

Total flavonoids in *Artemisia absinthium* L. and evaluation of its anticancer activity

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Abstract

Aim of the study:

To optimize the extracting parameters of total flavonoids in *Artemisia absinthium* L. by ultrasound-assist combined with enzymatic hydrolysis and to combine molecular docking and network pharmacology to predict its core constituent targets and thus evaluate its antitumor activity.

Methods

Reaction surface methodology was used to investigate the univariate effects of enzyme ratio, enzyme amount, enzyme hydrolysis temperature, enzyme hydrolysis time, pH, solid-liquid ratio, ethanol concentration, and sonication temperature on total flavonoid yield in *A. absinthium* L.. On this basis, a three-factor, three-level experimental approach was adopted with solid-liquid ratio, enzymatic hydrolysis temperature, and ethanol concentration as independent variables and total flavonoids as response values, and then combined with Box-Behnken design (BBD) to optimize the extraction conditions. The quantitative and qualitative analysis of the main components was performed by UHPLC-MS. The inhibitory effect of flavonoids and their active components on the proliferation of cervical cancer HeLa cells was detected by MTT. Molecular docking and network pharmacology were used to predict the molecular mechanism of the main active components (Cynaroside and Astragalin) and to decipher the overall mechanism of total flavonoids against cervical cancer.. pharmMapper and SwissTargetPrediction databases were used to sort out the potential targets of the main chemical components. Targets related to cervical cancer were collected from OMIM and DrugBank.

Results

The content of total flavonoids in *A. absinthium* L. reached $3.80 \pm 0.13\%$, and the main components included Astragalin, Cynaroside, Ononin, Rutin, Kaempferol-3-O-rutinoside, Diosmetin, Isorhamnetin, and Luteolin. Cynaroside and Astragalin exert their cervical cancer inhibitory functions by regulating several signaling proteins (e.g. EGFR, STAT3, CCND1, IGFIR, ESR1). GO and KEGG analyses showed that the anti-cancer of both compounds was associated with ErbB signaling pathway and FoxO signaling pathway. MTT results showed that total flavonoids of *A. absinthium* L. and its active components (Cynaroside and Astragalin) significantly inhibited the growth of HeLa cells in a concentration-dependent manner with IC_{50} of $396.0 \pm 54.2 \mu\text{g/mL}$ and $449.0 \pm 54.8 \mu\text{g/mL}$, respectively.

Conclusion

The optimal process conditions for obtaining total flavonoids by ultrasound-assisted enzymatic digestion were: enzyme ratio 3:2, enzyme dosage 2%, enzymatic hydrolysis temperature 45°C, enzymatic hydrolysis time 105 min, pH 3.5, solid-liquid ratio 1:15, ethanol concentration 85%, sonication temperature 30°C. Results from network pharmacology and molecular docking indicate that EGFR and SRC are the key targets of the two core components of total flavonoids against cervical cancer, the optimal procedure for extracting total flavonoids from *A. absinthium* L. was fixed and the total flavonoids showed good anti-cervical cancer activity. Furthermore, its active components can mediate apoptosis by inducing the accumulation of ROS.

Introduction

Cervical cancer is one of the most common gynecologic malignancies worldwide and is a serious health risk for women due to its extremely high morbidity and mortality (Marquina et al., 2018). 604,000 new cases and 342,000 deaths occurred worldwide in 2020 (Sung et al., 2021). Persistence of high-risk human papillomavirus (HPV) infection is a major factor in the development of cervical cancer (Hu and Ma, 2018). Currently, the main treatment options for cervical cancer include surgical resection, radiation therapy, chemotherapy, locally targeted therapy, and immunotherapy (Eifel, 2006; Kumar et al., 2018; Mauricio et al., 2021). Although conventional treatments are effective for early-stage cervical cancer, they have limited efficacy for locally staged and metastatic cervical cancer (Liontos et al., 2019). Most importantly, long-term use of chemotherapeutic agents, such as cisplatin, adriamycin, paclitaxel, and bleomycin, is prone to toxic side effects and drug resistance (Ali et al., 2020; Wei et al., 2019). Therefore, biomedical researchers are also constantly searching for an alternative drug with better safety and effectiveness as a new strategy for cancer treatment.

Artemisia is a genus of small herbs and shrubs found in the northern temperate regions (Bora and Sharma, 2011). The species is often reported as a valuable additive for spice plants and the alcohol industry. In addition, *Artemisia* species is rich in essential nutrients such as fatty acids, carbohydrates, dietary fiber, proteins, essential amino acids, vitamins, and minerals and thus is widely used as traditional food, tea, and dietary supplements (Szopa et al., 2020; Trendafilova et al., 2020). Notably, the herbs of this species are also considered raw materials for oil extraction and have a high food value, as many of them are used in cooking (Allen, 2010; Parada et al., 2011).

A. absinthium L. belongs to Campanulales, family Asteraceae, and is commonly known as Wormwood (Moacă et al., 2019). The medicinal perennial mainly distributes in Xinjiang, Sichuan, and Yunnan in China (Mohammadian et al., 2016), which is rich in secondary metabolites including flavonoids, alkaloids, phenols, terpenoids, and derivatives with various pharmacological activities, such as antibacterial, anti-inflammatory, antiviral, hepatoprotective, antioxidant and anticancer (Ahmad et al., 1992; Ali et al., 2021; Juteau et al., 2003; Koyuncu, 2018; Mishra et al., 2013; Szopa et al., 2020). In addition to its traditional medical applications, it has a high food value. Also, the most popular spirits of the 19th and 20th centuries, absinthe, whose main component is *A. absinthium* L., is a highly pure alcoholic beverage with psychoactive properties due to the high content of α - and β -tulip ketones (Kshirsagar and Rao, 2021; Mladenova, 1998).

Bioflavonoids, also known as flavonoids, are natural plant constituents (Cheng et al., 2021). More than 8000 species have been identified and isolated from plants, whose ($C_6-C_3-C_6$) structures are formed through two phenolic hydroxyl groups of benzene rings (A and B rings), interconnected by a three-carbon bond (Pietta, 2000; Zhang, Q. et al., 2020). They can be classified into six groups based on structural diversity, including flavones, isoflavones, flavanones, flavonols, flavanols, and anthocyanins (Choy et al., 2019). Many studies have reported that flavonoids have a significant role in treating and preventing tumours (Chanput et al., 2016; Iwashina, 2000; Luan et al., 2021; Maleki et al., 2019). The extraction methods of flavonoids mainly include heating, reflux extraction, soaking, organic solvent extraction, microwave-assisted extraction, ultrasound-assisted extraction, and enzymatic hydrolysis method (He et al., 2020; Liao et al., 2021; Liu et al., 2014). In the enzymatic hydrolysis method, cellulase is usually used to destroy the plant cell wall to release the intracellular material (Liu et al., 2017). Ultrasonic extraction can continuously shake the extract to accelerate the dissolution of intracellular substances and improve the extraction rate of total flavonoids and the utilization rate of raw materials (Lee et al., 2013; Yu et al., 2019). Although the traditional extraction method equipment is relatively simple, the extraction is time-consuming and inefficient. It is easy to destroy the thermally unstable components at high temperatures (Chaves et al., 2020; Chávez-González et al., 2020). To overcome the shortcomings of the traditional extraction method and consider the low content and complexity of total flavonoids in *A. absinthium* L., in this experiment, we adopted ultrasound-assisted enzymatic hydrolysis to improve the yield of total flavonoids, which has the characteristics of mildness and environmental protection (Daoying et al., 2019; DU Lei et al., 2022; Wang et al., 2008).

The response surface optimization method (RSM) has been widely used in process optimization based on a rational experimental design (Zou et al., 2011). A multiple quadratic regression equation is used to fit the functional relationship between the factors and the response values. Finally, the regression equation is analyzed and predicted to obtain the best process parameters. Box-Behnken design (BBD) is a statistical model of the response surface design method. BBD represents an independent quadratic design that does not contain embedded or fractional factorial designs and is easy to design and statistically analyze compared to other designs (Bezerra et al., 2008; Zhang et al., 2019). It can reduce experiments' number, cost, and time (Arya et al., 2021; Jing et al., 2015). The main components included Astragaloside, Cynaroside, Ononin, Rutin, Kaempferol-3-O-rutinoside, Diosmetin, Isorhamnetin, and Luteolin.

Network pharmacology is a systematic approach based on analysing networks of disease, gene, protein targets and drug interactions (Chen et al., 2018). The synergistic effects of components and multi-target drugs and their potential mechanisms are better elucidated by analyzing complex and multi-level interaction networks. It has been widely used to study the molecular mechanisms of herbal medicines, herbal drug pairs, and herbal complexes acting on diseases (Yuan et al., 2021). Molecular docking is a method to design drugs by simulating the interaction pattern between receptors and drugs. In recent years, the application of molecular docking to explain the relevant mechanism of action has become a trend in developing new drugs (Saikia and Bordoloi, 2019). In this study, we used a network pharmacology approach to explore the major bioactive components of total flavonoids in *A. absinthium* L. and predict their effective molecular targets and potential mechanisms in treating cervical cancer.

In summary, the ultrasound-assisted enzymatic hydrolysis was used to optimize the extraction process of total flavonoids from *A. absinthium* L. based on single-factor experiments combined with BBD experimental design and multiple linear regression and binomial fitting of the experimental data were used to perform predictive analysis of the best process. The antitumor activity of the total flavonoids and their active ingredients (Cynaroside and Astragaloside) obtained under the optimal extraction conditions were evaluated, which showed good anti-cervical cancer activity. Furthermore, its active components can mediate apoptosis by inducing the accumulation of ROS. This study provided a theoretical basis for the further development and comprehensive application of *A. absinthium* L..

Materials and Methods

2.1. Materials and Reagents

A. absinthium L. powder; ethanol, quercetin control (Shanghai Maclean Biochemical Technology Co., Ltd.), sodium nitrite, aluminium nitrate, sodium hydroxide, aluminium trichloride, potassium acetate, methanol, ethyl acetate, petroleum ether, isopropanol (Tianjin Xinbote Chemical Co., Ltd.).

2.2. Methods

2.2.1. Quercetin standard curve plotting

Quercetin 5.0 mg was weighed precisely and fixed to 25 mL with methanol to obtain a mass concentration of 0.2 mg/mL standard solution, which was stored in a refrigerator at 4°C for use. Were added 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL of quercetin control solution into 10 mL volumetric flasks, and were added 0.1 mol/mL AlCl_3 solution for 6 min, then were added 1 mol/mL $\text{C}_2\text{H}_3\text{KO}_2$ solution for 3 mL, and finally were fixed the volume with methanol solution to the scale. The corresponding solution without quercetin was used as a blank control, 100 μL was aspirated, and the absorbance at 415 nm was measured. The standard curve was plotted with absorbance (y) as the vertical coordinate and quercetin concentration (x) as the horizontal coordinate.

2.2.2. Extraction of Total Flavonoids

After weighing 2.0 g of *A. absinthium* L. powder in a 50 mL centrifuge tube, it was adding ethanol solution at a ratio of 1:10, adding a certain amount of complex enzymes (cellulase and pectinase) according to the mass ratio of *A. absinthium* L., carrying out enzymatic hydrolysis in a water bath at a certain pH and temperature (mixed once at 30 min), and then inactivating the enzyme in a water bath at 100°C for 5 min, then, adding 10 mL of ethanol solution at a concentration of 80%, and finally sonicating the above enzymatic solution at a certain power, sonication temperature and sonication time. Then, 10 mL of ethanol solution was added, and the above enzymatic solution was sonicated at a certain ultrasonic power, ultrasonic temperature, and ultrasonic time. finally, the supernatant was taken from the sonicated sample solution after centrifugation, and the absorbance of each solvent was measured at 415 nm with blank as the control, and the total flavonoid extraction rate and antitumour activity were calculated. (The blank was divided into those with and without enzyme, and the effect of enzyme addition on the yield and activity was evaluated before and after the enzyme addition).

2.2.3. Determination of total flavonoid content

Weigh accurately 2.0 g of the dried powder of *A. absinthium* L. crushed with a high-speed universal grinder, add a certain volume fraction of aqueous ethanol solution according to a certain solid-liquid ratio; extract with ultrasound for a certain time in an ultrasonic cleaner, filter, and concentrate the extract with a rotary evaporator under reduced pressure to remove the solvent. The concentrated solution was transferred to a 100 mL volumetric flask, and the volume was fixed with ethanol to the scale. Precisely measure 1.0 mL of *A. absinthium* L. flavonoids extract with aluminium trichloride-potassium acetate method to detect the absorbance value (A) of total flavonoids extract and bring into the standard. The curve regression equation calculated the total flavonoid concentration and then converted it into flavonoid content.

Total flavonoid extraction amount (mg/g) = $C \times V \times N / M$

C: concentration of total flavonoids measured (mg/mL);

V: volume of extraction solution (mL);

N: dilution times;

M: mass of raw material (g).

2.2.4. Single-factor experimental design

Weighing 2.0 g of *A. absinthium* L., the factors that affected the total flavonoid extraction were selected by the controlled variable method for the one-way experiment. One of the factors is ethanol concentration, enzyme complex ratio, enzyme complex amount, pH, material-liquid ratio, enzyme hydrolysis temperature, enzyme hydrolysis time, sonication temperature and sonication time. The influence of other factors on the extraction rate of flavonoids from *A. absinthium* L. was investigated using a single-factor experimental design.

2.3. Optimization of Extraction Conditions of Total Flavonoids by Response Surface Methodology

The results of the single-factor experiments were combined. Three factors, including solid-liquid ratio, enzymatic hydrolysis temperature and ethanol content, were selected to significantly affect the extraction rate of total flavonoids from *A. absinthium* L. Regression analysis was performed using DesignExpert 8.0 software to calculate the optimal extraction conditions using a regression equation, and validation test was performed three times in parallel to obtain the optimal extraction conditions by the response surface method and complete analysis of each factor.

2.4. LC-MS

2.4.1. Metabolites Extraction

The sample was thawed on ice. After 30 s vortex, 200 μ L sample was dried under gentle nitrogen flow. The sample was then reconstituted in 200 μ L of 50% methanol containing 0.1% formic acid. After vortexing for 30 seconds, it was subjected to ultrasound in an ice bath for 15 minutes. The sample was then centrifuged at 12000 rpm (RCF=13800($\times$ g), R=8.6 cm) for 15 minutes at 4°C. The resulting supernatant was passed through a 0.22 μ m filter membrane. It was then transferred to 2 ml glass vials and stored at -80 °C until analysis by UHPLC-MS/MS.

2.4.2. MS Analysis

AB Sciex QTrap 6500+ mass spectrometer was applied for assay development. Typical ion source parameters were: IonSpray Voltage: +5000/-4500 V, Curtain Gas: 35 psi, Temperature: 500 °C, Ion Source Gas 1: 55 psi, Ion Source Gas 2: 60 psi.

2.4.3. Screening of core active ingredients and targets

PharmMapper (<http://lilab-ecust.cn/pharmmapper/>) and SwissTargetPrediction databases (<http://www.swisstargetprediction.ch/>) were used to retrieve the gene targets of the active ingredients. The obtained targets were then mapped to the UniProt database (<https://www.uniprot.org/>) for normalization.

2.4.4. Disease-related gene mining

Targets associated with cervical cancer were collected from the OMIM database (<https://www.omim.org/>) and DrugBank database (<https://www.drugbank.ca/>). Normalization of gene names and definition of species as "human" was performed using the UniProt database. Venn diagrams of active ingredients and cervical cancer targets were mapped using a bioinformatics platform.

2.4.5. Protein–protein interaction network construction and analysis

A target PPI network was obtained by compiling potential targets from the STRING database (<https://string-db.org/>). The species selection parameter was set to "Homo sapiens" and the minimum required confidence in the interaction score was set to 0.900. The PPI network was then visualized by Cytoscape software.

2.4.6. Gene ontology (GO) and the Kyoto encyclopedia of genes and genomes (KEGG) enrichment

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichments were performed using Bioconductor Cluster Profiler for core drug group ontology (GO) enrichment analysis of potential target genes and KEGG pathway enrichment analysis of tissues. Pathways were ranked according to the number of molecules in the pathway with a critical value ($p < 0.05$).

2.4.7. Network construction

Compound target pathway networks were constructed using Cytoscape 3.7.2. Topological properties were analyzed using Cytoscape's Network Analyzer plug-in to identify key components and targets.

2.4.8. Molecular docking makes sure the interaction between targets and key compounds

Molecular docking ensures interactions between targets and key compounds. Target protein structures were obtained from the RCSB PDB database (<http://www.rcsb.org/>). 2D structures of ginseng leaf actives were sought from the PubChem database. ChemBio Draw 3D and Autodock Tool were applied to optimize the structures of key compounds and targets, including 3D chemical structure creation, energy minimization, and format conversion. PyMol was used to process proteins, including removal of ligands, correction of protein structures,

and removal of water. Docking was done by R 4.1.3 software and Autodock Vina to select the molecule with the lowest binding energy in the docked conformation and observe binding effects by matching to the original ligand and intermolecular interactions.

2.5. Cell Culture

HeLa, SiHa and H8 cells were obtained from the Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, Xinjiang University (Urumqi, Xinjiang, China) and were cultured in RPMI1640 with DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium having 10% fetal bovine serum (MRC, Changzhou, China), 50 U/mL each of penicillin and streptomycin (MRC, Changzhou, China), respectively, at 37°C in a humidified environment with 5% CO₂.

2.6. MTT

HeLa and SiHa cells at the logarithmic stage were inoculated with 5×10^3 cells/mL in 96-well plates and treated with different concentrations of total flavonoids (100, 200, 400, 500, 600, and 800 µg/mL), Cynaroside and Astragalin (100, 150, 200, 250, 300, 350, and 400 µg/mL). Control (blank), 0.3%DMSO (negative Control) and 35 µg/mL cisplatin (positive Control) were set, with 6 replicates in each group. After 24 h of incubation, the waste liquid was discarded by centrifugation, and 100 µL MTT (0.5 mg/mL) was added to each of them and incubated at 37°C for 4 h under dark conditions. The supernatant was removed by centrifugation, dissolved with 150 µL DMSO, and detected the OD value of each well at 490 nm. The following formula calculated cell survival rate: Cell viability (%) = $(OD_{\text{treated}}/OD_{\text{control}}) \times 100\%$.

2.7. Apoptosis

HeLa cells were inoculated with 1×10^5 cells/dish in 60 mm culture dishes, and the cells were treated with different concentrations of total flavonoids (200, 400, and 600 µg/mL), Cynaroside and Astragalin (200, 300 and 400 µg/mL) for 24 h, respectively. The digested cells were collected from each group, centrifuged at 1200 r/min for 7 min, washed twice with PBS (4-5 mL), and discarded the supernatant. 100 µL of binding buffer (1×binding buffer) was added to each group to resuspend the cells. Cells were resuspended by adding 2.5 µL Annexin V-FITC and 5 µL PI, mixed and shaken for 10 min in an ice bath, resuspended by 300 µL of 1×binding buffer, and detected by FACS. The data were analyzed by FlowJo7.6 software.

2.8. Hoechst

HeLa cells were inoculated 1×10^5 cells/well in 6-well plates and incubated for 24 h. Cells were treated with different concentrations of flavonoids, cynaroside and astragalin and incubated at 37 °C for 24 hours. The supernatant was discarded and washed twice with precooled PBS buffer, followed by fixation with precooled 4% paraformaldehyde for 10 min at 4°C. After washing with PBS buffer, the cells were stained with Hoechst 33258 dye for 20 min in the dark. After washing with PBS, the cells were stained with Hoechst 33258 for 20 min under low light. After dyeing for 20 min, the cells were slowly washed twice with PBS for 3 min each time and photographed with an inverted fluorescent microscope to observe the changes in the karyotype.

2.9. ROS

Logarithmic growth stage HeLa cells were inoculated at a density of 1×10^5 /mL in 60 mm dishes for 24 h for cell apposition. HeLa cells were treated with various concentrations of total flavonoids, Cynaroside and Astragalin and incubated at 37°C for 24 h. Cells were collected, and the supernatant was discarded. Cells were suspended in the DCFH-DA dilution solution and incubated for 20 min at 37°C. Cells were shaken every 3-5 min. The cells were washed 3 times with serum-free medium to adequately remove the unbound DCFH-DA fluorescent probe, which was detected by flow cytometry and analysed by FlowJo 7.6 software.

2.10. Statistics

The experimental results were processed using Graphpad prism 8 software, and the Box-Behnken experimental design and data analysis were performed using DesignExpert 8.0 software. All data were expressed as the mean ± standard deviation (S.D.). $p < 0.05$ was considered statistically significant.

Results

3.1. Standard Curve

Based on the measurement results, the standard curve was plotted with the absorbance at 415 nm as the vertical coordinate and the quercetin concentration as the horizontal coordinate. The linear regression equation of total flavonoids in *A. absinthium* L. was obtained as: $y=9.7675x-0.0164$, and the linear correlation coefficient $R^2=0.9974$, suggesting that the standard had a good linear relationship.

3.2. Single-factor Experiments

3.1.1. Effect of Compound Enzyme Ratio on the Extraction Rate of Total Flavonoids

The fixed solid-liquid ratio was 1:10, the amount of the enzyme was 1.0%, pH was 4.0, the enzymatic hydrolysis temperature was 50°C, the enzymatic hydrolysis time was 90 min, and the sonication time was 20 min. The effects of cellulase and pectinase at different ratios (1:3, 2:3, 1:1, 3:2, 3:1 g/g) on extracting total flavonoids in *A. absinthium* L. were investigated. As shown in Figure 1 (A), the total flavonoid yield showed a trend of increasing and then decreasing with the increased cellulase ratio. When the ratio of cellulase to pectinase was 3:2, the total flavonoid yield reached 2.68%. It is possible that the increased cellulase ratio may have a stronger effect on the damage of cell wall and intercellular matrix of *A. absinthium* L., which leads to effectively release intracellular components. However, when cellulase ratio reached a certain degree, some fibres were attached to the surface of *A. absinthium* L., which prevented the release of total flavonoids, resulting in the decrease of the total flavonoids yield. Therefore, the ratio of cellulase to pectinase was chosen to be 3:2.

3.1.2. Effect of Enzyme Dosage on the Extraction Rate of Total Flavonoids

Based on the above-optimized compound enzyme ratio, the solid-liquid ratio was fixed at 1:10, pH was 4.0, the enzymatic hydrolysis temperature was 50°C, the enzymatic hydrolysis time was 90 min and the sonication time was 20 min. The effects of the amount of compound enzyme (0.5%, 1.0%, 1.5%, 2.0%, 2.5%) on the total flavonoids yield of *A. absinthium* L. were investigated. As shown in Figure 1 (B), the total flavonoid yield significantly increased with the increase of the amount of the enzyme compound and then decreased. When the amount of enzyme was 2.0%, the total flavonoid yield reached the maximum value of 2.72%. Therefore, the optimal amount of the enzyme is 2.0%.

3.1.3. Effect of pH on the Extraction Rate of Total Flavonoids

The optimum pH of cellulase for the experiment was between 4.0 and 6.5, and the optimum pH of pectinase was between 3.0 and 6.0. Based on the above-optimized ratio and amount of the complex enzymes, the solid-liquid ratio was fixed at 1:10, the enzymatic hydrolysis temperature was 50°C, the enzymatic hydrolysis time was 90 min, and the sonication time was 20 min. The effects of pH (3.0, 3.5, 4.0, 4.5, 5.0) on the total flavonoid yield of *A. absinthium* L. were investigated. As shown in Figure 1 (C), the total flavonoid yield showed a trend of increasing and then decreasing with the increase of pH from 3.0 to 5.0. When the pH was 3.5, the total flavonoids yield reached a maximum of 3.07%. Therefore, a pH of 3.5 was chosen for the enzymatic hydrolysis.

3.1.4. Effect of Ethanol Concentration on the Extraction Rate of Total Flavonoids

Based on the above-optimized ratio and dosage of complex enzymes and pH, the solid-liquid ratio was fixed at 1:10, enzymatic hydrolysis temperature was 50°C, enzymatic hydrolysis time was 90 min, and sonication time was 20 min. the effects of ethanol concentrations (10%, 30%, 50%, 70%, 85%, and 100%) on the yield of total flavonoids in *A. absinthium* L. were investigated. As shown in Figure 1 (D), a lower ethanol concentration is generally suitable for extracting polar flavonoids, and a higher ethanol concentration is ideal for extracting non-polar flavonoids. When the concentration of ethanol increased from 10% to 85%, the extraction amount of total flavonoids was increased and reached the highest amount at 85% ethanol. Therefore, the optimum ethanol dosage was 85%.

3.1.5. Effect of Solid-liquid Ratio on the Extraction Rate of Total Flavonoids

Based on the above-optimized ratio and amount of complex enzymes, pH and ethanol concentration, the enzymatic hydrolysis temperature was 50°C, the enzymatic hydrolysis time was 90 min, and the sonication time was 20 min. The effects of solid-liquid ratios (1:5, 1:8, 1:10, 1:12, 1:15, 1:20 g/mL) on the total flavonoid yield of *A. absinthium* L. were investigated (Figure 1E). When the solid-liquid ratio was 1:10 (g/mL), it was difficult for the material to be completely immersed in the solvent and resulted in incomplete extraction. When the solid-liquid ratio was 1:15 (g/mL), the maximum yield of total flavonoids reached 1.24%. Therefore, 1:15 (g/mL) was chosen as the best solid-liquid ratio.

3.1.6. Effect of Enzymatic Hydrolysis Temperature on the Extraction Rate of Total Flavonoids

Based on the above-optimized compound enzyme ratio and dosage, pH, ethanol concentration and solid-liquid ratio, the enzymatic hydrolysis time was 90 min and the sonication time was 20 min. The effect of enzymatic hydrolysis temperature (30, 40, 50, 60, 70°C) on

the yield of total flavonoids in *A. absinthium* L. was investigated. As shown in Figure 1 (F), the total flavonoid yield showed a trend of increasing and then decreasing with the increase of enzymatic hydrolysis temperature. When the enzymatic hydrolysis temperature was 45°C, the total flavonoid yield reached 3.51%. Therefore, the optimal temperature of enzymatic hydrolysis was 45°C.

3.1.7. Effect of Enzymatic Hydrolysis Time on the Extraction Rate of Total Flavonoids

Based on the above-optimized compound enzyme ratio and amount, pH, ethanol concentration, solid-liquid ratio, and enzymatic hydrolysis temperature, the effect of enzymatic hydrolysis time (45, 60, 75, 90, 105, 120 min) on the yield of total flavonoids in *A. absinthium* L. was investigated by fixing the sonication time of 20 min. The compound enzyme failed to destroy the cell wall sufficiently under shorter enzymatic hydrolysis time, which resulted in low total flavonoid yield. When the enzymatic hydrolysis time was extended to 105 min, the total flavonoid yield reached 4.59% (Figure 1G). Therefore, the enzymatic hydrolysis time of 105 min was chosen.

3.1.8. Effect of Ultrasonic Temperature on the Extraction Rate of Total Flavonoids

Based on the above-optimized ratio and dosage of complex enzymes, pH, ethanol concentration, solid-liquid ratio, and enzymatic hydrolysis temperature and time, the effects of sonication temperature (20°C, 40°C, 60°C, 80°C, 100°C, 120°C) on the yield of total flavonoids in *A. absinthium* L. were investigated. The increase of sonication temperature has a certain promotion effect on the extraction of total flavonoids within 10°C and 30°C. The highest total flavonoid yield reached 4.75% at 30°C (Figure 1H). Therefore, the optimal sonication temperature of 30°C was selected.

Figure 1. Effect of single factors on the extraction of total flavonoids from *A. absinthium* L.. (A) Enzyme ratio; (B) enzyme dosage; (C) pH; (D) ethanol concentration; (E) solid-liquid ratio; (F) enzymatic hydrolysis temperature; (G) enzymatic hydrolysis time; (H) ultrasonic temperature.

3.3. Analysis of Extraction Parameters Using Response Surface Method

3.3.1. Response Surface Experimental Design

Response surface methodology is an important method in condition optimization, and only fewer experiments are required to obtain statistically acceptable results. Based on the single-factor experiments, three factors (solid-liquid ratio (A), enzymatic hydrolysis temperature (B), and ethanol concentration (C)) with significant effects on the extraction rate of total flavonoids from *A. absinthium* L. were selected for the 3-level response surface method experiments (Table 1). The 17 response values for different combinations were shown in Table 2. Applying a quadratic linear regression fitted to the data and the significance and variance analysis yielded the following binary, multinomial regression model. $Y=3.81-0.19A+3.424E-003B+0.16C-0.27AB+0.11AC-0.026BC-0.68A^2-0.47B^2-0.45C^2$. As shown in Table 3, the regression model was highly significant ($p<0.01$), and the out-of-fit term $p=0.1379$ was not significant (confidence level of 95%), indicating a good fit of the model. The correlation coefficient of the regression model equation $R^2=0.9373$ indicates that the correlation of the predicted and true values is high, and the error is small, which can well reflect the experimental data. The CV value indicates the accuracy of the test, and the model's CV value is 7.14%, suggesting the test is reliable. One equation $A<0.05$ is significant, B and $C>0.05$ is not significant, binomial A^2 , B^2 , and $C^2<0.01$ is highly significant, interaction term $AB<0.05$ is significant, while AC and $BC>0.05$ is not significant. It indicates that the effect of each experimental factor on the response value is not a simple linear relationship but a non-linear relationship. Examining the magnitude of the p-value and F-value, the degree of influence of each factor on the total flavonoid yield can be obtained as $A>C>B$.

Table 1. Experiment design of response surface factor.

	A	B	C
Level	Solid-to-liquid ratio (g/mL)	Enzymatic hydrolysis temperature (°C)	Ethanol concentration (%)
-1	12	40	70
0	15	45	85
1	18	50	100

Table 2. Response surface design and response results.

Tests	A-Solid-to-liquid Ratio (g/mL)	B-Enzymatic hydrolysis temperature (°C)	C-Ethanol concentration (%)	Extraction yield (%)
1	-1	-1	0	2.4968
2	1	1	0	2.29127
3	-1	0	-1	2.72895
4	1	0	1	2.84924
5	0	0	0	3.62145
6	1	-1	0	2.51907
7	-1	0	1	2.85513
8	0	1	1	2.86716
9	0	-1	-1	2.8646
10	0	0	0	3.99514
11	0	0	0	3.69056
12	0	0	0	3.91323
13	1	0	-1	2.27592
14	-1	1	0	3.36115
15	0	0	0	3.83645
16	0	1	-1	2.61121
17	0	-1	1	3.22293

Table 3. Significance test for each regression coefficient and variance analysis.

Sources of variation	Sum of squares	Degree of freedom (DOF)	Mean square	F Value	p Value	Significance
Model	4.99	9	0.55	11.63	0.0019	significant
A	0.28	1	0.28	5.95	0.0448	
B	9.38E-05	1	9.38E-05	1.97E-03	0.9659	
C	0.22	1	0.22	4.53	0.0709	
AB	0.3	1	0.3	6.26	0.0409	
AC	0.05	1	0.05	1.05	0.3399	
BC	2.62E-03	1	2.62E-03	0.055	0.8213	
A ²	1.94	1	1.94	40.76	0.0004	
B ²	0.91	1	0.91	19.11	0.0033	
C ²	0.87	1	0.87	18.27	0.0037	
Residual	0.33	7	0.048			
Lack of Fit	0.24	3	0.079	3.33	0.1379	not significant
Pure Error	0.095	4	0.024			
Cor Total	5.32	16				
R ²	0.9373					
CV%	7.14					

Note: P<0.05 indicates a significant difference, and P<0.01 indicates a highly significant difference.

3.3.2. Response Surface Analysis

The steeper response surface means the greater effect of the factor on the total flavonoid yield and the more sensitive to the operating conditions. The contour lines can reflect the significance of the interaction between the factors. The oval shape indicates that the interaction between the two elements is significant, while the circle shows no significant change. As shown in Figure 2A, the response surface of the interaction term AB is steeper. The elliptical shape of the contour line shows that there is a significant interaction between the temperature of the enzymatic hydrolysis and the solid-liquid ratio that influences the rate of extraction of total flavonoids from *A. absinthium* L. Instead of increasing initially and then declining with an increase in enzymatic hydrolysis temperature, the extraction rate of total flavonoids increased progressively with the increase in solid-liquid ratio when the enzymatic hydrolysis temperature was fixed. The interaction terms AC (Figure 2B) and BC (Figure 2C) were relatively smooth, i.e., the interaction was weak, and the contours were approximately circular, indicating the interaction between solid-liquid ratio and ethanol concentration, enzymatic hydrolysis temperature and ethanol concentration was not significant. The effect on the extraction rate of total flavonoids was not significant. The effect on the extraction rate of total flavonoids was negligible. The optimal conditions for extraction of total flavonoids from absinthe were: solid-liquid ratio 1:14.6, ethanol concentration 87.45%, ultrasonic temperature 30°C, enzyme ratio 3:2, enzyme dosage 2%, enzymatic hydrolysis temperature 45.19°C, enzymatic hydrolysis time 105 min, enzymatic hydrolysis pH 3.5. In order to facilitate the experimental operation, the ideal extraction conditions were established: solid-liquid ratio 1:15, ethanol concentration 85%, ultrasonic temperature 30°C, enzyme ratio 3:2, enzyme dosage 2%, enzymatic hydrolysis temperature 45°C, enzymatic hydrolysis time 105 min, enzymatic hydrolysis pH 3.5. The results showed that the yield of total flavonoid content was 3.8%. The results showed that the total flavonoid content of A can be extracted by the surface reaction method under optimum technical conditions.

Figure 2. Response surface plots (3D) show different extraction parameters' effects on the total phenolic yield. (A) Solid-liquid ratio and enzymatic hydrolysis temperature; (B) solid-to-liquid ratio and ethanol concentration; (C) enzymatic hydrolysis temperature and volume fraction of ethanol.

3.4. LC-MS

LC-MS was used to qualitatively and quantitatively analyze the main components of total flavonoids in *A. absinthium*. The total ion flow chromatogram of total flavonoids showed that 41 major peaks were well separated (Figure 3). Based on the information of the excimer ion peaks given by the primary mass spectrometry and the structural fragmentation information of the secondary mass spectrometry, combined with the retention time of UPLC and the references, the possible structures of 41 chemical components were deduced, including 37 flavonoids, 2 phenolic compounds, 1 terpenoid and 1 amino acid and its derivatives (Figure 3B, Table 4).

Figure 3. (A) Ion flow chromatogram of total flavonoid extract from *A. absinthium* L.. (B) Structural formulae of compounds in the total flavonoid extract of *A. absinthium* L..

Table 4. Compounds are contained in total flavonoids and MS parameters.

Number	Compound name	Structured	Compound class	Active	Tumor Type	Mechanism	References
1	Galangin	C ₁₅ H ₁₀ O ₅	Flavonoids	Anticancer	Ovarian cancer, colon cancer, liver cancer, gastric cancer, breast cancer,	The mitochondrial pathway induces apoptosis, inhibits angiogenesis and cell cycle arrest	(Ha et al., 2013; Huang et al., 2015; Liang et al., 2021; Liu et al., 2018; Zhang et al., 2010)
2	Sinensetin	C ₂₀ H ₂₀ O ₇	Flavonoids	Anticancer	Gastric cancer, liver cancer, breast cancer	Regulation of AMPK / mTOR pathway	(Dong et al., 2011; Kim et al., 2020; Rezakhani et al., 2020)
3	Orientin	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Anticancer	Breast cancer, colon cancer, bladder cancer, cervical cancer	Inhibition of cell cycle arrest, inhibition of migration, and invasion	(Guo et al., 2014; Kim, S.J. et al., 2018; Thangaraj et al., 2019; Tian et al., 2019)
4	Hydroxygenkwanin	C ₁₆ H ₁₂ O ₆	Flavonoids	Anticancer	Liver cancer, breast cancer, lung cancer	Induces apoptosis and inhibits cell proliferation	(Ao et al., 2020; Chou et al., 2019; Leu et al., 2020)
5	Glycitein	C ₁₆ H ₁₂ O ₅	Flavonoids	Anticancer	Gastric cancer, glioma, breast cancer	NF-κB/AP-1-dependent and non-dependent pathways inhibit cell invasion and cell cycle arrest	(Lee et al., 2010; Zang et al., 2019; Zhang et al., 2015)
6	Acacetin	C ₁₆ H ₁₂ O ₅	Flavonoids	Anticancer	Breast cancer, liver cancer, prostate cancer, lung cancer	Inhibition of cell cycle arrest and cell migration	(Hsu et al., 2004a; Hsu et al., 2004b; Shen et al., 2010; Shim et al., 2007)
7	Tiliroside	C ₃₀ H ₂₆ O ₁₃	Flavonoids	Anticancer	Liver cancer, breast cancer	Inhibits cell migration and invasion	(Da'i et al., 2016; Han et al., 2021)
8	Narcissoside	C ₂₈ H ₃₂ O ₁₆	Flavonoids	Antidiabetic			(Liu et al., 2020)
9	Tectochrysin	C ₁₆ H ₁₂ O ₄	Flavonoids	Anticancer	Colon cancer, lung cancer, prostate cancer	The death receptor pathway induces apoptosis	(Oh et al., 2014; Park et al., 2015; Wang, Y. et al., 2019)
10	Daidzin	C ₂₁ H ₂₀ O ₉	Flavonoids	Anticancer	Cervical cancer	JAK2/STAT3 inhibits cell growth and induces apoptosis	(Yao et al., 2021)
11	Chrysin	C ₁₅ H ₁₀ O ₄	Flavonoids	Anticancer	Prostate cancer,	Downregulation of PI3K-AKT and	(Lima et al., 2020;

					gastric cancer, bladder cancer, colorectal cancer	ERK inhibits cell proliferation, cell	Ronnekleiv-Kelly et al., 2016; Ryu et al., 2017; Zhong et al., 2020)
12	Liquiritin	C ₂₁ H ₂₂ O ₉	Flavonoids	Antiinflammatory, anticancer	cervical cancer, gastric cancer, liver cancer	Upregulation of p53 and p21 induces apoptosis	(He et al., 2017; Ronnekleiv-Kelly et al., 2016; Wang et al., 2020; Xie et al., 2017; Zhou and Ho, 2014)
13	Quercetin	C ₁₅ H ₁₀ O ₇	Flavonoids	Anticancer	Gastric cancer, breast cancer, ovarian cancer, prostate cancer, liver cancer	Inhibition of cell cycle arrest, induction of apoptosis	(Choi et al., 2001; Ghafouri-Fard et al., 2021; Shafabakhsh and Asemi, 2019; Yoshida et al., 1990; Zhou et al., 2017)
14	Pinocembrin	C ₁₅ H ₁₂ O ₄	Flavonoids	Anticancer	Colon cancer, melanoma, ovarian cancer, lung cancer	Inhibition of cell autophagy, proliferation, and migration	(Gao et al., 2019; Gong, 2021; Kumar et al., 2007; Zheng et al., 2018)
15	Genkwanin	C ₁₆ H ₁₂ O ₅	Flavonoids	Anticancer	Lung cancer	Inhibition of cell cycle blockade	(Wei et al., 2021)
16	Isoliquiritigenin	C ₁₅ H ₁₂ O ₄	Flavonoids	Anticancer	Prostate cancer, breast cancer, lung cancer, cervical cancer	Inhibition of cell cycle blockade	(Hirchaud et al., 2013; li et al., 2004; Kanazawa et al., 2003; Maggiolini et al., 2002)
17	Chalconaringenin	C ₂₀ H ₁₄ O ₅	Terpenoids	Antioxidant			(Fernandes et al., 2021)
18	Homoorientin	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Anticancer	Colon cancer	Inhibition of cell proliferation and induction of apoptosis	(Vadde et al., 2015)
19	Vitexin	C ₂₁ H ₂₀ O ₁₀	Flavonoids	Anticancer	Lung cancer, ovarian cancer, gastric cancer	Downregulation of p-p38/p38 and p-ERK1/2/ERK1/2; Bcl-2/Bax ratios	(Liu et al., 2019; Zhao et al., 2020; Zhou et al., 2021)
20	Eriodictyol	C ₁₅ H ₁₂ O ₆	Flavonoids	Anticancer	Lung cancer, cervical cancer	Inhibition of mTOR/PI3K/AKT signaling pathway and cell cycle blockade	(Yu and Liu, 2020; Zhang, Y. et al., 2020)
21	Genistein	C ₁₅ H ₁₀ O ₅	Flavonoids	Anticancer	Ovarian cancer, breast cancer, cervical cancer	Inhibition of cell cycle arrest, induction of apoptosis	(Gossner et al., 2007; Kim et al., 2009; Pagliacci et al., 1994)

22	Tectorigenin	C ₁₆ H ₁₂ O ₆	Flavonoids	Anticancer	Ovarian cancer, breast cancer, liver cancer	Inactivation of AKT/IKK/IκB/NF-κB signaling pathway induces apoptosis	(Jiang et al., 2012; Yang et al., 2012; Zeng et al., 2018)
23	Rhoifolin	C ₂₇ H ₃₀ O ₁₄	Flavonoids	Anticancer	Breast cancer	Suppression of EMT	(Xiong et al., 2021)
24	Apigenin	C ₁₅ H ₁₀ O ₅	Flavonoids	Anticancer	Pancreatic cancer, lung cancer, cervical cancer	Induction of cell cycle arrest and apoptosis	(Liu et al., 2005; Oh et al., 2008; Ujiki et al., 2006)
25	Schaftoside	C ₂₆ H ₂₈ O ₁₄	Flavonoids	Anticancer	Melanoma	Increased cellular autophagy	(Kim, P.S. et al., 2018)
26	Diosmin	C ₂₈ H ₃₂ O ₁₅	Flavonoids	Anticancer	Breast cancer, colorectal cancer	Inhibition of cell cycle arrest, induction of apoptosis	(Helmy et al., 2020; Lewinska et al., 2017)
27	Kaempferol	C ₁₅ H ₁₀ O ₆	Flavonoids	Anticancer	Ovarian cancer, colon cancer, breast cancer	Downregulation of PI3K/AKT and hTERT induces apoptosis and senescence	(Luo et al., 2011; Wang, X. et al., 2019; Yoshida et al., 2008)
28	Isoquercitrin	C ₂₁ H ₂₀ O ₁₂	Flavonoids	Anticancer	Colon cancer, liver cancer	Downregulation of the PI3K-AKT/mTOR signaling pathway inhibits cell proliferation	(Amado et al., 2014; Huang et al., 2014)
29	Luteolin	C ₁₅ H ₁₀ O ₆	Flavonoids	Anticancer	Cervical cancer, breast cancer, colorectal cancer	Cell cycle arrest, inhibition of cell growth, and invasion	(Cook, 2018; Horinaka et al., 2005; Pandurangan and Esa, 2014)
30	Hyperoside	C ₂₁ H ₂₀ O ₁₂	Flavonoids	Anticancer	breast cancer, Lung cancer	AKT /mTOR-p70S6K signaling pathway induces autophagy	(Fu et al., 2016; Qiu et al., 2019)
31	Isorhamnetin	C ₁₆ H ₁₂ O ₇	Flavonoids	Anticancer	Lung cancer, colorectal cancer	Inhibition of PI3K AKT/mTOR signaling	(Li et al., 2015; Saud et al., 2013)
						pathway to suppress cell proliferation	
32	Diosmetin	C ₁₆ H ₁₂ O ₆	Flavonoids	Anticancer	Liver cancer	Inhibits cell cycle arrest and induces apoptosis	(Ma and Zhang, 2020)
33	Protocatechualdehyde	C ₇ H ₆ O ₃	Phenols	Anticancer	Breast cancer	Cell cycle arrest	(Choi et al., 2014)
34	Kaempferol-3-O-rutinoside	C ₂₇ H ₃₀ O ₁₅	Flavonoids	Antiinflammatory	Lung cancer	Inhibition of cancer cell growth through calcium signaling pathway calcium	(Li et al., 2021)

						signaling pathway	
35	protocatechuic acid	C ₇ H ₆ O ₄	Phenols	Anticancer	Lung cancer	Induction of apoptosis	(Yee Kuen et al., 2020)
36	Rutin	C ₂₇ H ₃₀ O ₁₆	Flavonoids	Anticancer	Colon cancer, lung cancer	Induction of apoptosis	(Nafees et al., 2018; Paudel et al., 2021)
37	Ononin	C ₂₂ H ₂₂ O ₉	Flavonoids	Anticancer	Breast cancer	Promote cells apoptosis, decrease cells invasion and migration	(ZUO, 2020)
38	Cynaroside	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Anticancer	Gastric cancer.	Cell cycle arrest	(Ji et al., 2021)
39	Astragalin	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Anticancer	Lung cancer, gastric cancer	Inhibits cell proliferation, cell migration and invasion	(Chen et al., 2017; Wang, Z. et al., 2021)
40	2-Chloro-L-phenylalanine	C ₉ H ₁₀ ClNO ₂	Amino acids and their derivatives	Antioxidant			(Wang, C. et al., 2021)
41	Naringenin	C ₁₅ H ₁₂ O ₅	Flavonoids	Anti-inflammatory, Anticancer	Lung cancer, breast Cancer	Inhibition of cell proliferation, cell cycle arrest	(Bodet et al., 2008; Lim et al., 2017; Shi et al., 2021; Zhang et al., 2016)

3.5. Screening mutual targets of drugs and diseases

The core active ingredient targets of total flavonoids were matched with cervical cancer targets, from which the two main active ingredient compounds (Cynaroside and Astragalin) were selected to have 6 and 5 compound targets, respectively (Figure 4A, 4C). The target interaction PPI network graph (Cynaroside) was obtained by STRING online analysis platform with 46 nodes and 331 edges, and the average node degree value was 14.4. The PPI network showed (Figure 4B) that EGFR, ESR, HSP90AA1, EP300, STAT3, EGF and CCND1 were the main targets for cervical cancer. The PPI network graph (Figure 4D, Astragalin) had a total of 45 nodes 279 edges with an average node degree value of 12.4. The PPI network showed that SRC, EGFR, ESR1, STAT3, EGF, CCND1, CDH1 and IGF1R were the main targets of cervical cancer.

Figure 4. (A) Venn diagram of the total flavonoid component Cynaroside and cervical cancer target genes. (B) Venn diagram of the total flavonoid component Astragalin and cervical cancer target genes. (B, D) Shared Target Interactive PPI Network.

3.6. Enrichment analysis of GO and KEGG pathway

GO functional enrichment analysis of key targets, GO terms were screened according to P (P<0.05, Q<0.05) values. There were 590, 40 and 67 GO terms associated with biological processes, cellular components and molecular functions (Cynaroside), respectively. As shown in Figure 5A, the first 20 biological processes were associated with ERBB2 signaling pathway, epidermal growth factor, Cellular response to estrogen stimulus and Mammary gland epithelial cell proliferation, among others. For cellular components, the targets are enriched in Chromatin, Nuclear chromatin. Molecular functional analysis revealed that including Type iii transforming growth factor beta receptor binding, Type i transforming growth factor beta receptor binding, Nitric-oxide synthase regulator activity, Transcription regulator complex. Another active component had 418, 45 and 50 GO terms related to biological processes, cellular components and molecular functions, respectively (Astragalin). As shown in Figure 5B, the first 20 biological processes and ERBB2 signaling pathway, Entry of bacterium into host cell, Regulation of epithelial cell proliferation involved in prostate gland development, Epithelial cell differentiation involved in prostate gland development, Positive regulation of intracellular steroid hormone receptor signaling pathway related. For cellular components, the targets are enriched in Flotillin complex, Fascia adherens, Beta-catenin destruction complex, beta-catenin-TCF complex, Wnt signalosome. Molecular functional analyses revealed that including Heat shock protein binding, Beta-catenin binding, Ephrin receptor binding, Hsp90 protein binding, Tau protein binding. KEGG signaling pathway enrichment analysis contained 93 pathways (Cynaroside)

and 83 pathways (Astragalin), respectively. Screening enrichment of the top 20 pathways, Bladder cancer, Pancreatic cancer, Endocrine resistance, Glioma, Chronic myeloid leukemia, Non-small cell lung cancer, Prostate cancer, Colorectal cancer, and Melanoma (Figure 5 C); Bladder cancer, Endometrial cancer, Prostate cancer, Glioma, Thyroid cancer, and ErbB signaling pathway (Figure 5 D).

Figure 5. (A, B) The GO enrichment analysis of core nodes. Including cellular components, molecular functions, and biological processes. (C) The top 20 pathways for KEGG enrichment analysis of the targets of Cynaroside. (D) The top 20 pathways for KEGG enrichment analysis of the targets of Astragalin.

3.7. Active ingredients-shared key targets-signal pathway network diagram construction

A component-target-channel network diagram was formed by Cytoscape 3.7.2 to comprehensively clarify the mechanism of active ingredients in cervical cancer (Figure 6). In the figure, the yellow diamond represents the component in the drug, the green oval represents the target of the component, and the pink V represents the pathway enriched in the target. It shows that each active compound can act on multiple targets, that each target can also respond to multiple active ingredients, and that each target can have an effect on multiple pathways.

Figure 6. (A) Total Flavonoid Component Cynaroside-Target-Disease Network Diagram. (B) Total Flavonoid Component Astragalin-Target-Disease Network Diagram.

3.8. Key active ingredients and core target molecular docking results

Molecular docking results of key active ingredients and core targets. Molecular docking was performed for the core targets with the highest degree of PPI network and their corresponding active ingredients. The docked protein data were downloaded from the PDB database for EGFR (PDB ID 5wb7), SRC (PDB ID 4hxj). The 3D structures were imported into AutoDock software and docked with the active compounds. The core targets in the PPI network and their corresponding small molecule drug ligands were docked using AutoDock. The lower the binding energy, the better the ligand will bind to the protein. The molecules with the lowest binding energy were visualized using Pymol software (Figure 7). The main form of interaction is hydrogen bonding. This result suggests that their combination may play an important role in the treatment of cervical cancer with total flavonoids.

Figure 7. Detailed target-compound interactions. (A) EGFR-Cynaroside. (B) SRC-Astragalin.

3.9. Total flavonoids and active ingredients can inhibit cell proliferation and induce apoptosis

To evaluate the antitumor effect of total flavonoids from *A. absinthium* L., HeLa cells were treated with different concentrations of total flavonoids and their active ingredients for 24 h. The morphological changes of the cells were observed by inverted microscopy. HeLa cells showed a small and round morphology, with a significant decrease of cell number. The active ingredients Cynaroside and Astragalin also showed similar results (Figure 8A). In addition, the inhibitory effect of total flavonoids on the proliferation of HeLa, SiHa, and H8 cells was examined, and the activity of Cynaroside and Astragalin were also examined against HeLa, respectively. As shown in Figure 8B, total flavonoids inhibited the growth of Human cervical cancer cells HeLa and SiHa in a dose-dependent manner and the 24 h semi-inhibitory concentration (IC_{50}) values were 396.0 ± 54.2 $\mu\text{g/mL}$ and 449.0 ± 54.8 $\mu\text{g/mL}$, respectively. Interestingly, total flavonoids had a weak effect on the growth of human immortalized human cervical normal epithelial cells H8. Meanwhile, Cynaroside and Astragalin also inhibited the activity of HeLa cells in a dose-dependent manner with IC_{50} of 263.4 ± 3.7 $\mu\text{g/mL}$ and 282.3 ± 5.0 $\mu\text{g/mL}$ (Figure 8B). The effect of total flavonoids on HeLa cell apoptosis was further detected. After Annexin V-FITC/PI staining, the assay was performed by flow cytometry. The total flavonoids significantly induced HeLa cell apoptosis in a dose-dependent manner while caused low level of necrosis. The results suggested that total flavonoids of *A. absinthium* L. had good anti-cervical cancer activity. Subsequently, we further tested whether the inhibitory effect of Cynaroside and Astragalin on the growth of HeLa cells was also associated with the induction of apoptosis. The results showed that low and high concentrations inhibited the proliferation of HeLa cells. Cynaroside and Astragalin significantly increased the percentage of apoptotic HeLa cells in a concentration-dependent manner compared to the control group (Figure 8C). Apoptosis of HeLa cells was further observed by Hoechst 33342 staining after 24 h with Cynaroside and Astragalin. The results revealed that most untreated cells showed round, intact and uniformly stained nuclei. In contrast, the drug-treated group showed a bright blue color with increasing concentration, as the nucleus structure was altered and the chromatin of the nucleus underwent concentration-dependent fixation and breakage (Figure 8D).

Figure 8. Total flavonoids and active ingredients can inhibit cell proliferation and induce apoptosis. Cells were treated with different concentrations of total flavonoids, Cynaroside and Astragalin for 24 hours, respectively. (A) The viability of HeLa cells was detected by

MTT assay. (B) The morphology of HeLa cells was observed by inverted microscopy. (C) After staining with Annexin V and PI, HeLa cells were analyzed by flow cytometry. (D) The effect of Hoechst 33342 staining on cell chromatin was observed under an inverted fluorescence microscope.

3.10. Induction of ROS accumulation by active ingredients of total flavonoids

Reactive oxygen species (ROS), a signalling molecule in cell regulation, can induce apoptosis. ROS dose plays an important role not only in energy metabolism but also in cellular physiological processes. Therefore, DCF-DA, a specific fluorescent dye for ROS detection, was used as a fluorescent probe to assess the changes in intracellular ROS levels after Cynaroside and Astragalin treatment of HeLa cells. DCF-DA rapidly crosses the cell membrane and is converted to DCFH by intracellular lipases. After staining, Cynaroside and Astragalin-treated cells showed green fluorescence in a dose-dependent manner (Figure 9A). Flow cytometry results showed a dramatic increase in cell fluorescence after 24 h of treatment compared to the Control group (Figure 9B). Together, these results suggest that ROS play an essential role in Cynaroside and Astragalin-induced apoptosis in HeLa cells.

Discussion

To date, significant efforts have been made to develop antitumor drugs. Natural products have been paid attention due to their considerable bioactivities and safety. The extraction methods of natural components mainly include heating, reflux, immersion, organic solvent extraction, microwave-assisted extraction, enzymatic hydrolysis method, etc. In order to improve the extraction rate of total flavonoids in *A. absinthium* L., the combination of the ultrasonic extraction method with the enzymatic hydrolysis method was performed in this study, which has been widely used in the extraction of active ingredients from plants due to the advantages including convenient, efficient, and environment friendly. Cellulase can decompose plant tissues under mild conditions and accelerate the release of active ingredients, and ultrasonic can accelerate the dissolution of intracellular substances. Therefore, the combination of the two methods greatly improves the extraction efficiency of plant active ingredients. To a certain extent, it also significantly reduces the extraction cost and is greener and healthier. In addition, we conducted the design test and data analysis of ultrasound-assisted enzymatic extraction of total flavonoids by a single-factor test based on response surface optimization. It overcomes the drawback that the orthogonal test design can only deal with discrete level values but cannot find the best combination of factors and the optimal response face value over the whole region. At the same time, it makes up for the lack of interaction effects and other mixed effects. It can also reduce the number of experiments and time consumption. Similarly, Saliha et al. used a response surface optimization ultrasound-assisted extraction method to extract total phenols from *A. absinthium* L., determined the optimal conditions for maximum extraction of active ingredients (Sahin et al., 2013). Dai et al. (Dai et al., 2020) extracted flavonoids from *Saussurea involucre* by ultrasonication and Xu et al. (Yuxia and Huabin, 2013) extracted total flavonoids from sea red fruits by enzymatic hydrolysis. Yun et al. (Yun et al., 2022) found that the extraction of flavonoids baicalein and wogonin from *Scutellaria baicalensis* roots by ultrasound-assisted enzymatic hydrolysis almost doubled the conversion rate and significantly increased the productivity compared to the enzymatic hydrolysis method. Thus, we effectively extracted the total flavonoids from *A. absinthium* L. by ultrasound-assisted enzymatic digestion in a time-saving manner.

Furthermore, total flavonoids of *A. absinthium* L. was prepared using the optimal extraction conditions and its antitumor activity was evaluated. The results showed that total flavonoids of *A. absinthium* L. had good anti-cervical cancer activity. Sultan et al. found that extracts of *A. absinthium* L. could promote apoptosis in colon cancer cells through activation of the mitochondrial pathway (Nazeri et al., 2020). It was also reported that extracts of *A. absinthium* L. were able to mediate the growth and apoptosis of hepatocellular carcinoma cells through endoplasmic reticulum stress and mitochondria-dependent pathways (Wei et al., 2019). In addition, extracts of *A. absinthium* L. have anticancer and antioxidant effects on various tumors by increasing the intracellular amount of free radicals on cancer cells, which leads to DNA damage and, consequently, apoptosis (Koyuncu, 2018). As such, the anticancer activity of the active components of total flavonoids was also evaluated. In previous studies, it was confirmed that apoptosis is a physiological process of cells. Cancer occurs when this balance is disrupted, either by an increase in cell proliferation or a decrease in cell death (Gerl and Vaux, 2005). Moreover, apoptosis induction is considered the primary pathway by which biological agents inhibit the proliferation of tumor cells. Thus, our study tested whether the inhibition of the proliferation of cervical cancer HeLa cells by Cynaroside and Astragalin was achieved through apoptosis. As we expected, Cynaroside and Astragalin significantly increased the rate of apoptosis in HeLa cells in a dose-dependent manner. The results suggest that Cynaroside and Astragalin inhibit the proliferation of cervical cancer cells by inducing apoptosis. In addition, we found that Cynaroside and Astragalin apoptosis induction may be associated with intracellular ROS levels in HeLa cells, and the excessive production of ROS leads directly to apoptosis. Therefore, ROS accumulation is considered one of the main targets of cancer therapy. Yuan et al. (Yuan et al., 2012) found that Isoliquiritigenin induced apoptosis by increasing intracellular ROS levels in HeLa cells. Lim et al. (Lim et al., 2021) found that indole-3-carbinol mediated apoptosis in lung cancer H1299 cells by increasing

ROS production. These are consistent with our results, which showed that Cynaroside and Astragalin inhibit the development and progression of cervical cancer HeLa cells by increasing the accumulation of ROS.

Conclusion

In summary, *Artemisia L.* is traditionally used in food, spices, condiments and beverages. Its leaves are the most commonly used edible part, and the above-ground part known as "herb" is also widely used. Thus, we have extracted and optimized the extract of *A. absinthium L.* The optimal extraction conditions of the process were: enzyme ratio 3:2, enzyme dosage 2%, enzymatic hydrolysis temperature 45°C, enzymatic hydrolysis time 105 min, pH 3.5, solid-liquid ratio 1:15, ethanol volume percentage 85%, and sonication temperature 30°C. The actual extraction rate of total flavonoids under these conditions could reach 3.80±0.13%, which was similar with the total flavonoids extraction predicted by the model (3.84%). The optimized extraction process was simple, stable, and feasible to provide a theoretical basis for the further development and comprehensive application of *A. absinthium L.* Moreover, results from network pharmacology and molecular docking indicate that EGFR and SRC are the key targets of the two core components of total flavonoids against cervical cancer, the total flavonoids of *A. absinthium L.* might be used to treat cervical cancer. In addition, both Cynaroside and Astragalin inhibited the proliferation of HeLa cells and induced apoptosis by increasing the production of ROS.

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Declarations

Author Contributions: M.H. and K.Y. performed experiments, analyzed data and prepared figures. Investigation, M.H., K.Y. and H.Z. L.X. and J.L. designed the experiments and wrote the manuscript. All authors reviewed the manuscript.

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Data availability: The datasets presented in the current study are available from the corresponding author on reasonable request.

Ethics approval: Not applicable.

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Figures

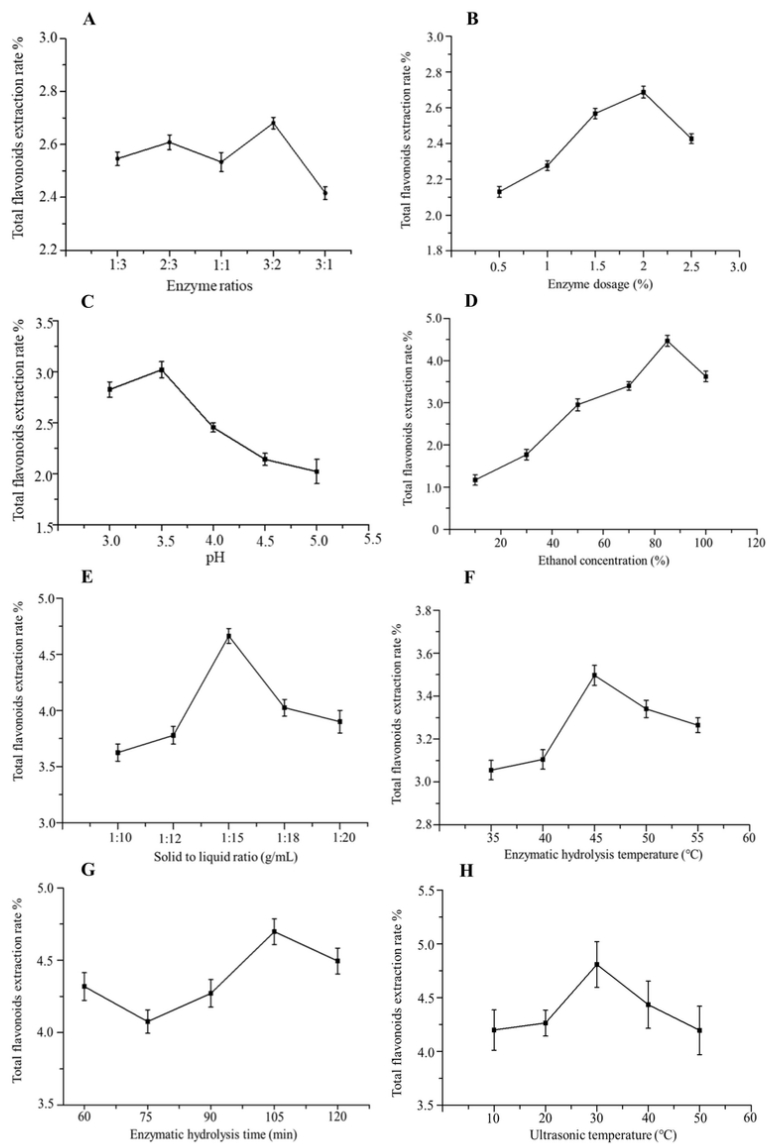


Figure 1

Effect of single factors on the extraction of total flavonoids from *A. absinthium* L.. (A) Enzyme ratio; (B) enzyme dosage; (C) pH; (D) ethanol concentration; (E) solid-liquid ratio; (F) enzymatic hydrolysis temperature; (G) enzymatic hydrolysis time; (H) ultrasonic temperature.

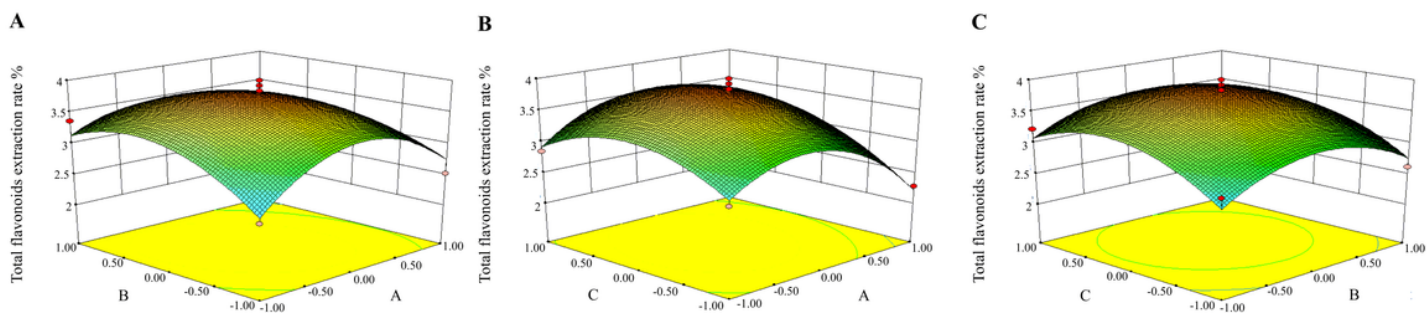


Figure 2

Response surface plots (3D) show different extraction parameters' effects on the total phenolic yield. (A) Solid-liquid ratio and enzymatic hydrolysis temperature; (B) solid-to-liquid ratio and ethanol concentration; (C) enzymatic hydrolysis temperature and volume fraction of ethanol.

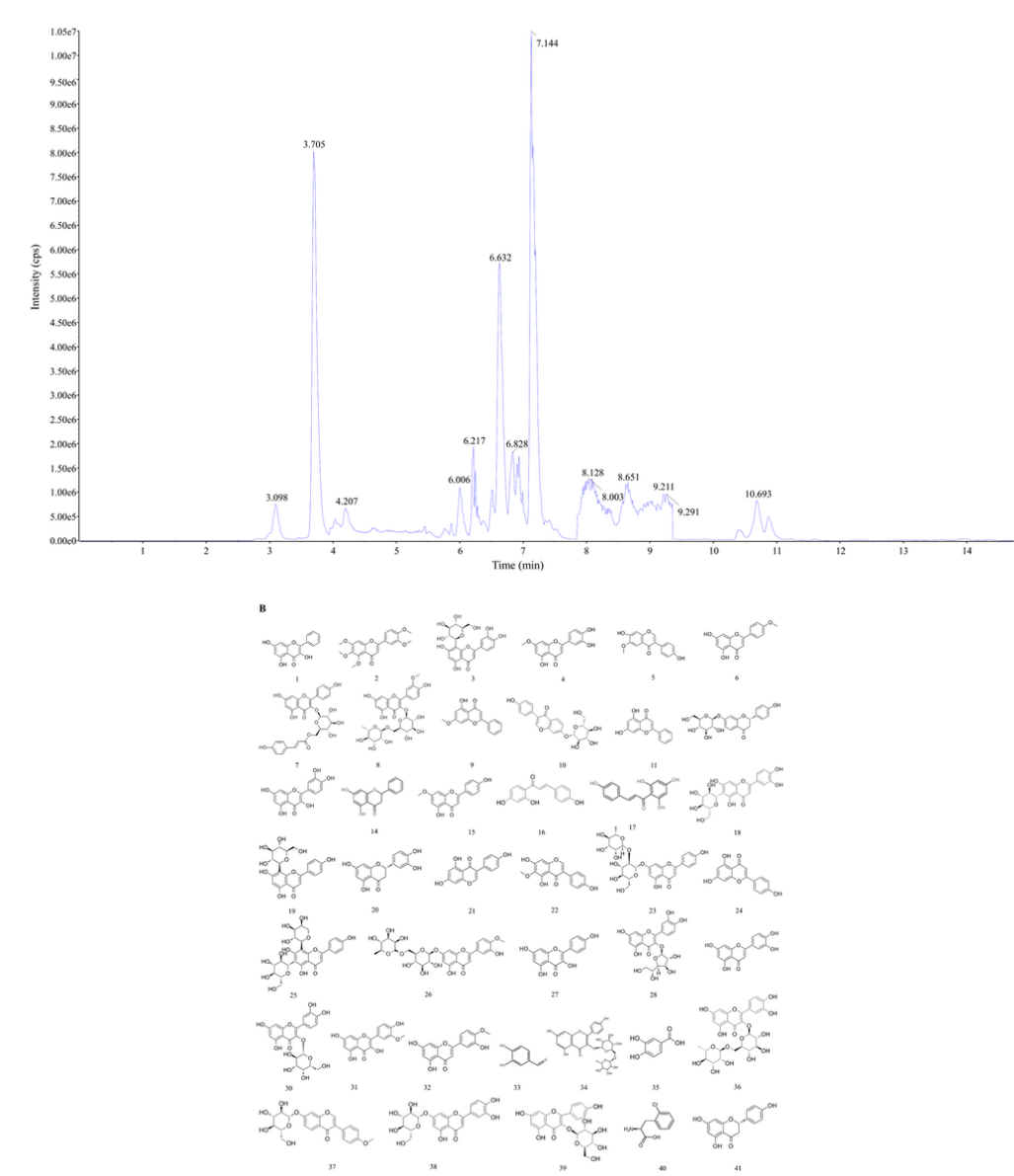


Figure 3

(A) Ion flow chromatogram of total flavonoid extract from *A. absinthium* L.. (B) Structural formulae of compounds in the total flavonoid extract of *A. absinthium* L..

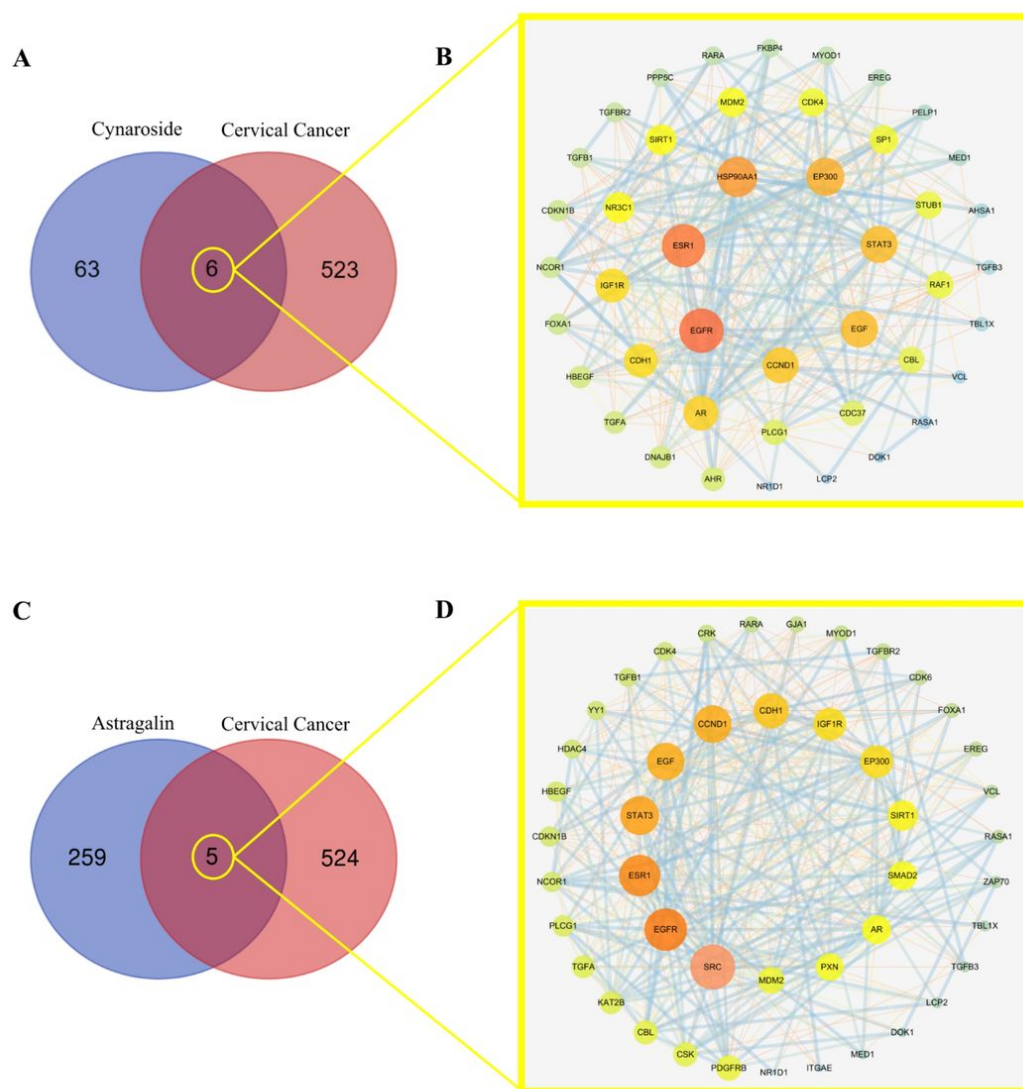


Figure 4

(A) Venn diagram of the total flavonoid component Cynaroside and cervical cancer target genes. (B) Venn diagram of the total flavonoid component Astragaloside and cervical cancer target genes. (B, D) Shared Target Interactive PPI Network.

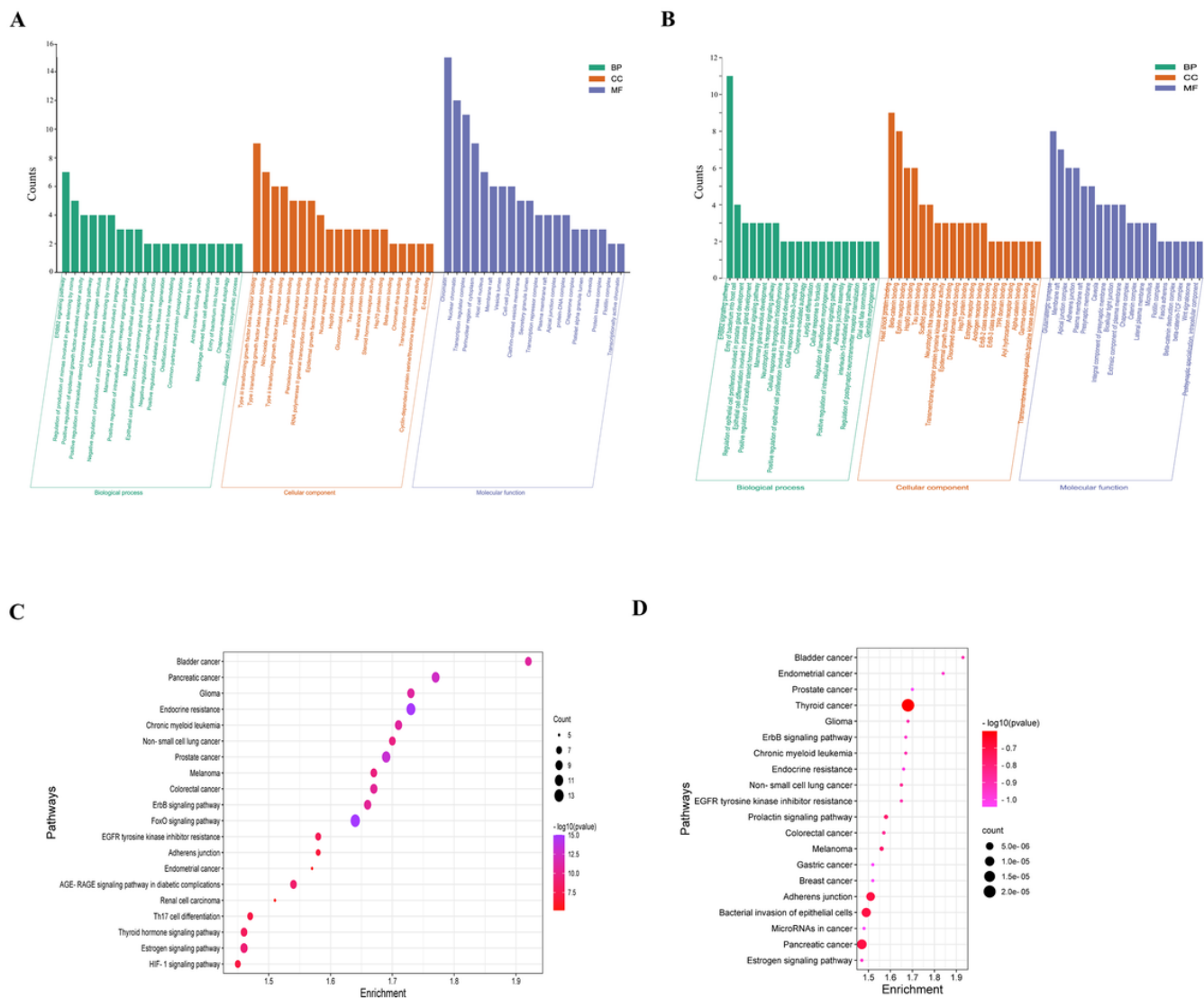
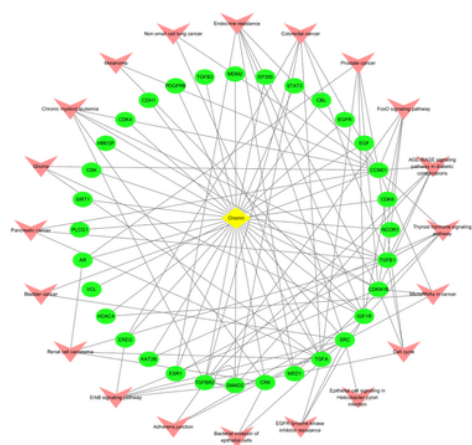
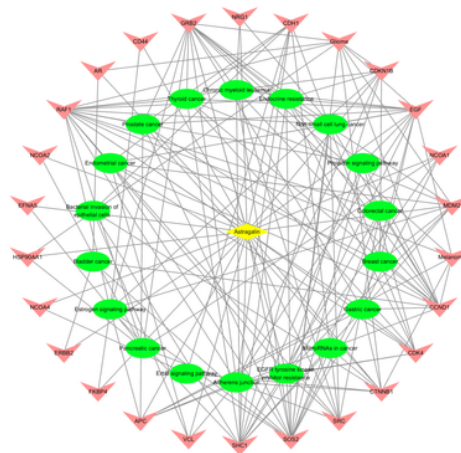


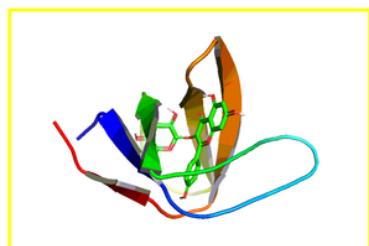
Figure 5

(A, B) The GO enrichment analysis of core nodes. Including cellular components, molecular functions, and biological processes. (C) The top 20 pathways for KEGG enrichment analysis of the targets of Cynaroside. (D) The top 20 pathways for KEGG enrichment analysis of the targets of Astragaline.

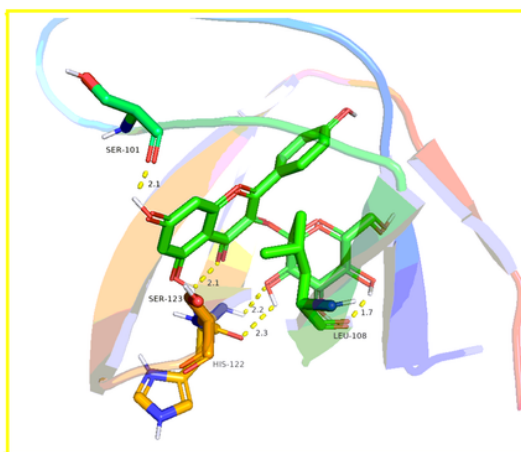
A**B****Figure 6**

(A) Total Flavonoid Component Cynaroside-Target-Disease Network Diagram. (B) Total Flavonoid Component Astragalin-Target-Disease Network Diagram.

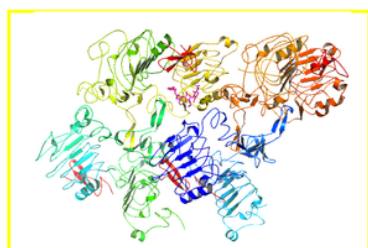
A



EGFR-Cynaroside



B



SRC-Astragalin

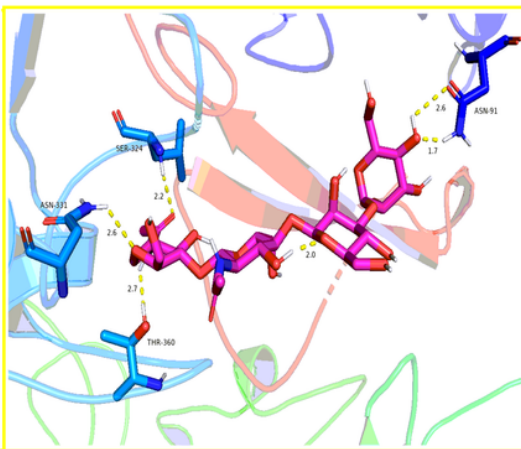


Figure 7

Detailed target-compound interactions. (A) EGFR-Cynaroside. (B) SRC-Astragalin.

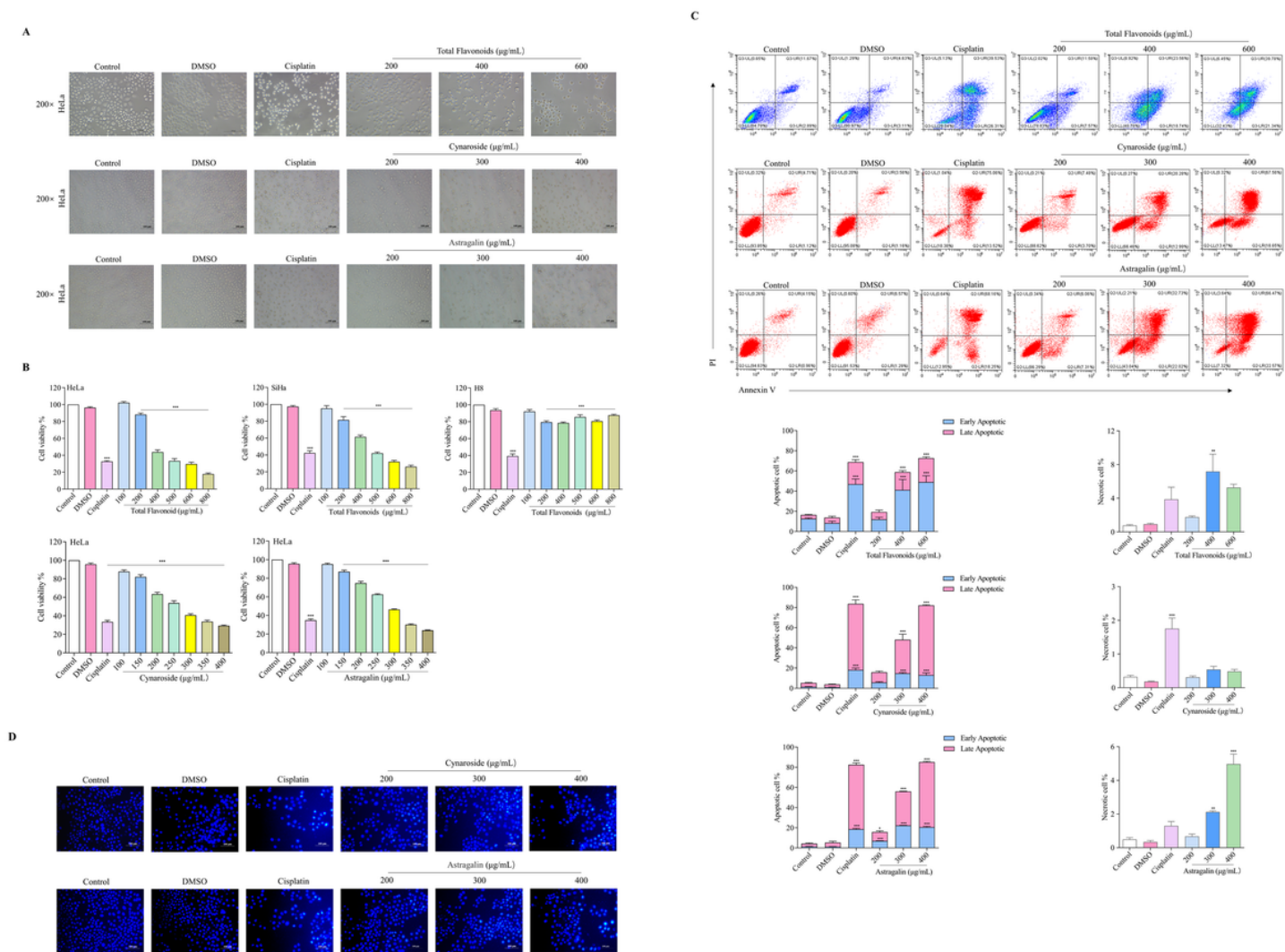
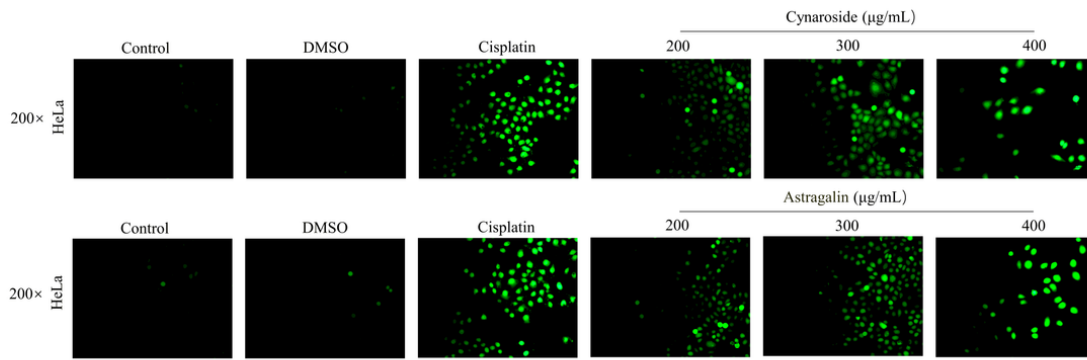


Figure 8

Total flavonoids and active ingredients can inhibit cell proliferation and induce apoptosis. Cells were treated with different concentrations of total flavonoids, Cynaroside and Astragaloside for 24 hours, respectively. (A) The viability of HeLa cells was detected by MTT assay. (B) The morphology of HeLa cells was observed by inverted microscopy. (C) After staining with Annexin V and PI, HeLa cells were analyzed by flow cytometry. (D) The effect of Hoechst 33342 staining on cell chromatin was observed under an inverted fluorescence microscope.

A



B

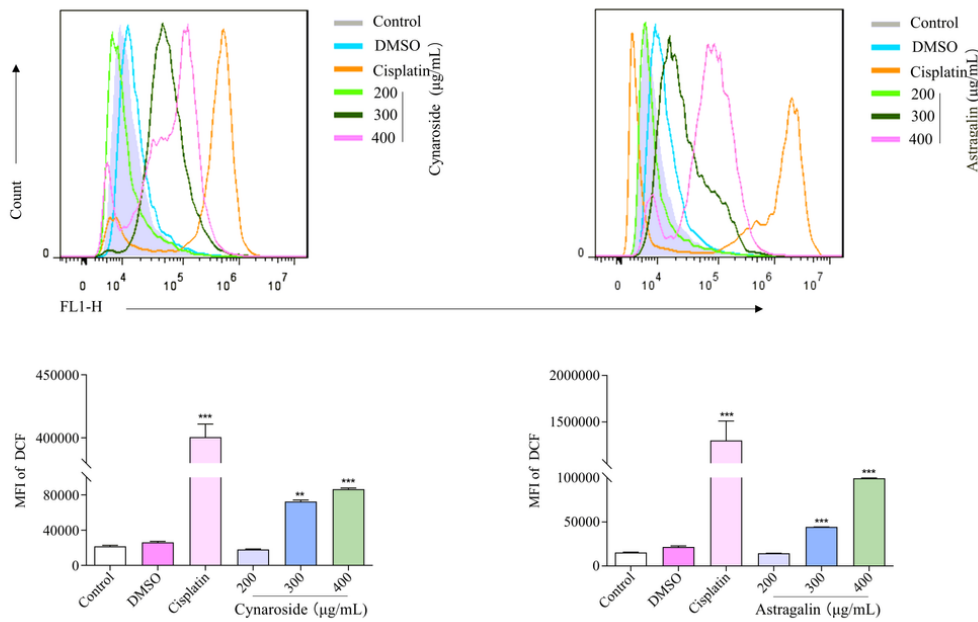


Figure 9

Induction of ROS accumulation by active ingredients of total flavonoids. Cells were treated with different concentrations of total flavonoids, Cynaroside and Astragalin for 24 hours, respectively. (A) fluorescence intensity of ROS was observed by inverted fluorescence microscopy; (B) cellular ROS levels were detected by flow cytometry.