. Co., Ltd., China). The morphology of apoptotic cells was examined using a fluorescent microscope (OLYMPUS, Japan). Early apoptotic cells showed yellow-green fluorescence stained by acridine orange staining and late apoptotic cells showed reddish orange fluorescence stained by ethidium bromide.

2.6 Flow cytometry analysis of cell apoptosis

Quantitative assessment of apoptosis cells induced by HEO was measured by flow cytometry using a Dead Cell Apoptosis Kit (Jiangsu KeyGEN BioTECH Co., Ltd. China). Briefly, SUDHL-4 cells were seeded in 6-well plates at an initial density of 5×10^5 cells/well. After overnight incubation, SUDHL-4 cells were treated with HEO (88, 176 and 264 µg/mL) for 48 h. The cells were washed with PBS and collected by centrifugation at $250 \times g$ for 5 min. Then, the cell pellets were resuspended in 500 µL annexin V-binding buffer containing 5 µL of fluorescein isothiocyanate conjugated annexin-V and 5 µL of PI (10 µg/ml). After incubation for 15 min at room temperature, fluorescence signals of FITC-Annexin V and PI were detected using a fluorescence activated cell sorter.

2.7 Animals

BALB/c-nude mice, aged between 4 to 6 weeks, were obtained from Beijing Vital River Laboratory Animal Technologies Co. Ltd (Beijing, China). The animals were maintained in a specific pathogen-free environment with a 12 h light/dark cycle at 20-26 °C with a relative humidity of 40–70 %. Sterilized food and water were freely available. All animals were treated according to the procedures outlined in the Guide for the Care and Use of Laboratory Animals (P. R. China), and experimental procedures were approved by the Animal Ethics Committee of Zhengzhou University.

2.8 Establishment of DLBCL tumor model and HEO treatment

To establish DLBCL xenograft mice model, SUDHL-4 cells (2×10^6 cells) in 0.2 mL were injected into nude mice by subcutaneous flank inoculation. The tumor-bearing mice were randomly divided into five groups ($n = 5$ animals each group) and received intraperitoneal injection of HEO (70, 150 and 230 mg/kg), vincristine (1 mg/kg) or normal saline once daily for 7 days, respectively. The mice weight and tumor volume were recorded every day. The tumor volume (mm^3) is calculated as $a \times b^2 \times 0.5$, where a is the tumor length and b is the tumor width.

2.9 Network pharmacology analysis

To investigate the potential mechanism of HEO against DLBCL, network pharmacology was conducted using a total of 27 constituents of HEO (**Supplementary Table S1**). The CAS numbers of these molecules were obtained from Huayuanwang and their structural information was acquired from PubChem database. Potential therapeutic targets were predicted using the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database. DLBCL-associated pathogenic targets were compiled through multi-source databases, including DrugBank (<https://go.drugbank.com/>), OMIM (<https://omim.org/>), and GeneCard (<https://www.genecards.org/>) using "DLBCL" as the search term. Interactive Venn diagram analysis (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) identified shared targets between HEO components and DLBCL pathogenesis. Protein-protein interaction (PPI) networks were constructed via STRING database with species restriction to Homo sapiens and a confidence level of 0.4. The core targets were subsequently filtered using Cytoscape 3.7.2 (degree value $> 2 \times \text{median}$). Functional annotation was performed through Metascape platform (<https://metascape.org>) for Gene Ontology (GO) enrichment analysis spanning biological process (BP), molecular function (MF), and cellular component (CC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping. Data visualization was executed using the bioinformatics platform (<http://www.bioinformatics.com.cn/>).

2.10 Phospho-specific protein microarray analysis

The expression profile of phospho-related proteins was detected and analyzed using a human Phospho antibody microarray kit (CSP100, Fullmoon Biosystems, Sunnyvale, CA, USA) containing 269 antibodies against 136 phosphorylated sites and 133 unphosphorylated sites. Phospho-array detection was performed in cooperation with Wayen Biotechnology (Shanghai, China). Briefly, SUDHL-4 cells were treated with HEO for 48 h and protein was extracted, purified and then labeled by biotinylation. The resulting biotin-labeled proteins were diluted 1:20 in coupling solution and incubated with antibody microarray. The conjugation-labeled protein was detected by Cy3-Streptavidin. For data analysis, the fold change in intensity was measured using GenePix Pro 6.0 software (Axon Instruments). The phosphorylation ratio of each protein was calculated as follows: phosphorylation ratio = phospho value/unphospho value.

2.11 Western blotting

SUDHL4 cells were harvested, lysed with RIPA lysis buffer (PS0013, Leagene, China) containing 1 % (v/v) protease inhibitor (phenylmethanesulfonyl fluoride) or 1 % (v/v) phosphatase inhibitor cocktail (CW2383S, Cwbio, China) for 30 min on ice, and centrifuged at 14000 rpm for 15 min at 4 °C to obtain the protein. The protein samples were boiled at 100 °C for 10 min and then electrophoresed on SDS-polyacrylamide gels. The separated proteins were transferred onto polyvinylidene fluoride membranes. The membranes were blocked with a 5 % fat-free milk solution (w/v) in Tris-buffered saline containing 0.1 % Tween 20 (TBST) for 2 h at room temperature. After blocking, the membranes were incubated with the primary antibody at 4 °C overnight. Subsequently, the membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The protein bands were visualized using a supersensitive multifunctional imaging system (GE Healthcare, USA). The primary antibodies used were anti-PI3K (Abcam, UK), anti-p-PI3K p110 delta (phospho-Tyr524, Abcam, UK), anti-Akt (Sanying, China), anti-p-Akt (phospho-Ser473, Sanying, China), anti-Bcl-2 (Signalway, USA), anti-caspase 3 (Signalway, USA), and anti-Bax (Signalway, USA).

2.12 Molecular docking

Molecular docking was performed to elucidate the molecular recognition patterns between HEO active molecules and therapeutic targets. The crystallographic coordinates of PI3K p110 δ and Akt were acquired from RCSB Protein Data Bank (v5.0, <https://www.rcsb.org/>). Protein structures underwent preprocessing in AutoDockTools 1.5.6, involving protonation state optimization, assignment of Gasteiger-Marsili atomic charges, and generation of polar-hydrogen-enriched ".pdbqt" files. Twenty-seven HEO-derived bioactive ligands were structurally curated via PubChem Compound Database (<https://pubchem.ncbi.nlm.nih.gov/>), generating ".pdbqt" file format by setting free rotatable and torsional bonds. Molecular docking simulations were executed using AutoDock Vina 1.1.2 with Lamarckian genetic algorithm optimization. Post-docking analysis in PyMOL 2.4.0 enabled visualization of binding poses. The ligand prioritization was conducted

based on binding affinity.

2.13 Statistical analysis

Statistical analyses were conducted with SPSS 20.0 (IBM, Somers, NY, USA), adopting a statistical significance of $p < 0.05$. Intergroup comparisons were assessed *via* a two-tailed independent Student's *t*-test for two groups. The differences among multiple groups were evaluated by one-way ANOVA with Duncan's post hoc analysis. Results are presented as means $\pm$ standard deviation (SD).

3 Results

3.1 Characterization of HEO by GC-MS

The chemical profiling of HEO was performed using gas chromatography tandem mass spectrometry (GC-MS) method. As illustrated in the typical total ions chromatogram (**Fig. 1**), a total of 27 compounds were identified in HEO, including terpenes, aldehydes, ketones, alcohols, lower fatty acids and esters (Supplementary Table S1). The major constituents were 2-undecanone (32.71 %), *n*-decanoic acid (18.38 %), 1-nonanol (2.69 %), bornyl acetate (2.66 %), and decanal (2.40 %), which accounted for 58.84 % of the total HEO.

3.2 HEO inhibited the proliferation of DLBCL

To evaluate the inhibitory effect of HEO on DLBCL, SUDHL-4 cells were treated with different concentrations of HEO, and the cell viability was measured by CCK-8 assay. The result showed that HEO exhibited significant inhibitory activity against DLBCL in a time and concentration-dependent manner. The IC_{50} values of HEO against DLBCL were 264 ± 3.8 μ g/mL, 200 ± 14.3 μ g/mL and 176 ± 14.3 μ g/mL for 24 h, 48 h and 72 h, respectively (**Fig. 2**). The result suggested that HEO exerts significant inhibitory effect on the proliferation of DLBCL.

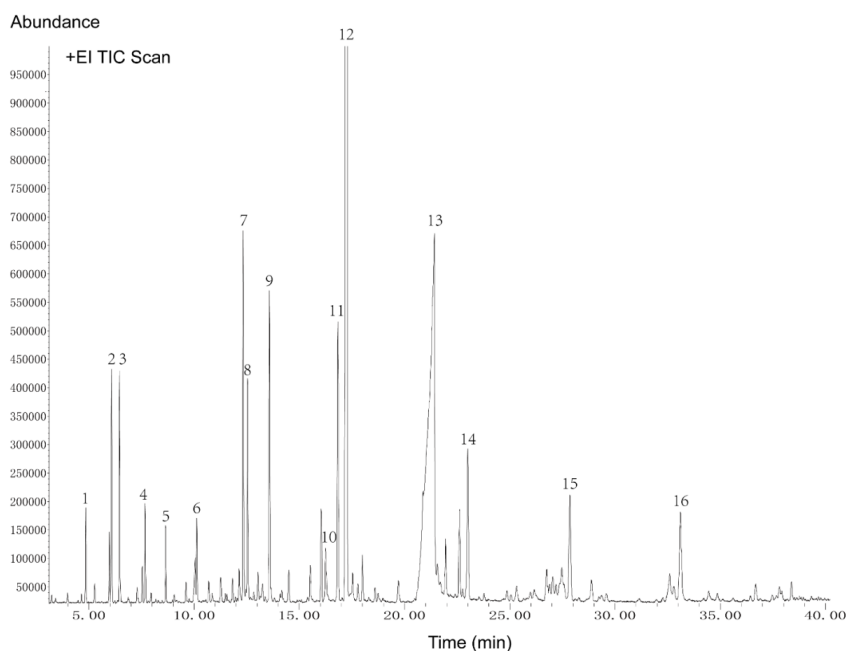


Fig. 1 The representative total ions chromatogram of HEO achieved by GC-MS. Peaks: (1) α -pinene (4.853 min); (2) β -pinene (6.080 min); (3) β -myrcene (6.460 min); (4) D-limonene (7.673 min); (5) 1,4-cyclohexadiene,1-methyl-4-(1-methylethyl)- (8.648 min); (6) nonanal (10.129 min); (7) 1-nonanol (12.328 min); (8) 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (*R*)- (12.543 min); (9) decanal (13.578 min); (10) cyclooctane (16.252 min); (11) bornyl acetate (16.833 min); (12) 2-undecanone (17.252 min); (13) 1-undecanol; *n*-decanoic acid; geranyl acetate (20.685-21.415 min); (14) caryophyllene (23.004 min); (15) 2-tridecanone (27.854 min); (16) caryophyllene oxide (33.105 min). (EI, electron ionization)

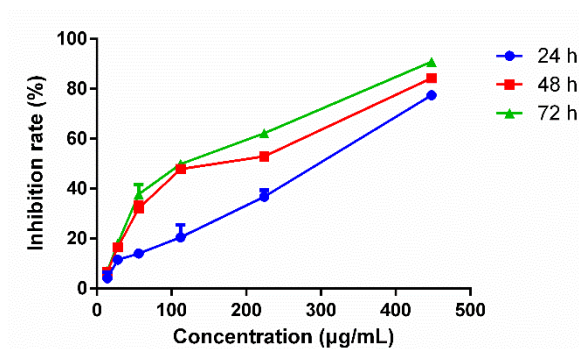


Fig. 2 HEO treatment inhibited cellular proliferation of SUDHL-4 cells.

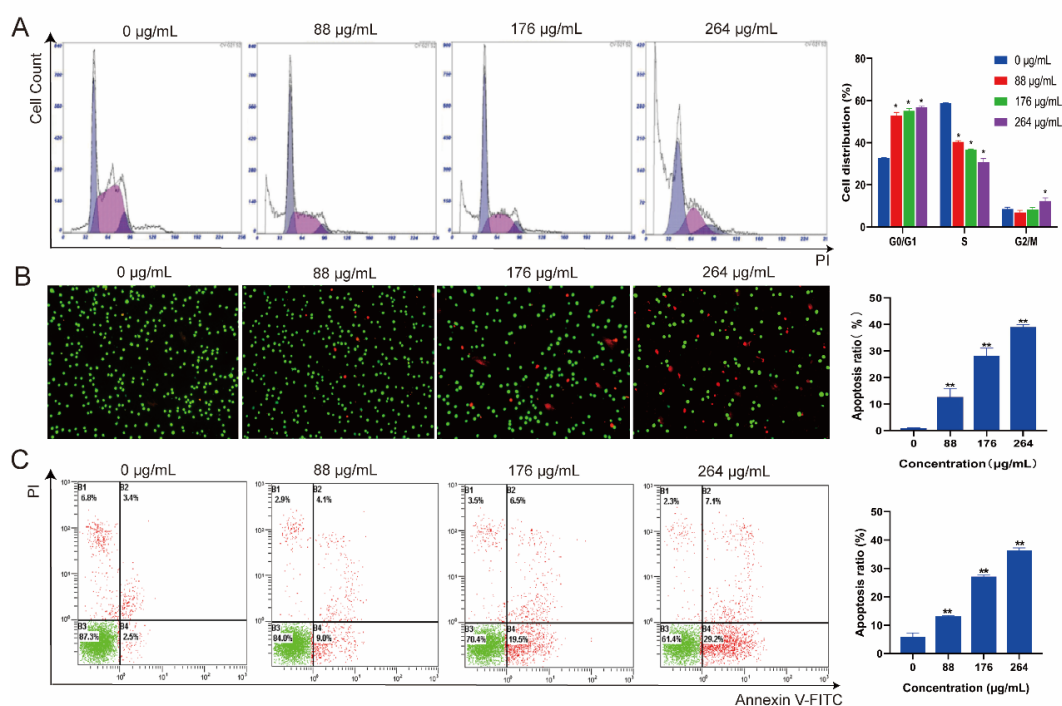


Fig. 3 HEO induced cell cycle arrest and apoptosis in DLBCL cells. (A) SUDHL-4 cells were treated with various concentrations of HEO for 48 h, and the cell cycle distribution was analyzed by flow cytometry. (B) Apoptosis analysis by AO/EB. Early apoptotic cells showed yellow-green fluorescence stained by acridine orange staining and late apoptotic cells showed reddish orange fluorescence stained by ethidium bromide. (C) SUDHL-4 cells were treated with various concentrations of HEO for 48 h and the cell apoptosis was analyzed by flow cytometry with annexin V-FITC/PI staining. * $p < 0.05$ and ** $p < 0.01$ vs. control group

3.3 HEO induced cell cycle arrest and apoptosis in DLBCL

To determine the effect of HEO on cell cycle arrest in DLBCL cells, the cell cycle distribution was analyzed by flow cytometry in SUDHL-4 cells treated with HEO. It was found that HEO decreased the population of SUDHL-4 cells in the S-phase and increased the cell population in the G0/G1 phase in a concentration-dependent manner, suggesting that HEO treatment induced G0/G1 phase accumulation. The result indicated HEO inhibits DLBCL proliferation by inducing cell cycle arrest at the G0/G1 phase (Fig. 3A).

To further investigate the induction of apoptosis by HEO, SUDHL-4 cells were treated with different concentrations of HEO for 48 h, and morphological changes of SUDHL-4 cells was evaluated by acridine orange/ethidium bromide (AO/EB) dual staining. The result showed that cell shrinkage, nuclear condensation and membrane blebbing were observed in HEO-treated SUDHL-4 cells. Also, the reddish orange labeling of EB characteristic of late apoptosis was

significantly detected in SUDHL-4 cells after treated with HEO at the concentration of 176 $\mu\text{g/mL}$ (Fig. 3B). To further confirm the morphological signs of apoptosis using AO/EB staining, the apoptosis rate of SUDHL-4 cells was measured by flow cytometry with annexin V-fluorescein isothiocyanate/propidium iodide (V-FITC/PI) staining. It was found that the apoptosis rate of SUDHL-4 cells was obviously increased in a concentration-dependent manner (Fig. 3C). These results indicated that HEO displayed cytotoxic effect on DLBCL *via* inducing cell cycle arrest and apoptosis.

3.4 HEO inhibited DLBCL growth in a xenograft mouse model

To further assess the effects of HEO on tumor growth *in vivo*, a xenograft mouse model was established in BALB/c athymic nude mice by subcutaneous flank inoculation of SUDHL-4 cells. Mice bearing tumors were intraperitoneally administered with HEO (70, 150 and 230 mg/kg) or vincristine (1 mg/kg) as the positive control once daily for 7 days. Compared to the untreated group, administration of HEO significantly inhibited tumor growth in nude mice in a dose-dependent manner (Figs. 4A and 4B). The tumor volume significantly decreased 14.11 %, 27.44 % and 50.32 % in the mice after treatment with HEO at the doses of 70, 150 and 230 mg/kg for 7 days, respectively (Fig. 4A). Over the course of the 7 days study, treatment of HEO did not cause significant toxicity in any of the three dosed groups. In the positive control group, vincristine (1 mg/kg) treatment inhibited tumor growth with an inhibitory rate of 55.81 %, but vincristine-treated mice exhibited obvious weight loss (Fig. 4B), which may be caused by drug systemic toxicity^{34,35}. These results demonstrated that HEO significantly suppressed tumor growth of DLBCL with no significant toxicity.

3.5 Potential mechanism of HEO against DLBCL

Network pharmacology approach was employed to investigate the potential targets and mechanisms of HEO against DLBCL. A total of 27 ingredients in HEO were determined using GC-MS method, while 266 potential targets were revealed by Traditional Chinese Medicine Systems Pharmacology database. Subsequently, 813 lymphoma-related genes were retrieved from GeneCard, DrugBank, and OMIM databases, generating 53 potential genes overlapping between disease and the potential targets. Protein-protein interaction mapping of these targets was performed through STRING database and visualized *via* Cytoscape 3.7.2 (Fig. 5A). Topological analysis showed that the top 10 targets (Degree > 2 $\times$ median) included epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (STAT3), heat shock protein 90 α family class A member 1 (HSP90AA1), non-receptor tyrosine kinase (SRC), Bcl-2, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA), protein tyrosine phosphatase receptor type C (PTPRC), matrix metalloproteinase-9 (MMP9), mammalian mitogen-activated protein kinase 14 (MAPK14), and kinase insert domain receptor (KDR) (Figs. 5B and 5C). Functional annotation was performed by GO enrichment analysis, and the results demonstrated that the top 10 BP were involved in peptidyl-tyrosine phosphorylation, protein autophosphorylation, peptidyl-tyrosine autophosphorylation, protein phosphorylation, etc. (Fig. 6A). Moreover, the top 10 signaling pathways of KEGG enrichment analysis performed by Metascape database included pathways in cancer, PI3K/Akt signaling pathway, lipid and atherosclerosis, etc. (Fig. 6B). GO BP terms highlighted protein phosphorylation, while KEGG analysis was largely associated with PI3K/Akt signaling pathway. These results suggested that HEO may regulate protein phosphorylation of PI3K/Akt signaling pathway.

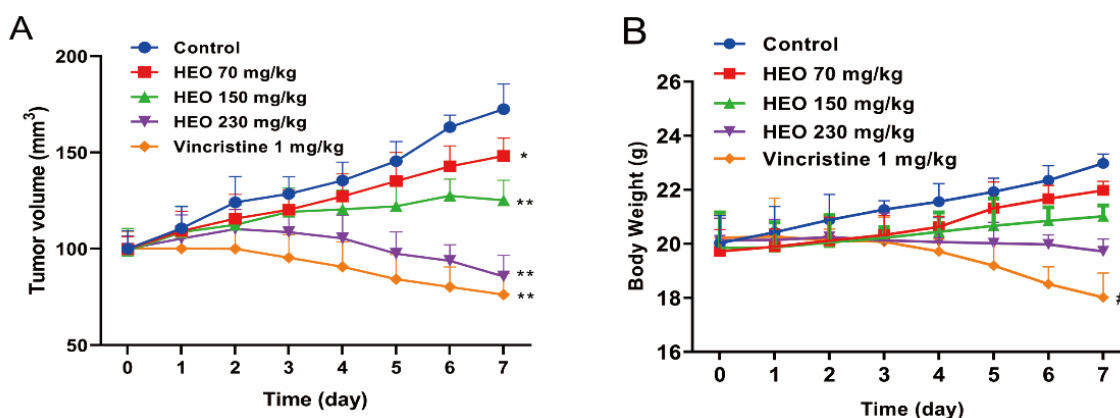


Fig. 4 HEO treatment inhibited tumor growth of DLBCL in nude mice bearing SUDHL-4 cells. The tumor-bearing nude mice were treated with HEO (70, 150 and 230 mg/kg), vincristine (1 mg/kg) or vehicle once daily for 7 days. The tumor volume (A) and body weight (B) of tumor-bearing mice from different groups were recorded during the treatment period. * $p < 0.05$ and ** $p < 0.01$ vs. control group. # $p < 0.05$ vs. day 0.

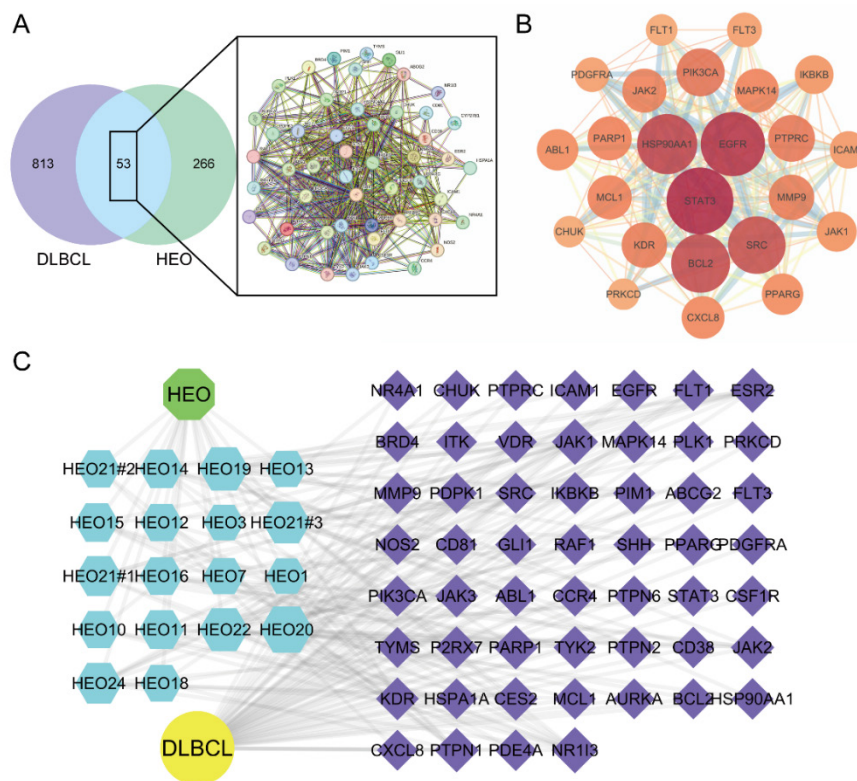


Fig. 5 (A) The overlap between the compound targets of HEO and DLBCL-related targets illustrated by Venn diagram, alongside PPI network constructed from the 53 overlapping targets. (B) A ranking of the PPI network targets, where bigger red nodes signify higher degrees of connection. (C) Compounds-overlapping targets network. The left and right nodes represent 18 potential compounds and 53 overlapping targets, respectively.

3.6 HEO inhibited DLBCL proliferation and induced apoptosis *via* suppressing PI3K/Akt signaling pathway

Network pharmacology results suggested that HEO may regulate protein phosphorylation in biological process and PI3K/Akt signaling pathway. To further investigate the mechanism of HEO against DLBCL, we applied a phospho-specific protein microarray containing 131 phosphoprotein antibodies to characterize the difference in phosphorylation patterns between HEO-treated and control SUDHL-4 cells (**Fig. 7A**). The results showed that the HEO-treated group exhibited 29 phosphorylation sites that significantly changed (26 phosphorylation sites significantly decreased and 3 phosphorylation sites increased compared to the control group) (**Fig. 7B**; fold change HEO-treated *vs.* Control ≥ 1.2). KEGG analysis revealed the top 20 signaling pathways regulated by HEO (**Fig. 7C**). Among these signaling pathways, PI3K/Akt signaling pathway is the most important signaling related to DLBCL³⁶. The PI3K/Akt signaling pathway is associated with numerous cellular functions, including cell viability, proliferation and apoptosis. Moreover, several studies have demonstrated that overexpression of phosphorylated Akt (p-Akt) is significantly correlated with poorer progression-free survival in $\sim 1/4$ th of DLBCL patients^{28, 29}. Therefore, further evaluation of HEO on the phosphorylation of PI3K/Akt signaling pathway was conducted.

SUDHL-4 cells were treated with HEO, and the protein expression levels of PI3K and Akt were measured by western blotting. Consistent with the results of antibody array, HEO significantly reduced the phosphorylation levels of PI3K and Akt in a concentration-dependent manner, while no significant change was observed in the total protein levels of PI3K and Akt (**Fig. 7D**), indicating HEO inhibited the PI3K/Akt signaling pathway by suppressing the phosphorylation of PI3K and Akt. It has been demonstrated that evasion of apoptosis through upregulating antiapoptotic Bcl-2 family members is one of most important pathogenic factors for DLBCL^{31, 30}. The ratio between Bcl-2 (antiapoptotic member) and Bax (pro-apoptotic member) is considered predictive of the forthcoming cell death²³⁻²⁵. The pro-apoptotic protein BAX can promote cell death by permeabilizing the outer mitochondrial membrane and subsequent initiation of the caspase cascade²⁶. In this study, we found that HEO treatment significantly inhibited Bcl-2 expression and increased the ratio of Bax/Bcl-2. Also, HEO treatment increased the protein expression level of downstream effector cleaved

caspase-3. These results indicated that HEO inhibited DLBCL proliferation and induced apoptosis by suppressing PI3K/Akt signaling pathway and Bcl-2.

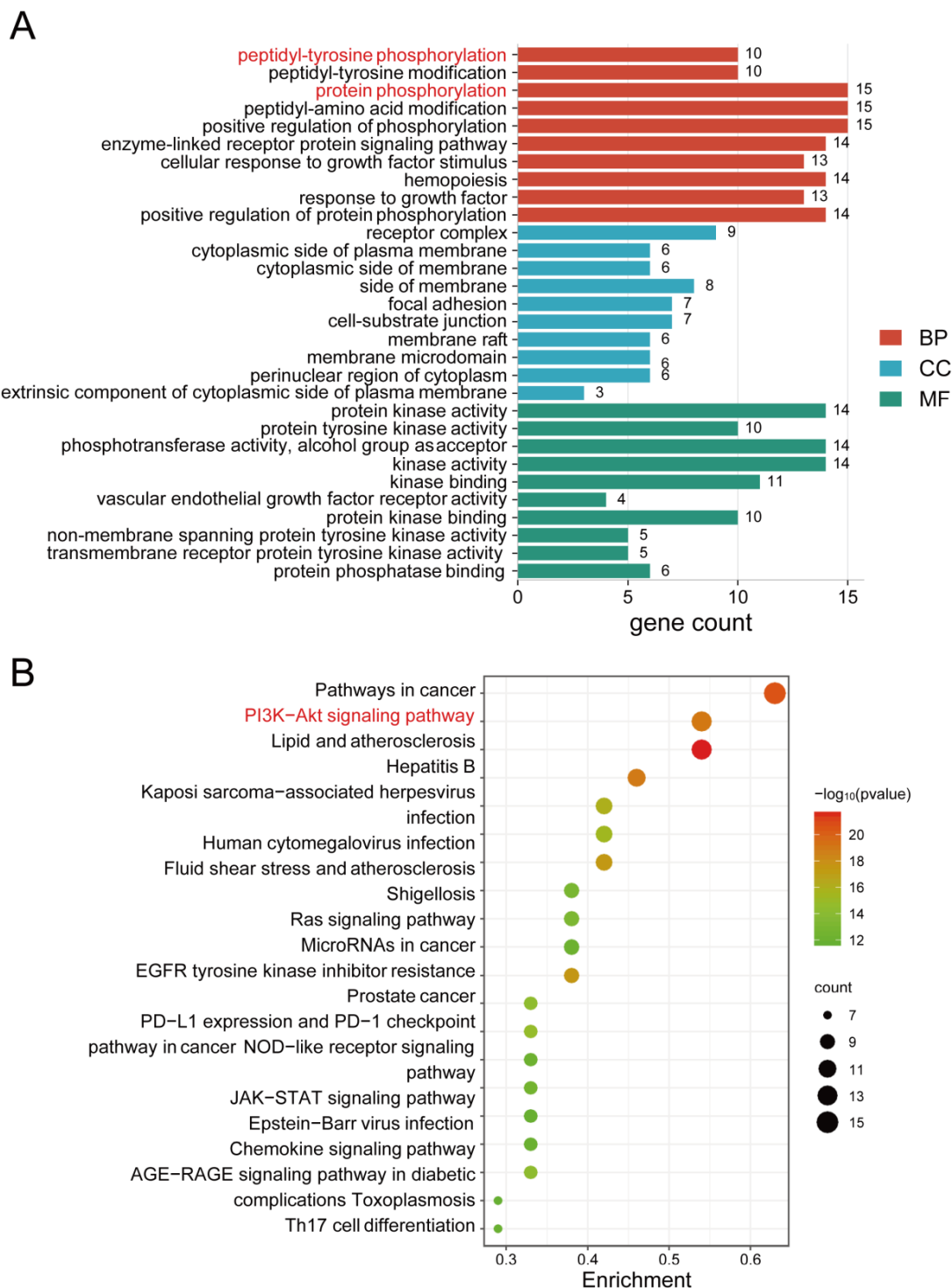


Fig. 6 GO analysis (A) and KEGG pathways (B). (BP, MF and CC represent biological process, molecular function, and cellular component, respectively)

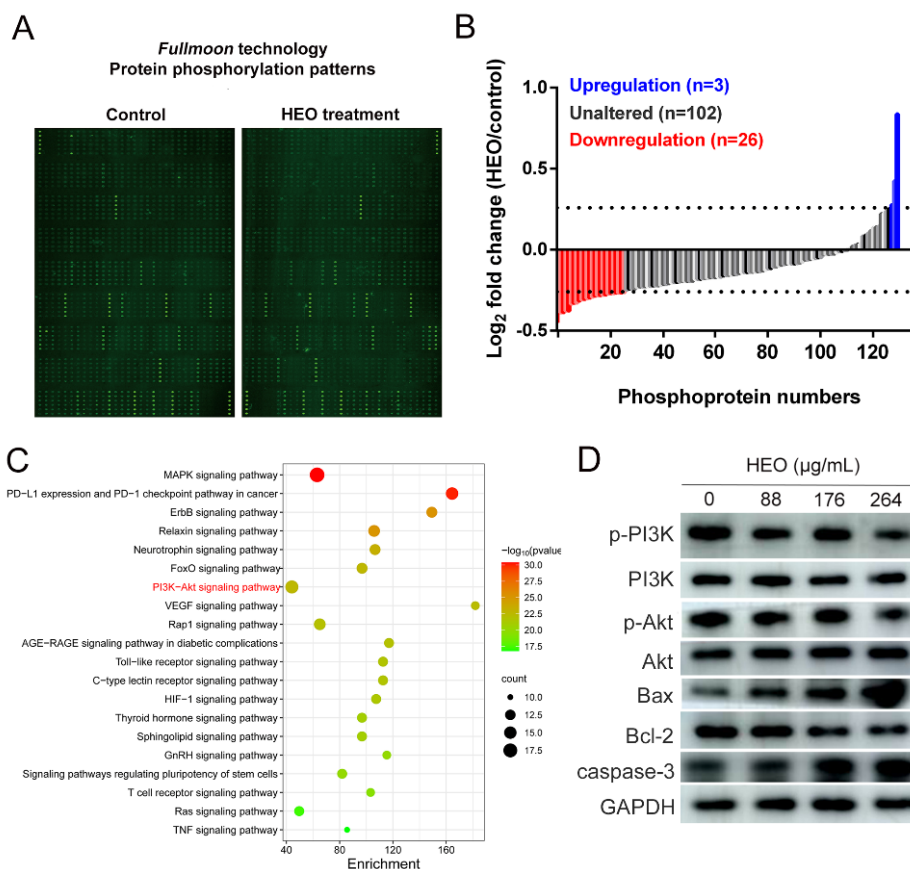


Fig. 7 (A) Phosphorylation of 131 proteins in HEO-treated SUDHL-4 cells using phospho-specific protein microarray. (B) A total of 29 phosphorylated proteins were significantly altered (26 decreased and 3 increased) in HEO-treated SUDHL-4 cells (fold-change HEO-treated vs. Control ≥ 1.2). (C) KEGG analysis of altered proteins in the phospho-specific protein microarray. (D) The protein expression levels of PI3K, Akt, p-PI3K, p-Akt, Bcl-2, Bax, and caspase-3 in SUDHL-4 cells after treatment with HEO.

3.7 Active molecules of HEO analysed by molecular docking

It has been demonstrated that PI3K/Akt pathway is effective therapeutic targets for DLBCL. In this study, HEO suppressed DLBCL proliferation and induced cell apoptosis through inhibiting PI3K/Akt signaling pathway. Based on GC-MS analysis, a total of 27 molecules were identified in HEO. The molecule-target interactions of these compounds with the two therapeutic targets were analyzed through molecular docking. AS showed in Supplementary Table S2 and S3, the docking score values represent the interactions of the active constituents with PI3K and AKT. Notably, the majority of compounds exhibited favorable docking scores (< -6.0 kcal/mol), indicating strong ligand-protein interactions. Structural visualization of the top two molecules for PI3K was demonstrated in **Figs. 8A** and **8B**. Bornyl acetate formed conventional hydrogen bond, combined with alkyl and pi-alkyl interactions (**Figs. 8B** and **8E**). Compared to bornyl acetate, caryophyllene oxide achieved the highest docking score (-7.6 kcal/mol) through van der Waal forces, carbon hydrogen bond, alkyl and pi-alkyl interactions (**Figs. 8A** and **8D**). Also, the protein-ligand analysis identified terpineol as the most potent ligand for AKT, with docking score of -6.7 kcal/mol. Terpineol was linked to the active site of AKT included conventional hydrogen bond, van der Waal forces, alkyl and pi-alkyl interactions (**Figs. 8C** and **8F**). Previous studies showed that bornyl acetate, caryophyllene oxide and terpineol possess significant anticancer activities by inhibiting PI3K/Akt/mTOR signaling pathway and decanal induces apoptosis strongly in leukemic cells^{37–41}. Therefore, HEO likely acts as a novel inhibitor of PI3K/Akt for the treatment of DLBCL. The main components of HEO (bornyl acetate and decanal), caryophyllene oxide and terpineol may contribute to the therapeutic effect.

4 Discussion

DLBCL is the most common lymphoma representing approximately one third of all non-Hodgkin lymphomas. Several approaches are used for the treatment of lymphoma. First-line treatment predominantly consists of six cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy. However, about 40 % of patients do not benefit from the standard first-line immune-chemotherapeutic treatment^{3, 4}. Moreover, elderly patients are frequently ineligible for high dose chemotherapy due to considerable drug toxicity. Therefore, effective agents with low toxicity are urgently required.

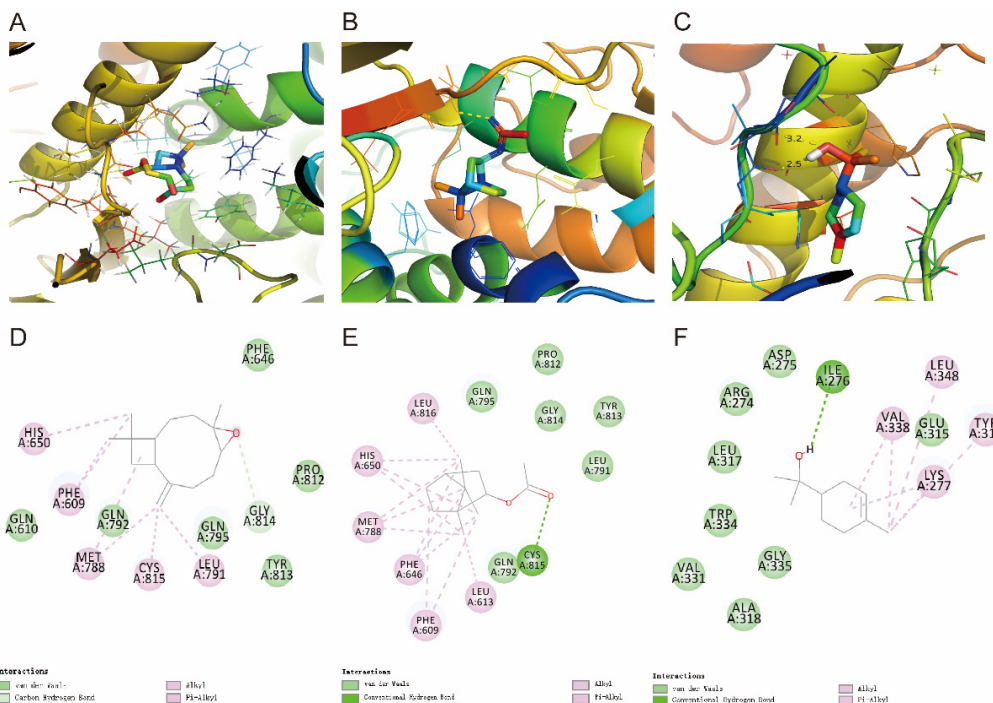


Fig. 8 Molecular interaction of the active compounds with PI3K and Akt crystal structures. 3D bioactive conformation of caryophyllene oxide (A), bornyl acetate (B) and terpineol (C) to residues of PI3K, PI3K, and AKT, respectively. 2D docking pattern of caryophyllene oxide (D), bornyl acetate (E) and terpineol (F) with amino acids of PI3K, PI3K, and AKT, respectively.

Phytochemical-based therapeutics demonstrated significant advantages in biomedical development, attributed to their favorable safety profiles⁴². Notably, natural product-derived pharmacophores constitute the predominant source of approved antitumor drugs, underscoring their therapeutic prominence in oncology⁴³. *H. cordata* is a well-known Asian medicinal plant with biological activities for the treatment of inflammation, pneumonia, and severe acute respiratory syndrome, etc⁴⁴. Recent studies have indicated that it exhibits antitumor properties across various types of cancers⁴⁵. It has been demonstrated that the water extract of *H. cordata* displayed obvious antileukemic activity on five leukemic cells, including K562, U937, P3HR1, L1210 and Raji⁴⁶. Also, the alcoholic extract of *H. cordata* demonstrated significantly cytotoxic effect to human acute lymphoblastic leukemia Molt-4 cells by inducing cell apoptosis⁴⁷. Furthermore, an *in vitro* study demonstrated that both ethanolic and water extracts of *H. cordata* significantly inhibited the cell viability of multiple types of cancer cells and exhibited the highest sensitivity in T-cell acute lymphoblastic leukemia Jurkat cells⁴⁸. Our previous study suggested that HEO, the important active constituents in *H. cordata*, displayed anti-tumor activity in Burkitt lymphoma (Raji) cells by promoting cells cycle arrest in the G0/G1 phase and inducing cell apoptosis²¹. In the present study, we further evaluated the anti-tumor activities of HEO on DLBCL.

Our results showed that HEO significantly suppressed SUDHL-4 cell proliferation by promoting cell cycle arrest and inducing apoptosis. Further *in vivo* investigations demonstrated that it exhibited potent anti-tumor activity in a dose-dependent manner. Clinical data have suggested that the efficacy of vincristine is relatively limited in patients with DLBCL. Moreover, high frequency side effects occurred in patients receiving vincristine, including mechanical allodynia, sensory/tactile disorders, and numbness in the hands and feet. Peripheral neuropathy induced by vincristine not only

restricts the drug dose and leads to treatment cessation, but also causes serious physical and mental damage^{34,35}). In the present study, although vincristine displayed higher antitumor activity than HEO, vincristine-treated mice showed significant weight loss caused by drug systemic toxicity. Notably, HEO significantly inhibited tumor growth and did not cause obvious weight loss over the course of therapy, indicating low toxicity of HEO as illustrated in previous studies⁹).

Network pharmacology is a discipline that integrates comprehensive network analysis with pharmacological impacts, employed to investigate "compound-proteins/genes-disease" pathways. This approach is adept at delineating the intricate relationships among biological systems, drugs, and diseases from a network perspective. In this study, network pharmacology was used to predict the potential mechanism of HEO against DLBCL. GO BP terms were mainly enriched in protein phosphorylation. KEGG analysis was largely associated with PI3K/Akt signaling pathway and pathways in cancer. We further applied a phospho-specific protein microarray containing 131 phosphoprotein antibodies to investigate the effect of HEO on phosphorylation of multiple signaling pathways and found that HEO regulated the phosphorylation of multiple signaling pathways, such as PI3K/Akt signaling pathway, which is the most important signaling pathway related to DLBCL³⁶). Subsequently, the protein expression levels of PI3K, p-PI3K, Akt, and p-Akt were confirmed by western blotting. Consistent with the results of antibody array, HEO significantly reduced the protein expression of p-PI3K and p-Akt, while no obvious change was observed in the total protein expression of PI3K and Akt. Moreover, HEO decreased the expression levels of antiapoptotic protein Bcl-2 and increased the expression levels of proapoptotic proteins Bax and caspase-3. These results indicated that HEO inhibited DLBCL proliferation and induced apoptosis through downregulating PI3K/Akt signaling pathway, increasing Bax/Bcl-2 and consequent activation of caspase-mediated downstream cell death.

PI3K/Akt is an important signaling pathway involved in cell proliferation, apoptosis, survival, invasion, migration, glucose metabolism and DNA repair. Abnormal activation of PI3K is associated with the development and progression of human malignant tumors, including B cell malignancy⁴⁹). Inhibition of PI3K/Akt pathway has been reported to exert beneficial therapeutic effects in DLBCL^{50,36}). Recently, many approaches and pharmacologic inhibitors have been developed to block PI3K/Akt signaling for the treatment of DLBCL and improve the outcomes of these patients⁵¹⁻⁵³). In the present study, HEO significantly inhibited the protein expression of p-PI3K and p-Akt. Furthermore, molecular docking was employed to predict ligand-protein interaction and clarify the mechanism of molecular recognition between active molecules in HEO and the two signaling proteins. Our studies revealed that the majority of the active molecules in HEO exhibited robust interactions with PI3K and Akt, suggesting HEO may be a potential inhibitor of PI3K/Akt signaling pathway for the treatment of DLBCL.

5 Conclusions

In summary, HEO obviously inhibited tumor growth of DLBCL in vitro and in vivo. Further investigation revealed that HEO promoted DLBCL cell cycle arrest and induced cell apoptosis through suppression of phosphorylation of PI3K/Akt signaling pathway. These findings demonstrated that HEO likely acts as a novel inhibitor of PI3K/Akt signaling pathway for treatment of DLBCL.

Authors' Contributions

Youmei Peng, Zhuangli Zhang and Weihong Zhang conceived and designed the study. Shangkun Liu, Yinfeng Xie, Wenhuan Li and Jiayi Chen performed the experiments. Zhihong Zhao, Jian Wang, Xiaozhe Rong and Wenhui Yang analyzed the data. Xiaoqian Fu conducted network pharmacology and revised the manuscript. Yarong Wang performed molecular docking. All authors approved the manuscript.

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Conflicts of Interest

All authors declare that they have no conflicts of interest regarding the publication of this paper.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess25089

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