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ORIGINAL RESEARCH

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Ecliptae Herba extracts suppress cell proliferation and induce cell apoptosis of ovarian cancer cells by inactivating PI3K/AKT signaling pathway

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Abstract

Background: Among all the gynaecologic malignancies, ovarian cancer (OV) has the highest mortality rate, and herbal plants have been regarded as an alternative strategy for ovarian cancer therapy. Accumulating evidence indicates that the aboveground part of *Eclipta prostrata* (L.) L., known as Ecliptae Herba (EH), has anti-cancer activity and can contribute to the management of gynaecological disorders treatment. However, the application and the mechanism of EH against OV remain unexplored. **Methods**: A cell proliferation assay was first performed to validate the inhibitory effect of EH extracts on ovarian cancer cells. Network pharmacology was then employed to recognize potential bioactive compounds and target genes of EH. A Venn diagram was built to obtain the common targets of EH and OV and the protein-protein interaction (PPI) network of EH-OV correlated target genes was established by STRING. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment approaches were adopted to further reveal the molecular mechanism. Drug-compound-target-pathway-disease network of EH in OV treatment was constructed. The expression of the predicted targets, cell cycle changes and cell apoptosis levels were analyzed to confirm the predicted mechanism of EH. **Results**: EH extracts suppressed the proliferation of human ovarian cancer cells in a concentration- and time-dependent manner. Ten bioactive compounds and 290 compound-associated targets were collected for EH-OV common targets exploration. A total of 74 EH-OV correlated genes were gathered and enrichment analysis identified phosphatidylinositide 3-kinases (PI3K)/AKT signaling pathway as the main machinery. Drug-compound-target-pathway-disease network indicated the polypharmacological effect of EH. Finally, EH extracts were confirmed to inhibit cell proliferation and induce cell apoptosis of ovarian cancer cells by inactivating PI3K/AKT signaling pathway. **Conclusions**: This study demonstrated the anti-ovarian cancer activity of whole EH extracts and indicated that EH should be considered as a potential therapeutic strategy for ovarian cancer.

Keywords

Ecliptae Herba; Ovarian cancer; Network pharmacology; Bioactive compounds; Cell proliferation; Cell apoptosis; PI3K/AKT signaling pathway

1. Introduction

Ovarian cancer (OV) is the second most common type of gynaecological cancer with a lifetime risk of around 1.3% among women globally [1, 2]. Although many histopathological types are found, about 90% of ovarian cancer patients are diagnosed with epithelial ovarian cancer in the clinic, and the mortality rate for it ranks highest among gynaecologic malignancies [1–3]. Due to a lack of effective prediction and prevention strategies, most epithelial ovarian cancer patients are discovered at a late stage with the presence of distant metastasis, leading to a five-year survival rate of only around 29% [1, 2, 4]. The current treatment method for ovarian cancer is cytoreductive surgery followed by platinum-based chemotherapy. However, recurrence is a common phenomenon due to chemoresistance [2]. Although current immunotherapy holds promise in improving some patients' survival, epithelial ovarian cancer remains a clinical challenge due to its immunosuppressive tumor microenvironment. The development of new strategies is thus still in urgent need to provide treatment options with new reagents or effective combination regimens [5–7].

Ecliptae Herba (EH) is the aerial part of *Ecliptae prostrate* (L.) L. with wide distribution in Asia [8]. For years, Ecliptae Herba has been used as a traditional medicine with "haemostasis" and "kidney-nourishing" effects [9]. The main components of this plant include coumestans, flavonoids

and triterpene saponins [10]. Ecliptae Herba has currently been proven to possess anti-asthma, anti-osteoporotic, anti-angiogenic and immunomodulatory activities [11–17]. More interestingly, both crude extracts and some compounds of this plant have been found to exhibit anti-cancer activity in recent reports. Lirdprapamongkol *et al.* [18] demonstrated that Ecliptae Herba juice could inhibit the cell migration of liver, lung and breast cancer cells. Pan *et al.* [19] proved that Wedelolactone isolated from this plant is the active ingredient with anti-hepatocellular carcinoma cell proliferation activity. Zou *et al.* [20] reported that the ethyl acetate extract of Ecliptae Herba inhibited the migration of head and neck squamous cancer cells *via* the aryl hydrocarbon receptor (AhR) pathway. In 2015, Kim *et al.* [21] isolated 20 components of Ecliptae Herba and confirmed their cytotoxic effects on ovarian cancer cell line Skov3 by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assays, indicating its polypharmacological effect on ovarian cancer. However, it is worth noting that the exact mechanism of Ecliptae Herba in ovarian cancer remains missing and thus impeded its further application in clinical treatment.

In this study, we set out to explore the underlying mechanism of Ecliptae Herba on ovarian cancer. After isolating the whole extracts of Ecliptae Herba, we validated the consequence of the extracts on ovarian cancer cell proliferation and then collected integrated data from network pharmacology and enrichment investigation to identify the working mode. Finally, we utilized biological experiments to verify the predicted mechanism and provided confirmative evidence for applying Ecliptae Herba in ovarian cancer treatment.

2. Materials and methods

2.1 Cell lines and cell culture

Human ovarian cancer cells A2780, Caov3 and OVCAR3 were purchased from the Chinese Academy of Sciences Committee (Shanghai, China). A2780 cells were cultured in complete medium consisting of Roswell Park Memorial Institute (RPMI)-1640 medium (11875101, Gibco, Shanghai, China) and 10% fetal bovine serum (16140071, Gibco, Shanghai, China), Caov3 cells were cultured in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) medium (11965092, Gibco, Shanghai, China) and 10% fetal bovine serum and OVCAR3 cells were cultured in complete medium consisting of RPMI-1640 medium and 20% fetal bovine serum. All the medium contains 1% penicillin-streptomycin (penicillin-streptomycin, Gibco, Shanghai, China). Cells were incubated in a 5% carbon dioxide (CO_2) incubator at 37 degrees.

2.2 Preparation of Ecliptae Herba extracts

The dried aerial parts of *Eclipta prostrata* (L.) L. (CQMPC2020072601) was purchased from a local market of Chinese medicinal materials (Chongqing, China) in July 2020. The purchased materials were identified by Associate Professor Yu Dai from the department of Traditional Chinese Medicine, Chongqing Medical and Pharmaceutical College, China. For preparation, 50 g of the dried aerial parts of *Eclipta prostrata* (L.) L. were refluxed and extracted with 60% ethanol for 2 h. The extracted solution was then concentrated and dried under reduced pressure, yielding 6.2 g dry solid extracts. EH extracts were redissolved in dimethyl sulfoxide (DMSO) with cell culture medium diluted to indicated concentrations for different assays.

2.3 Cell proliferation assay

Ovarian cancer cells were seeded in 96-well plate (4000 cells/well) 24 h before EH extracts treatment. Selected wells of seeded cells were treated in complete medium with EH extracts dissolved at different concentrations (0, 200, 400, 600, 1000 μg/mL). The untreated control groups were kept in complete medium with DMSO. Cell Counting Kit-8 assay (CCK8, CK04, Dongren Chemical Technology, Shanghai, China) were performed at different time points (24, 48, 72 h) to detect cell proliferation. The 450 nm optical density was measured and cell viability in percentage was calculated for each time point.

2.4 Colony formation assay

Ovarian cancer cells were plated in 12-well plates at a density of 2000 cells/well. Cells were treated with EH extracts at 0, 200, 400 μg/mL. After incubation of 7–8 days, cell fixation and staining with crystal violet were conducted for final analysis. Three biological replicates were performed for each cell line.

2.5 Identification of bioactive compounds and target genes of Ecliptae Herba

The Traditional Chinese Medicine Systems Pharmacology Database (https://old.tcmsp-e.com/tcmsp.php) [22] was used to identify the bioactive components of Ecliptae Herba. The results were further filtered based on conditions of "oral bioactivity (OB) $\geq$30%" and "drug-likeness (DL) $\geq$0.18". Next, molecular structures for each filtered compound were collected from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The SwissTargetPrediction Webtool (http://swisstargetprediction.ch/) was employed to predict target genes for each compounds.

2.6 Acquisition of genes associated with ovarian cancer

Human ovarian cancer-associated genes were acquired from databases: GeneCards (https://www.genecards.org/) and Online Mendelian Inheritance in Man (OMIM, https://www.omim.org/) by searching for the key word "ovarian cancer". Genes with relevance $\geq$20 were screened from GeneCards. Duplicate values have been removed.

2.7 Network construction and Protein-Protein Interaction analysis

Compound-target network of Ecliptae Herba was visualized by using the Cytoscape 3.9.1 software. The Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) was adopted to obtain the common genes between Ecliptae Herba targets and ovarian cancer genes. Then STRING database (https://string-

db.org) was utilized to establish the PPI network of the common genes and the PPI network was then visualized by the Cytoscape 3.9.1 software. The interaction score for PPI network was set with default value of medium confidence (0.400).

2.8 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for EH-OV correlated-genes

The list of EH-OV correlated genes was inputted to DAVID online database (https://david.ncifcrf.gov/summary.jsp) for GO enrichment analysis and KEGG pathway analysis. Molecular function (MF), cellular component (CC) and biological process (BP) of GO analysis were demonstrated to illustrate gene functions. Results of KEGG pathway enrichment revealed important regulatory pathways of Ecliptae Herba against ovarian cancer.

2.9 RT-qPCR

Guided by the manufacturer's instructions of TRIZOL reagent (9108, Takara, Dalian, China), total RNA of each sample was isolated and reversely transcribed into complementary DNA (cDNA) with a reverse transcription kit (RR047A, Takara, Dalian, China). For quantitative real-time polymerase chain reaction (qRT-PCR) analysis, the relative quantification of gene expression was computed by comparative cycle threshold (CT) method ($\Delta\Delta$CT). The gene-specific primers were as follows: *CCND1* (Cyclin D1): Forward: 5′-AGCTGTGCATCTACACCGAC-3′, Reverse: 5′-GAAATCGTGCGGGGTCATTG-3′; *p21* (Cyclin-Dependent Kinase Inhibitor 1A): Forward: 5′-TGGCACCTCACCTGCTCTG-3′, Reverse: 5′-TCCTCTTGGAGAAGATCAGC-3′; *BAX* (Bcl-2 Associated X-protein): Forward: 5′-TCAGGATGCGTCCACCAAGAAG-3′, Reverse: 5′-TGTGTCCACGGCGGCAATCATC-3′; *BCL2* (BCL2 Apoptosis Regulator): Forward: 5′-GACTGAGTACCTGAACCGGC-3′, Reverse: 5′-ACAGTTCCACAAAGGCATCC-3′; *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase): Forward: 5′-AGCCACATCGCTCAGACAC-3′, Reverse: 5′-TTAAAAGCAGCCCTGGTGAC-3′. The experiments were performed in triplicate.

2.10 Western blotting

Ovarian cancer cells with EH extracts treatment were washed twice with cold phosphate buffer saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (P0013B, Beyotime Biotechnology, Beijing, China). Protein concentration was detected by BCA assay (P0010, Beyotime Biotechnology, Beijing, China). After denaturation at 95 °C for 5 min, samples were loaded into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked in 5% non-fat dry milk at room temperature for 1 h and incubated with the following primary antibodies at 4 °C overnight: anti-PI3K (4292S, Cell Signaling Technology, 1:1000), anti-phospho-PI3K (Tyr607) (ab182651, abcam, 1:1000) anti-AKT (4691S, Cell Signaling Technology, 1:1000), anti-phospho-AKT (Ser473) (4060S, Cell Signaling Technology, 1:1000), anti-Cyclin D1 (BM4272, Boster Bio, 1:1000), anti-p21 (2947S, Cell Signaling Technology, 1:1000), anti-GAPDH (10494-1-AP, Proteintech, 1:1000), anti-BAX (BA0315, Boster Bio, 1:1000), anti-BCL2 (BM0200, Boster Bio, 1:1000). The blots were then incubated with corresponding second antibodies for 1 h at room temperature. Blots were washed three times in tris buffered saline with 1‰ tween-20 (TBST) for 5 min each time and detected using enhanced chemiluminescence reagents and ChemiDoc imaging system (12003153, Bio-Rad, Hercules, CA, USA). The experiments were conducted in triplicate.

2.11 Cell cycle assay

Caov3 and A2780 cells were treated with EH extracts at indicated concentrations for 48 h and harvested to determine the cell cycle changes. Cells were washed twice and fixed with 70% ethanol at −20 °C overnight. Cells were treated with RNase for 30 min at 37 °C and subsequently stained with Propidium Iodide (PI) solution (421301, BioLegend, San Diego, CA, USA) at 4 °C for 30 min. Cell cycle distribution was detected by Flow cytometry (BD Biosciences). The experiments were performed in triplicate.

2.12 Cell apoptosis assay

Caov3 and A2780 cells were treated with EH extracts at indicated concentrations for 48 h and harvested to detect the cell apoptosis. Cells were washed twice and stained with Annexin V and PI solution (BioLegend) at room temperature in the dark for 15 min. Flow cytometry (BD Biosciences) was used for detection and analysis. The experiments were conducted in triplicate.

2.13 Statistical analysis

GraphPad Prism software (version 7.0, GraphPad Software, Boston, MA, USA) was adopted for statistical analysis. The data were tested for normality before analysis. All data from *in vitro* study performed in triplicates were presented as the mean ± standard deviation (SD). Variations between two groups were analyzed by using two-tailed Student's *t*-test. Changes with p-values < 0.05 were identified as statistical significance.

3. Results

3.1 EH extracts suppressed ovarian cancer cell proliferation in a concentration- and time-dependent manner

To examine the effects of EH on ovarian cancer cell proliferation, we first obtained the ethanol extracts of EH by using the method described in Materials and Methods. Then, we treated all human ovarian cancer cell lines in the lab with EH extracts of various concentrations (0, 200, 400, 600 and 1000 μg/mL) and measured the proliferation status at different time points. As displayed in Fig. 1A–C, the proliferation of Caov3, A2780 and OVCAR3, respectively, were suppressed

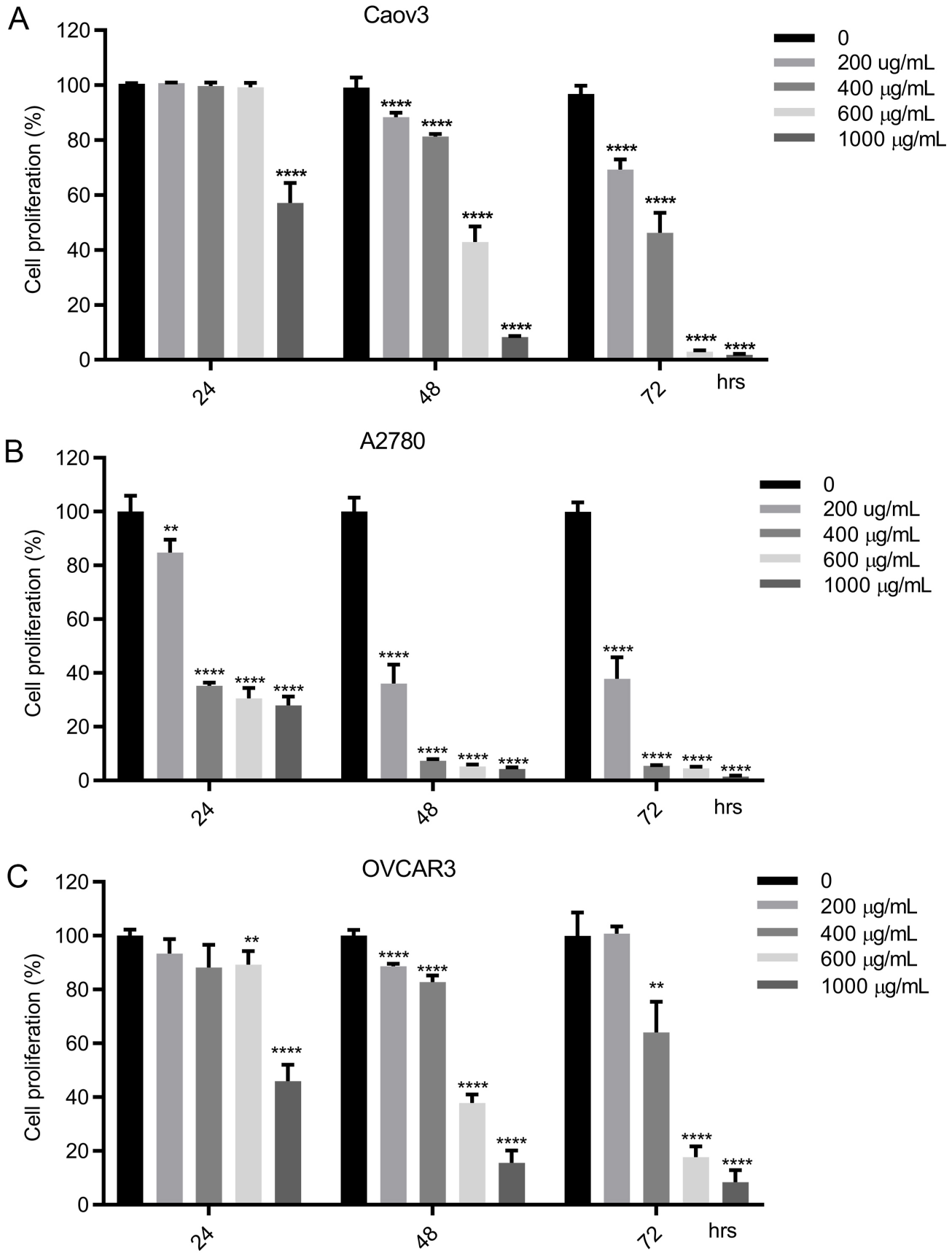

FIGURE 1. EH suppresses ovarian cancer cell proliferation in a concentration- and time-dependent manner. Ovarian cancer cells Caov3 (A), A2780 (B), OVCAR3 (C) were treated with EH extracts of various concentrations (0, 200, 400, 600 and 1000 μg/mL). Cell proliferation assay was performed at different time points (24, 48 and 72 h). Data represent the mean ± SD. **$p < 0.01$; ****$p < 0.0001$; Student's *t*-test versus the untreated group.

in a concentration- and time-dependent manner with EH treatment. To further confirm these results, a colony formation assay was then conducted (Fig. 2). In agreement with the findings of the cell proliferation assay, incubation with EH extracts significantly decreased the colony number of three ovarian cancer cell lines. Overall, the data above proved the anti-ovarian cancer capacity of Ecliptae Herba.

3.2 Network pharmacology identified ten bioactive compounds and 290 target genes of EH

To determine the working mode of EH against ovarian cancer, we adopted the network pharmacology approach for further investigation. Therefore, we searched for "Ecliptae Herba" in the database of Traditional Chinese Medicine Systems Pharmacology, and 48 compounds were identified. We further screened the compounds with conditions of "oral bioactivity (OB) $\geq$30%" and "drug-likeness (DL) $\geq$0.18" to determine their activity, and ten qualified compounds were filtered. Then, we attempted to obtain the structures of the selected compounds in PubChem and collected their target genes through the SwissTargetPrediction Webtool. We finally identified ten bioactive compounds of EH (Acacetin, Demethylwedelolactone, 3′-D-Methylorobol, Luteolin, Wedelolactone, Pratensein, Quercetin, Butin, Linarin and 1,3,8,9-tetrahydroxybenzofurano[3,2-c]chromen-6-one). Meanwhile, by eliminating repetitions, 290 target genes of EH were gathered for these selected compounds (Fig. 3).

3.3 PPI network of EH-OV correlated genes revealed eight key targets in mediating the suppression effect of EH against ovarian cancer

To obtain common genes between targets of Ecliptae Herba and ovarian cancer, we first collected 1055 target genes for ovarian cancer therapy by eliminating repetitions from Genecards and OMIM databases. Next, we clustered the 290 EH-targets and the OV-related genes and identified 74 correlated target genes as shown in Fig. 4A. Then, we built a PPI network, consisting of 74 nodes and 1014 edges, by using the STRING database (data not shown). We then loaded the PPI network into Cytoscape 3.9.1 software and proceeded to visualization after eliminating unconnected nodes. As shown in Fig. 4B, node size and colour depth represent the degree of each node; a bigger node size and a darker red colour indicate association at a higher degree. The PPI network demonstrated eight key nodes: EGFR (epidermal growth factor receptor), AKT1 (AKT serine/threonine kinase 1), ESR1 (Estrogen receptor 1), HSP90AA1 (Heat shock protein HSP 90-alpha), TP53 (Tumor protein p53), CCND1 (Cyclin D1), ERBB2 (Receptor tyrosine-protein kinase erbB-2) and SRC (Proto-oncogene tyrosine-protein kinase Src). Therefore, we reasoned that these eight key genes are the main contributors to EH suppression of ovarian cancer.

3.4 GO and KEGG pathway enrichment analysis

To further explore the potential regulatory mechanisms for the anti-ovarian cancer activity of EH, we continued to analyze the functional enrichment of the 74 correlated target genes. By uploading the gene list to the DAVID database, GO and KEGG enrichment analyses were performed. As shown in Fig. 4C, the top ten BPs, MFs and CCs in GO enrichment were ranked based on *p*-values. The GO enrichment results indicated that cellular response to chemical stress, cell proliferation, cell cycle regulation and protein tyrosine kinase structure and function were important terms. Meanwhile, the top ten KEGG pathways annotation in Fig. 4D identified that PI3K/AKT signaling pathway plays a vital role in the inhibitory effect of EH on ovarian cancer.

3.5 Drug-compound-target-pathway-disease network of EH in OV treatment was visualized

By integrating the data above, we next constructed and demonstrated the drug-compound-target-pathway-disease network of EH in OV treatment by Cytoscape software. As presented in Fig. 5, each compound was involved in no less than 3 key nodes, indicating that EH is a multi-compound-multi-target herbal medicine with promising therapeutical application in ovarian cancer.

3.6 EH blocked cell cycle progression and induced cell apoptosis of ovarian cancer cells by inactivating the PI3K/AKT signaling pathway

As observed above, PI3K/AKT signaling pathway is the potential mediator in EH treatment against ovarian cancer. Based on its functions in cell cycle regulation and cell apoptosis induction, we speculated that EH may perturb cell cycle progression and induce cell apoptosis. Therefore, we first detected the cell cycle changes with EH extracts incubation at indicated concentrations. As shown in Fig. 6A, the cell cycle progression of both Caov3 and A2780 cells was blocked with EH treatment, though with different distribution patterns. Meanwhile, we also determined the cell apoptosis levels with the same treatment conditions. As the cell apoptosis assay demonstrates in Fig. 6B, EH extracts dramatically increased the apoptosis population of both cell lines, indicating that cell apoptosis is a contributor to the treatment of EH against ovarian cancer. In agreement with this observation, RT-qPCR analysis in Fig. 6C revealed that the mRNA expression of *CCND1* (Cyclin D1) was significantly reduced, whereas the mRNA expression level of *p21*, an inhibitor of cyclin-dependent kinase, was remarkably increased. In alignment with these results, an increase in pro-apoptotic *BAX* gene expression and a decrease in tendency in anti-apoptotic *BCL2* gene expression was observed after EH extracts treatment of both Caov3 and A2780 cells (Fig. 6C).

To further validate the predicted mechanism, the protein expression level changes were finally detected. As shown in Fig. 6D, the protein expression level of p-PI3K (Tyr607), p-

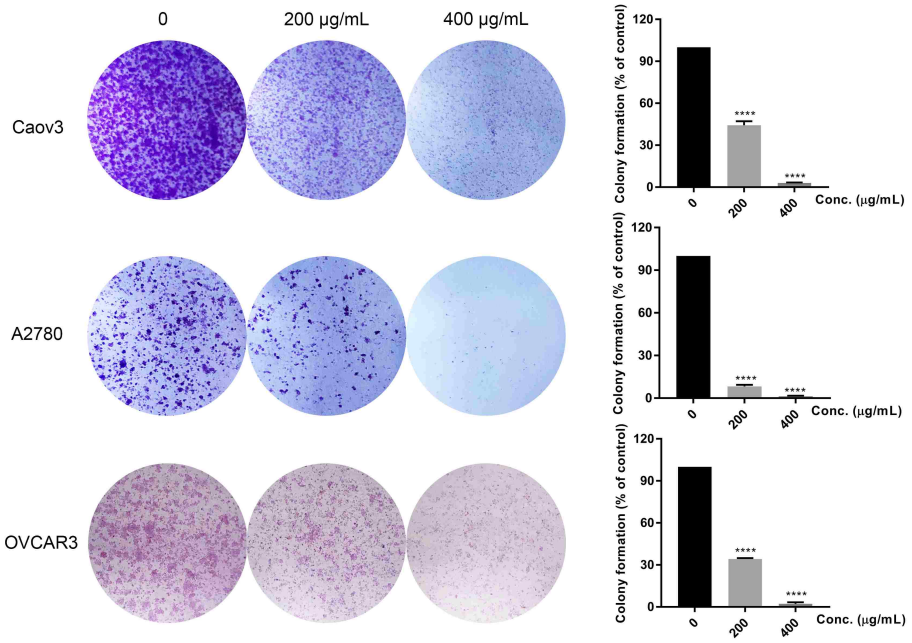

FIGURE 2. EH inhibits clonogenicity capability of ovarian cancer cells. Caov3, A2780 and OVCAR3 cells were treated with EH extracts of different concentrations (0, 200 and 400 μg/mL). A colony formation assay was conducted to validate the effect of EH extracts on cell clonogenicity capability. Data are represented by the mean ± SD. ****$p < 0.0001$; Student's *t*-test versus the untreated group.

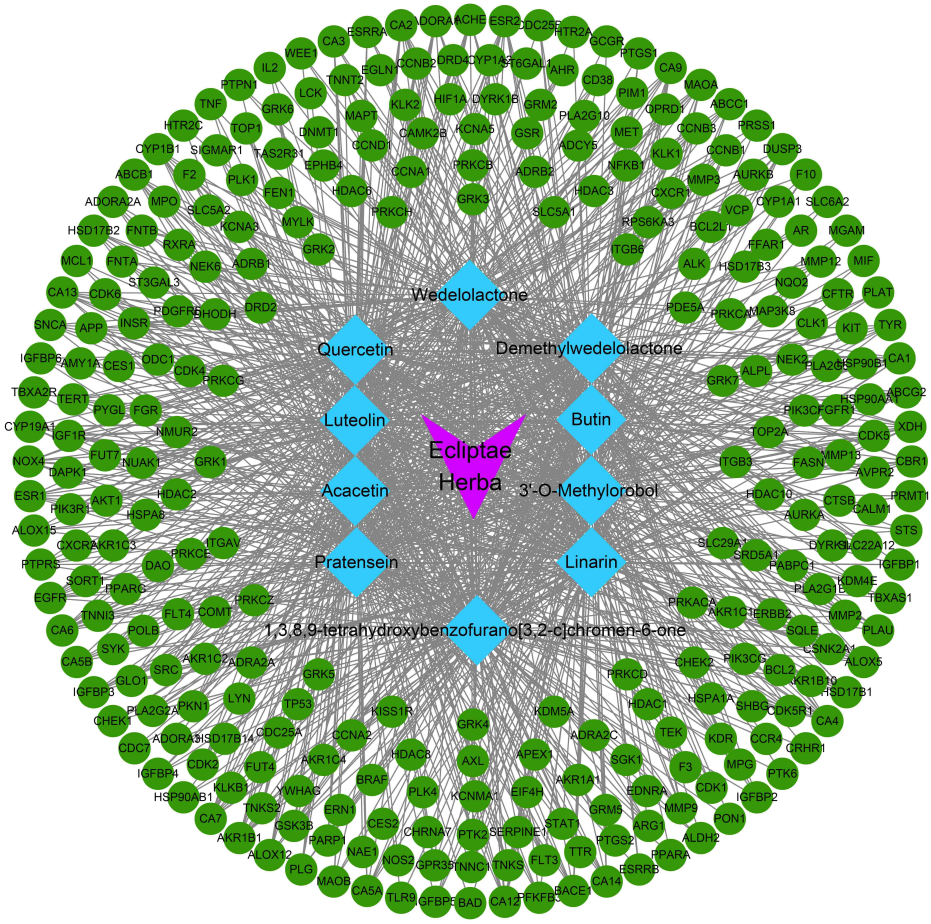

FIGURE 3. Network pharmacology identifies ten bioactive compounds and 290 target genes of Ecliptae Herba (EH). Purple node, EH; blue node, the bioactive compounds; green node, compound-related target genes; grey line, the interaction of each node.

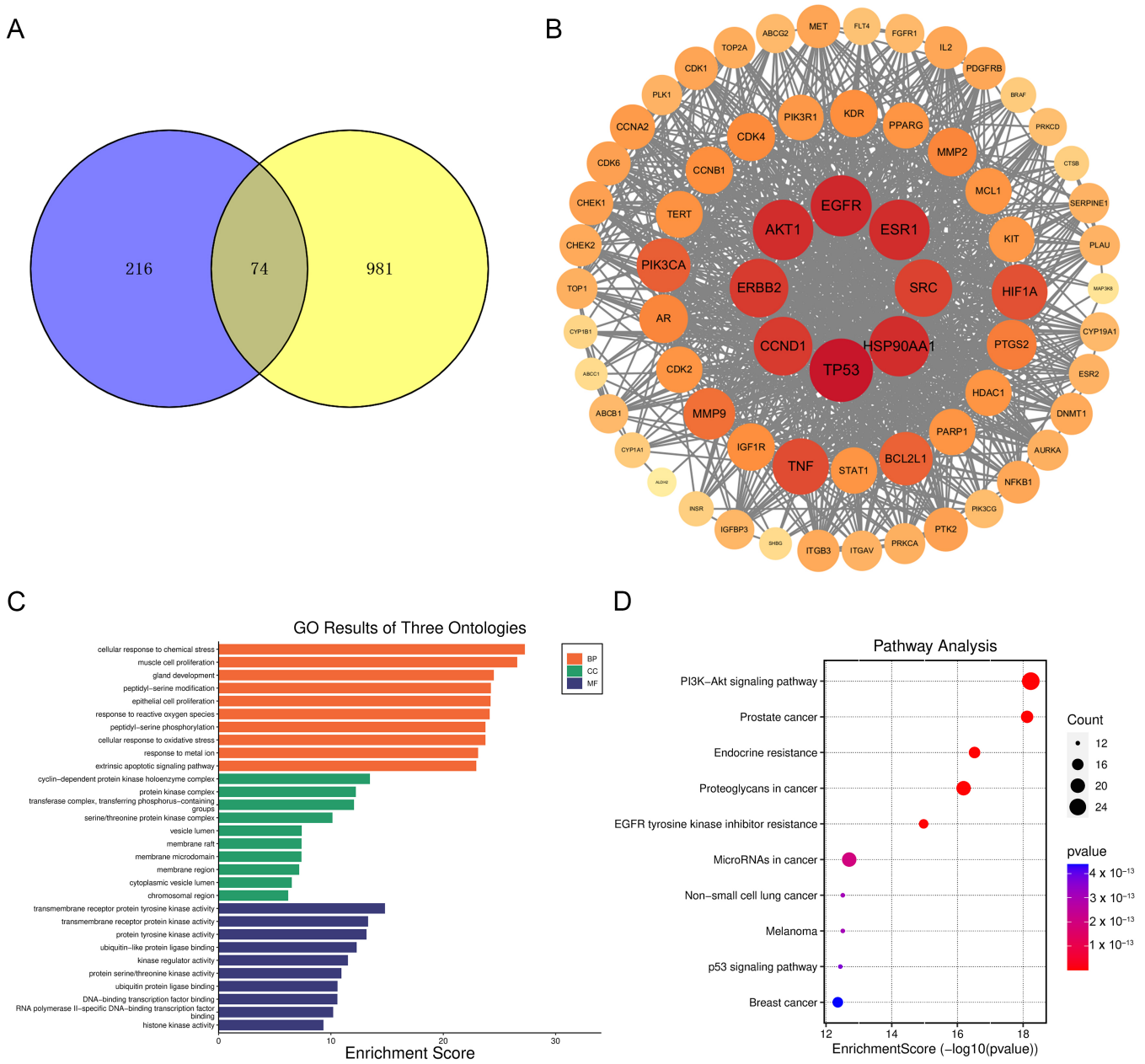

FIGURE 4. The protein-protein interaction (PPI) network, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of common genes between Ecliptae Herba (EH) targets and ovarian cancer-associated genes. (A) Venn diagram of EH targets (purple circle) and ovarian cancer-associated genes (yellow circle). (B) The PPI network of common genes. Bigger node sizes and darker red colors indicate association at a higher degree. (C) GO enrichment analysis. Terms of biological processes (BP), cellular components (CC) and molecular functions (MF) were analyzed. (D) KEGG pathways enrichment analysis. The top 10 pathways with lower *p*-values were visualized.

AKT (Ser473), Cyclin D1 and BCL2 were dramatically decreased by EH treatment. Additionally, the protein expression level of p21 and BAX was increased. These data suggest that Ecliptae Herba suppresses ovarian cancer cell proliferation and induces cell apoptosis by inactivating the PI3K/AKT signaling pathway.

4. Discussion

Ovarian cancer has the highest mortality rates among all gynaecologic tumors, and its disease process involves a complex system with distinct histological differentiation and molecular genetic heterogeneity [23]. The immunosuppressive tumor microenvironment characterizes ovarian cancer as a cold tumor. Current immunotherapy methods in clinic lead to low response rates. The development of chemoresistance is highly associ-

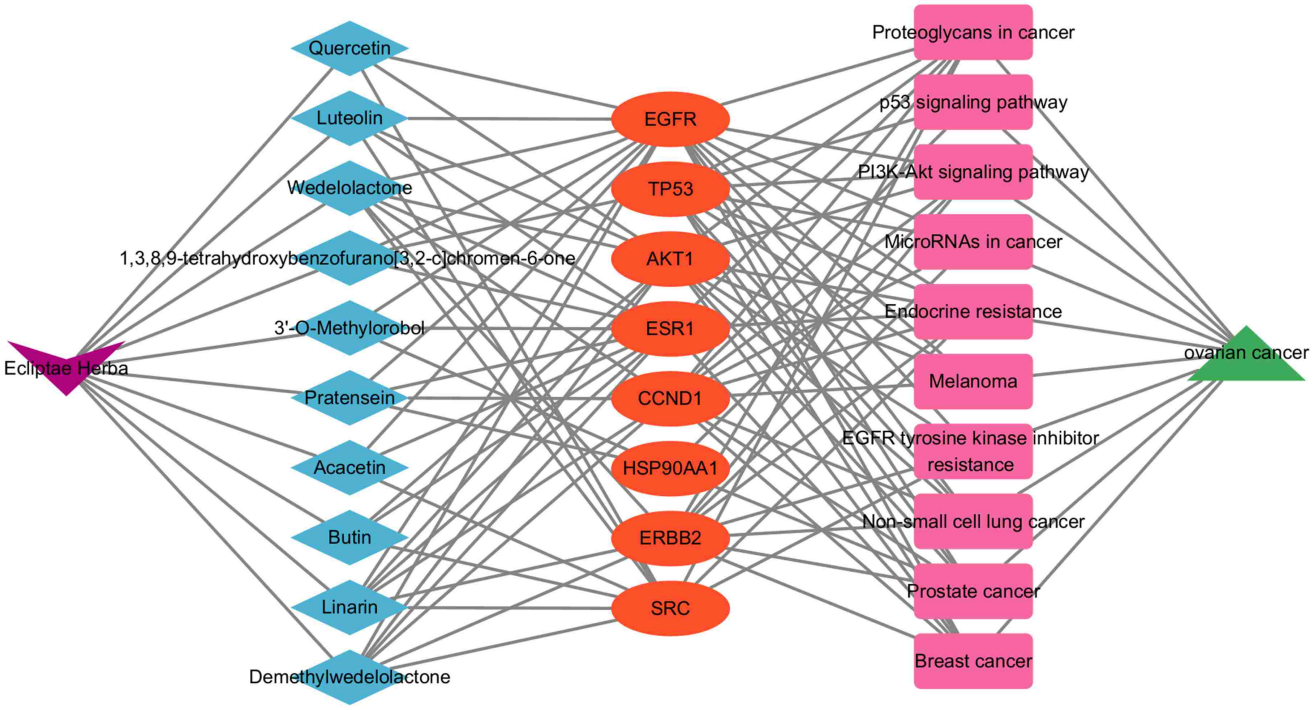

FIGURE 5. Drug-compound-target-pathway-disease network of Ecliptae Herba (EH) in Ovarian cancer treatment is visualized. Purple node, EH; blue node, the ten bioactive compounds; red node, eight key target genes; pink node, top ten Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways; green node, ovarian cancer; grey line, the interaction of each node.

ated with frequent recurrence and reduced overall survival rate [24]. New therapeutic approaches are urgently needed to provide new non-chemo drugs or novel regimens in combination with conventional therapies. Herbal medicine has been used for thousands of years; at present, it is extensively studied as an alternative treatment for cancer [25, 26]. The results of this study identified that whole Ecliptae Herba extracts can act as a potential treatment strategy for ovarian cancer, evidenced by its suppression effect on cell proliferation and the induction effect on cell apoptosis.

As a widely accepted traditional medicine, Ecliptae Herba is known to exert a liver protective function and contribute to apoptosis induction of Hela and endometrial cancer cells [27], whereas the components responsible for these actions need to be further explored. A previous report and our network pharmacology in this study screened out various bioactive compounds of Ecliptae Herba, mainly belonging to categories of coumestans and flavonoids, with a high probability of clinical application. Among these compounds, Luteolin and Quercetin are well-known anticancer agents that block tumor cells proliferation, modulate cell cycle progression and activate apoptotic processes *via* various signaling pathways, such as nuclear factor kappa B (NF-κB), PI3K/AKT and the tumor suppressor p53-induced apoptosis pathways [28–31]. In addition, Wedelolactone, as a main active constituent of Ecliptae Herba, is extensively elaborated in reports with cancer cell cytotoxicity; it can enhance the chemosensitivity of various human tumors by modulating the redox state of cancer cells [32]. Since the visualized drug-compound-target-pathway-disease network showed that Ecliptae Herba displays multi-compound-multi-target characteristics, it is thus possible that the whole Ecliptae Herba juice might be an option for clinical treatment. Accordingly, it is necessary to perform detailed quantity and effect explorations of Ecliptae Herba components in future studies.

The effect of Ecliptae Herba extracts has been tested on three ovarian cancer cell lines in this study. The results of the proliferation assay revealed that A2780 is sensitive to EH treatment, and both OVCAR3 and Caov3 cells exhibited resistance. This finding is in agreement with the source and characteristics of these cell lines. As reported, A2780 cells were derived from the tumor tissue of an untreated ovarian cancer patient recognized as endometroid adenocarcinoma. Multiple studies have validated its high sensitivity to cisplatin-induced cell apoptosis [33]. Data from *in vivo* xenograft models revealed that OVCAR3 and Caov3 cells display histological features of high-grade serous ovarian cancer (HGSOC) and Caov3 cells, carrying mutated *TP53*, show resistance to cisplatin-induced apoptosis with low p53 protein expression level in formed tumor tissues [34]. The apoptosis assay in our study further confirmed this finding. The apoptosis of Caov3 cells with EH extracts treatment in both assays indicated that Ecliptae Herba might induce ovarian cancer cell apoptosis *via* both p53-dependent and p53-independent pathways.

In the exploration of the mechanism underlying EH against ovarian cancer, the built PPI network revealed eight key contributors in cancer progression as top study candidates: EGFR, AKT1, ESR1, HSP90AA1, TP53, CCND1, ERBB2 and SRC. Knowledge of these key factors in ovarian cancer development and chemoresistance would provide more clues for the

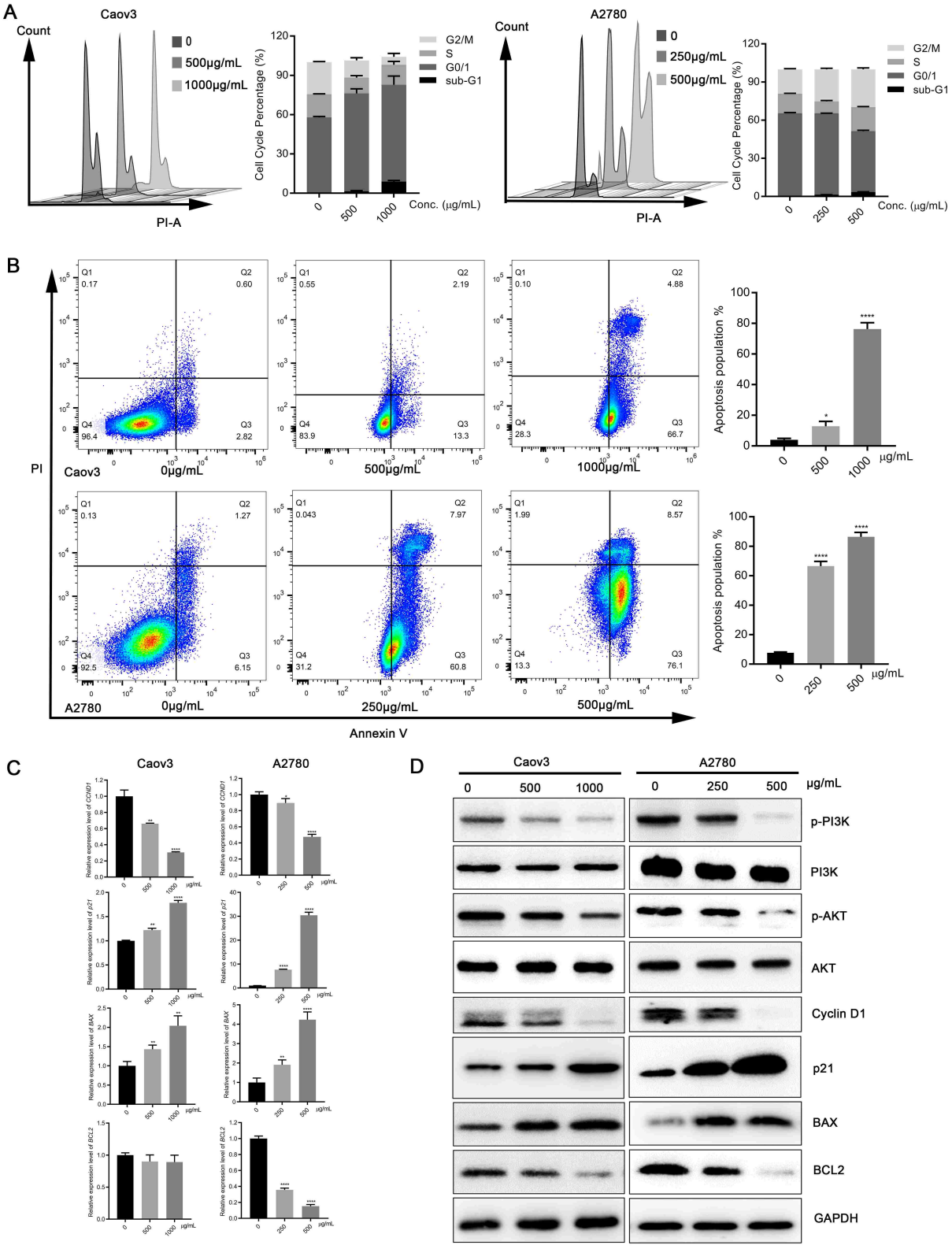

FIGURE 6. Ecliptae Herba (EH) blocks cell cycle progression and induced cell apoptosis of ovarian cancer cells by inactivating the phosphatidylinositide 3-kinases (PI3K)/AKT signaling pathway. (A) Cell cycle distributions of Caov3 and A2780 cells treated with EH extracts at indicated concentrations for 48hrs were detected with Propidium Iodide (PI) staining and illustrated in a histogram. The relative proportion of cell cycle phases was presented in mean ± standard deviation (SD). (B) Cell apoptosis levels of Caov3 and A2780 cells treated with EH extracts at indicated concentrations for 48 h were detected with Annexin V and PI staining and illustrated in a histogram. $*p < 0.05$; $****p < 0.0001$; Student's *t*-test versus the untreated group. (C) Gene expression analysis of *CCND1*, *p21*, *BAX* and *BCL2* in Caov3 and A2780 cells treated with EH extracts for 48 h. $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$; Student's *t*-test versus the untreated group. (D) Detection of protein expression level changes of Caov3 and A2780 cells treated with EH extracts (0, 500 and 1000 μg/mL) for 48 h.

following research. Among these modulators, EGFR, TP53, CCND1 and SRC act as proto-oncogenes to promote ovarian cancer development and progression by inducing a wide range of cancer cell behaviors, including growth, proliferation, adhesion, apoptosis, catabolism and oncogenic promotion [35–38]. The expressions of HSP90AA1, ESR1, AKT1 and ERBB2 are found to determine the chemosensitivity of cancer cells to chemotherapy drugs and thus show a positive correlation with ovarian cancer tumor grade [38–43]. The functions of these targets also further confirmed the polypharmacological effects of EH in ovarian cancer therapy.

Next, the cyclin-dependent protein kinase holoenzyme complex was highlighted through GO enrichment analysis, suggesting the abnormality of the cell cycle induced by EH compounds. KEGG investigation also unveiled that PI3K/AKT pathway is the crucial signaling participating in this action. Preclinical studies have reported that the PI3K/AKT pathway is commonly activated in ovarian cancers, resulting in hyperactive signaling cascades that are associated with multiple tumor cell behaviors, including proliferation, growth, survival, metabolism, angiogenesis and metastasis [44, 45]. Currently, inhibitors of the PI3K pathway are under clinical investigation, and their utility on ovarian cancer patients remains promising [46, 47]. As two common downstream regulatory genes of the PI3K/AKT pathway, Cyclin D1 and p21 act as tight regulators of cell cycle progression in energetically proliferating cells; their abnormal expression is significantly associated with chemosensitivity of ovarian cancer patients [48, 49]. It was speculated that the inhibitory effect of EH on ovarian cancer could be attributed to the inhibited PI3K/AKT pathway-induced cell cycle blockage. This hypothesis has been validated by *in vitro* biological experiments. Intriguingly, treatment with EH extracts leads to different distribution patterns of the cell cycle in the two tested cell lines, indicating that expressions of some unpredicted cell cycle-related proteins may be distinct and that cellular genotype could affect the cell cycle responses of ovarian cancer cells. In addition, as a member of the BCL2 family, the pro-apoptotic protein Bax could modulate mitochondrial membrane permeability and induce cell apoptosis [50]. Many researchers reported that phosphorylation of AKT is usually associated with increased expression of BCL2 and decreased expression of BAX [51]. With the induced BCL2/BAX ratio, the PI3K/AKT signaling pathway thus displays anti-apoptotic activity and regulates the expression of various cancers [52]. Our data presented that the EH extracts diminished the phosphorylation of AKT and reduced the ratio of BCL2/BAX, indicating the participation of cell apoptosis in the action mode of EH. Taken together, our findings showed that Ecliptae Herba extracts can suppress cell proliferation and induce cell apoptosis of ovarian cancer cells by the inactivating PI3K/AKT signaling pathway.

Although this study has confirmed the anti-ovarian cancer potential of Ecliptae Herba, several limitations should be noted. Firstly, only *in vitro* experiments were conducted in this work, so *in vivo* studies on tumor growth inhibition are needed for further confirmation. Secondly, the cytotoxic effects of Ecliptae Herba in *in vivo* studies are not covered in this work, so it is desirable to include this experimental design in future research. Thirdly, a combination with platinum-based chemotherapy should be explored to provide more evidence for further application of Ecliptae Herba in ovarian cancer treatment.

5. Conclusions

In summary, we detected the anti-cancer effects of Ecliptae Herba on three ovarian cancer cell lines and utilized network pharmacology to uncover the molecular mechanism underlying these effects. Ten bioactive compounds of Ecliptae Herba were identified and 74 EH-OV correlated targets were gathered for enrichment exploration. In agreement with GO and KEGG analysis, the *in vitro* biological experiments demonstrated that Ecliptae Herba can induce ovarian cancer cell cycle blockage and cell apoptosis by modulating PI3K/AKT signaling pathway. Taken together, the findings of this study support the possibility of using the Ecliptae Herba herbal medicine as a therapeutic strategy for ovarian cancer.

ABBREVIATIONS

OV, Ovarian cancer; EH, Ecliptae Herba; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; MF, Molecular Function; CC, cellular component; BP, Biological Process; PI, Propidium Iodide; OMIM, Online Mendelian Inheritance in Man; SD, standard deviation; OB, oral bioactivity; DL, drug-likeness; CCK8, Cell Counting Kit-8 assay; HGSOC, high-grade serous ovarian cancer; PI3K, phosphatidylinositide 3-kinases; AhR, aryl hydrocarbon receptor; RPMI, Roswell Park Memorial Institute; DMEM, Dulbecco's Modified Eagle Medium; CO_2, carbon dioxide; qRT-PCR, quantitative real-time polymerase chain reaction; CT, cycle threshold; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffer saline; RIPA; radioimmunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; EGFR, epidermal growth factor receptor; ESR1, Estrogen receptor 1; HSP90AA1, Heat shock protein HSP 90-alpha; ERBB2, Receptor tyrosine-protein kinase erbB-2; *CCND1*, Cyclin D1; NF-κB, nuclear factor kappa B.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

AUTHOR CONTRIBUTIONS

XQZ—designed, collected, analysed the data and wrote the original manuscript. XQZ, DY and ZQM—performed the research. CT and HY—provided help and advice on data collection and analysis. All authors provided inputs in the manuscript editing. All authors approved the submission of the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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