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Phytochemical Analysis and Cytotoxic Effects of Phyllanthus polygonoides Leaf Extracts on Hepatocellular Carcinoma: A Combined In-Vitro and In-Silico Approach

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Abstract

Hepatocellular carcinoma (HCC) is the most prevalent kind of liver cancer and a leading cause of cancer-related deaths worldwide. Treatment resistance and adverse effects often render HCC worse. With an emphasis on phytochemical analysis, antioxidant activity, and cytotoxic effects, this study investigates the possible anticancer characteristics of leaf extracts from *Phyllanthus polygonoides*. The methanolic extract showed considerable antioxidant activity by DPPH scavenging and notable cytotoxic effects in MTT tests. It also included flavonoids, phenols, tannins, alkaloids, quinones, and cardiac glycosides. The extract's capacity to fight cancer was validated using a wound-healing experiment. 25 phytocompounds were found using gas chromatography-mass spectrometry (GC-MS). Eight of the bioactive compounds were chosen for molecular docking based on toxicity and druggability criteria. Tests for hemolytic potential using human erythrocytes revealed significant anti-hemolytic action. Using Fourier-Transform Infrared Spectroscopy (FTIR), functional groups such as carboxylic acids, alcohols, amides, and phenols were identified. Strong binding affinities of important drugs were shown by molecular docking experiments, which were corroborated by positive ADMET profiles. By identifying active chemicals and pertinent signaling pathways, the study demonstrates the multi-target effects of P. polygonoides on HCC through the use of network pharmacology, molecular docking, and molecular dynamics simulations. These results point to the potential benefits of *P. polygonoides* phytochemicals as potential treatments for liver cancer and call for more research.

Keywords: Hepatocellular Carcinoma (HCC), Phyllanthus polygonoides, Anti-Cancer Activity, Phytochemical Analysis, Molecular Docking

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Introduction

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Liver cancer is defined as the formation of malignant tumors inside the liver's tissues. Liver cancer is the most prevalent type of cancer which is the most frequent cause of deaths worldwide, accounting for 75-85% of all occurrences.[1]. Hepatitis B and C virus infections are the leading causes of HCC, accounting for more than 75% of all cases globally.[2]. Excessive and long-term alcohol intake, liver cirrhosis, and metabolic syndromes, including nonalcoholic fatty liver disease, can all considerably cause the risk of HCC [3, 4].

Genetic disorders such as hemochromatosis and alpha-1 antitrypsin deficiency, which are associated with liver damage, also impact the development of HCC [4]. The global incidence of HCC has been rapidly increasing, in tandem with the growing prevalence of its established risk factors. The World Health Organization (WHO) currently recognizes HCC as the fourth leading cause of cancer-related deaths and the seventh most prevalent form of cancer, resulting in over 750,000 fatalities annually [5]. The absence of symptoms in the initial stages are the primary contributors to its unfavorable outcomes. This results in a delayed diagnosis and limited therapeutic options.

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Liver resection or liver transplantation can potentially provide a cure for early-detected HCC [6]. However, less than 30% of patients require surgery due to tumor size or impaired liver function at the time of diagnosis [3]. Locoregional therapy such as radiofrequency ablation, microwave ablation, or ethanol injection may effectively manage localized illness [1, 2, 4]. However, these therapies do not provide any survival advantage compared to the best supportive care for tumors that are present in many locations. HCC can be treated with medicines that target specific molecules, like sorafenib; mTOR inhibitors, like everolimus; and multi-kinase inhibitors, like regorafenib [7]. Nevertheless, the clinical advantages of these treatments are limited, as they are only successful in a small number of patients [8]. Furthermore, they are poorly tolerated because of their hazardous side effects, which significantly diminish the quality of life [9]. The development of resistance to these drugs not only reduces treatment time but also affects the disease's outcomes [10].

Medicinal plants have always been recognized as valuable sources of anticancer treatments. They produce a wide range of specialized metabolites, which have different medicinal properties including anti-cancer, anti-inflammatory, and anti-oxidant [11-13]. *Catharanthus roseus* and *Taxus brevifolia* are plants that produce specific compounds used in chemotherapy. *C. roseus* produces vincristine, and *Taxus brevifolia* produces paclitaxel. Both of these compounds are well-known and widely used in cancer treatment [14]. Additional botanical derivatives, have demonstrated preclinical effectiveness

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against HCC cell lines and in vivo models including Curcumin from *Curcuma longa*, resveratrol from grapes, Quercetin from different fruits and vegetables, Berberine from the various plants [15], and *Tanshinone IIA* from *Salvia miltiorrhiza* are all examples of bioactive compounds [16]. They modulate molecular targets abnormally activated during liver cancer development, such as inflammatory pathways (NF-B, COX-2, Nrf2) [17], cell survival and proliferation pathways (EGFR, PI3K/Akt, Wnt/-catenin), invasion and metastasis regulators (MMPs), and reactive oxygen species (ROS) [18, 19].

Phyllanthus polygonoides bioactive compounds can be repurposed to enhance the effectiveness of current treatments for HCC [9]. Nevertheless, the process of applying these findings in a clinical setting necessitates the need to address and overcome any current gaps through meticulously planned and executed investigations [11]. Plants exert multi-faceted effects by synergistically combining several natural components to counteract interconnected networks of pathogens [8]. Researchers are recognizing thoroughly researched species as additional and alternative treatments to traditional care regimens. It is evident that natural sources have the potential to be effective in enhancing HCC management [6, 7, 10]. The present study evaluated the anticancer effectiveness of *Phyllanthus polygonoides* against HCC, utilizing both experimental and computational approaches. *P. polygonoides* has long been used in indigenous medical practices to treat liver-related diseases.

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Network pharmacology is emerging field in drug discovery and development by combining systematic medicine with information science. . It has become a widely used technique in modern drug discovery. It is an integrative in silico approach that constructs a "protein–compound/disease–gene" network to uncover the mechanisms behind the combined therapeutic effects of traditional medicines. This development has transitioned the paradigm from a "one-target, one-drug" approach to a "network-target, multi-component therapy" model. Nevertheless, ongoing scientific research is gradually uncovering more comprehensive insights into the potential anti-hepatocellular properties of this substance.

The objective of this study was to assess the cytotoxic effects of *P. polygonoides* extract on HepG2 cell line. Computational techniques were used to identify the compounds and predict their molecular targets. Network pharmacology and molecular docking were utilized to obtain mechanistic insights into possible anticancer pathways. Further experiments should examine the active components and

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their molecular interactions, as the findings could support further exploration of their potential as a liver cancer treatment.

Method and material

2.1 Preparation of extracts

The leaves from the collected plant were washed with water then 70% ethanol to clean them. After air drying for 2-3 weeks, the dried leaves were ground into a fine powder. The ratio of powdered plant in solvent was 1:3 (1 gram of powder in 3 ml solvent) and dissolved over 3-4 days with stirring. The solvent containing dissolved compounds was filtered. The residue was re-added to the solvent and this dissolve-filter process was repeated thrice. The collected filtrate was evaporated to a residue which was scraped off into 50ml tubes.

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Stock and dose preparation

To prepare stocks, 40mg plant extract was dissolved in 1ml DMSO. This was serially diluted 2-fold, making 7 stocks (40, 20, 10, 5, 2.5, 1.25, 0.625 mg/ml). Working doses were made by taking 0.5µl from each stock and dissolving in media, yielding concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12 µg/ml. Stocks and doses were stored at 4°C until use. Doses for hemolytic assay were made in PBS.

Cell culture

HepG2 cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were grown at 37°C with 5% CO2 in a humidified incubator. Cells were subcultured at preconfluent densities using trypsin-EDTA.

MTT assay

The cytotoxicity of *Phyllanthus polygonoides* extract was tested on hepG2 cell lines. Cells were seeded in 96-well plates and incubated for 24 hours to form a monolayer at 80% confluence. Seven plant doses (3.12, 6.25, 12.5, 25, 50, 100, 200 µg/ml) were added to wells with 5% FBS DMEM. After 24 hours incubation, the MTT solution was added and incubated again for 4 hours, then DMSO was added to dissolve formazan crystals. Absorbance readings were taken at 570 nm and 620 nm wavelengths [6].

Cell viability was calculated as follows:

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Percent cell viability: (Test 570nm-620nm / control 570nm -620nm) * 100

Hemolytic assay

Hemolytic assay was performed to examine the impact of a plant extract on human blood. The hemolytic activity of *Phyllanthus polygonoides* was studied using the method described by Parnham and Wetzig [20]. In this assay, red blood cells were incubated with the test extract. After incubation, the extent of hemolysis is measured using a spectrophotometer. Triton-X and PBS used as positive and negative controls, respectively. The plant extract was dissolved in PBS, and these seven doses were tested in the hemolytic assay [21].

DPPH Assay

The DPPH assay evaluates the free radical scavenging antioxidant capacity [22]. Arbaayan et al protocol was followed with certain modifications [23]. Ascorbic acid used as positive control with same concentration as that of plant extract. The alteration in absorbance at 517 nm was recorded after regimens for thirty minutes at room temperature. [24].

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$$I\,\% = \frac{A_0 - A}{A_0} \times 100$$

% scavenging = [(Abs control - Abs sample) / Abs control] x 100

Where I is the DPPH inhibition percentage, A0 is the absorbance of the control sample at the beginning of the reaction (t = 0 h), and A is the absorbance of the tested sample after completion of the reaction time (t = 1 h) [25].

Wound Healing assay

A wound healing assay was conducted to evaluate the effect of the plant extracts on the growth of HepG2 liver cancer cells. Doses of 100 µg/ml and 200 µg/ml were selected. The treated groups were compared against with the controls at 24 hours and 48 hours [26].

Sample	Area	Mean	Min	Max
Sample	23727	139.396	106	197
Control 1	12119	140.173	107	204

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Control 2	6588	149.597	117	215
Control 3	72316	143.888	97	211
100_1	24696	146.304	108	215
100_2	108.49	140.048	109	201
100_3	21703	142.814	100	206
200_1	22963	144.062	100	214
200_2	24363	138.965	110	196

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Statistical Analysis

All experiments were conducted with biological and technical triplicates . One way ANOVA was applied using GraphPad prism with the level of significance ≤ 0.05. Data is represented as mean ± SD.

GC-MS

The characterization of the phytochemicals in Phyllanthus polygonoides was determined using a Shimadzu QP2010 Plus GC-MS system. Identification was carried out using a thermal desorption system coupled to the GC-MS. The ionization voltage was 70eV. GC was performed on a Restek column at temperatures ranging from 80-290°C. The injector and interface temperatures were both 290°C. Samples were injected in split mode with helium carrier gas flow of 1.2 ml/min. Compound identification was based on retention times, mass spectra and library matching to NIST and WILEY libraries. Identification of active compounds was accomplished by comparing retention indices, mass spectra and published data. This enabled determination of compound names, formulas, structures and bioactivities [27, 28].

FTIR

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The Fourier transform infrared (FTIR) technique was employed to detect the distinctive functional groups present in the extract. Ttabhe absorption spectrum of a molecule can reveal valuable information about its structure. A little amount of *Phyllanthus polygonoides* extract was combined with dry potassium bromide (KBr). The mixture was meticulously blended in a mortar and compressed at a pressure of 6 bars for a duration of 2 minutes to create a thin disc made of KBr. Subsequently, the disk was inserted into a sample cup of a diffuse reflectance accessory. The IR spectrum was acquired using the Bruker Vertex 70 infrared spectrometer from Germany. The material underwent scanning spectroscopy from 4000 to 400 cm-1. The maximum values of the UV-VIS and FTIR spectra were recorded [29, 30] .

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Network pharmacology

Screening of chemical compounds of *Phyllanthus polygonoides*

The compounds of *Phyllanthus polygonoides* were obtained from published literature using Google scholar, PubMed, CNKI database, the KNApSAcK database (http://www.knapsackfamily.com/), and the traditional Chinese medicine system pharmacology (TCMSP) (https://old.tcmsp-e.com/) [31]. [32]. Compounds 3D structures and canonical SMILES were downloaded from PubChem (http://www.pubchem.ncbi.nlm.nih.gov/). The canonical SMILES were utilized ADMET analysis using the admetSAR and TCMSP database. Afterwards, the compounds that satisfied the ADMET criteria were selected as novel compounds and used for further screening [33].

Identification of compounds targets, disease genes and common genes

The compounds targets were identified using SwissTargetPrediction (http://www.swisstargetprediction.ch/) by using the canonical SMILES. "*Homo sapiens*" were selected, and the anticipated targets were retrieved. The disease targets were chosen from GeneCards (http://www.genecards.org/) and DisGeNET (http://www.disgenet.org/) using the keyword "HCC". A Venn diagram was created by overlapping the common genes of compound targets and disease genes. [34].

Gene Function Annotation

The DAVID functional enrichment database (http://david.ncifcrf.gov/) was used for gene function annotation through Gene ontology analysis, which covers biological process (BP), cellular component (CC), and molecular function (MF) and the KEGG analysis. Only those results were selected with $p \leq$

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0.05. GO Analysis was visualized using ShinyGO database [35].

Network Pharmacology Analysis

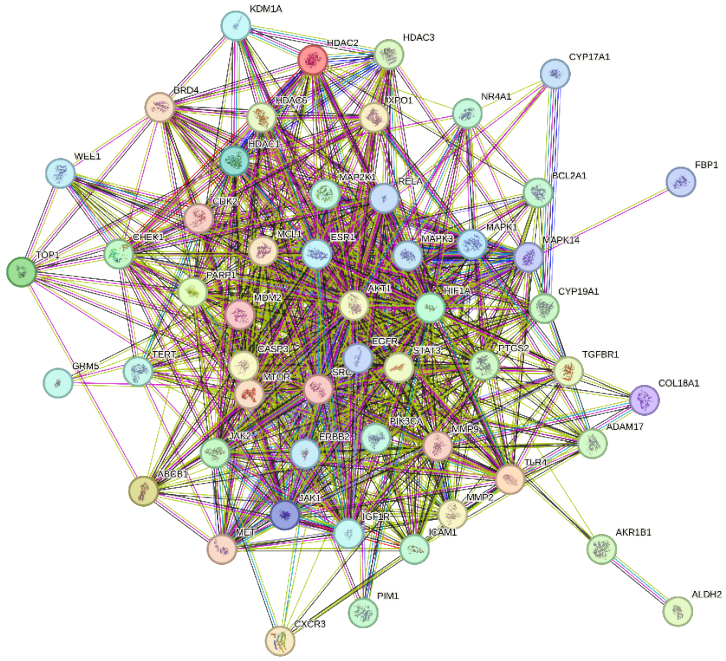
Compound-Target network

Cytoscape (version 3.8.2) was used to constract the network between potential targets of the disease and active compounds. (version 3.8.2) (http://cytoscape.org/). The network nodes represent the chemical components and targets, while the edges depict their interaction. The network analyzer was utilized to assess the fundamental characteristics of the network. The network was filtered based on the "degree" characteristic, which represents the number of connected nodes for each network node [36].

Prediction of Hub Genes and PPI Network

The therapeutic targets of HCC were assessed for correlation using the STRING database (https://string-db.org/). The specie was selected as "Homo sapiens," and P vale was set at 0.4 or above (Figure 2). The PPI network was visualized using Cytoscape. The CytoHubba plugin was utilized to identify the hub genes and nodes with a greater degree, which were subsequently examined in more detail. The greatest degree signifies a strong correlation between the targeted genes [37]. PPI network has been shown in Figure 1

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Figure 1. PPI Network of 58 intersected key targets(*P. polygonoides* and HCC).

Target Compound-Pathway Network Construction

The top 10 pathways were identified through KEGG pathway analysis using DAVID, and this information was utilized to construct a network of targets, compounds, and pathways utilizing Cytoscape[31, 32, 38].

Molecular docking

Molecular docking helps in identifying how ligands interact with their corresponding proteins. It was used to validate the results of the study. 3D structure of target compounds were downloaded in SDF formate from PubChem database. Hub genes were downloaded from Protein Data Bank (RCSB) PDB, (http://www.rcsb.org/). The best protein crystal structure, characterized by a smaller resolution value, completeness, and being a human protein, was chosen for docking. Chimera (version 1.15, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco) was used to remove ligands and water molecules and to correct the protein structure. The ligand and protein molecules were charged, hydrogenated, and normalized using AutoDockTool, and PDBQT file format was created. Molecular docking was performed between the refined ingredients and protein using AutoDock Vina. To calculate the binding energy, the Lamarckian genetic algorithm was selected, and Chimera software was used for the visualization of the docked results. This stage aimed to investigate the binding energy between the chemicals and their related targets by measuring their strong affinity; a higher affinity corresponds to a lower binding energy [31].

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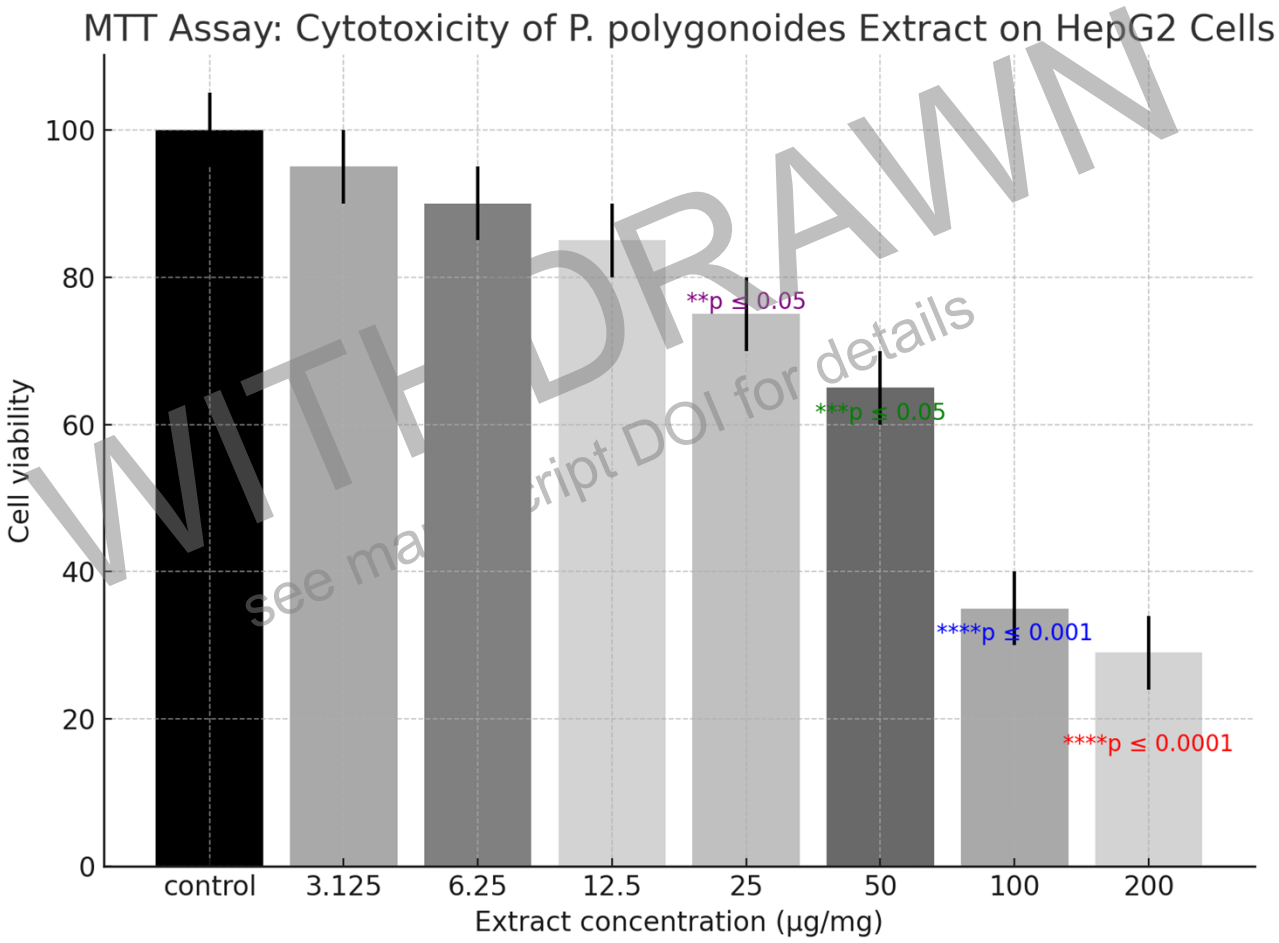
Results:

MTT assay

Phyllanthus polygonoides leaf extract significantly cytotoxic effects on HepG2 liver cancer cells were shown by the MTT assay analysis results. With increasing extract concentrations, there was a dose-dependent reduction in cell viability. Cell viability was reduced to 29% by the extract at the highest concentration of 200 µg/ml, indicating a strong cytotoxic effect (****$p \leq 0.0001$). Viability dropped to 35% (****$p < 0.001$) at 100 µg/ml. With viabilities of 65% and 75%, respectively, moderate cytotoxicity was seen at 50 µg/ml (***$p \leq 0.05$) and 25 µg/ml (**$p \leq 0.05$). The lower doses (3.125 to 12.5 µg/ml) demonstrated negligible cytotoxicity, keeping cell viability over 85%. The extract's

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potential as a source of anticancer agents is supported by these results, which indicate that it contains bioactive compounds that, at higher concentrations, can significantly inhibit HepG2 cell proliferation. More research is required to identify the active compounds and clarify their mechanisms of action. Results have been shown in Figure 2



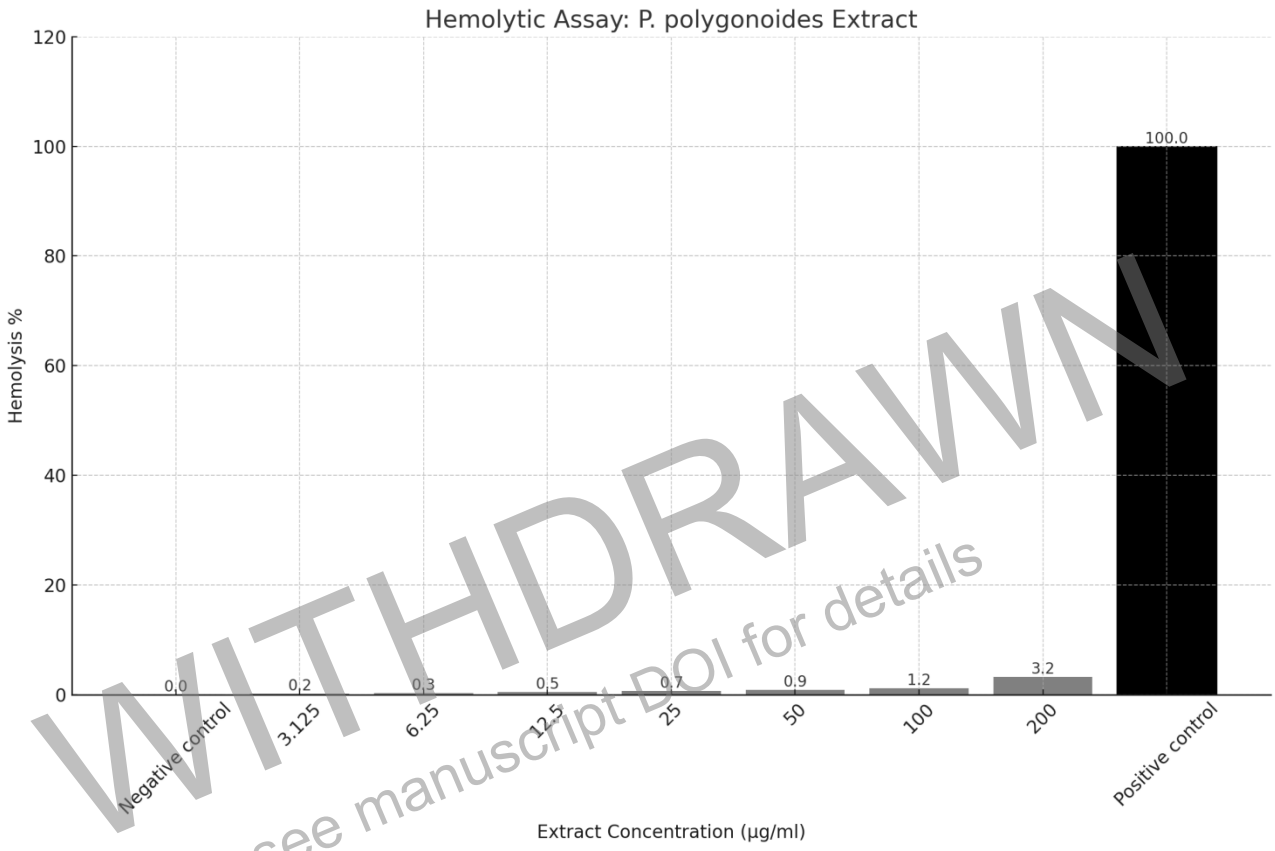
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Figure 2. Cytotoxic effect of *Phyllanthus polygonoides* extract against HepG2 cell line

Hemolytic assay

No hemolysis exceeding 10% was observed at any dose of *P.polygonoides* extract. The hemolysis observed at a concentration of 200 µg/ml of *P.polygonoides* plant extract was 3.2%. Results shown in Figure 3.

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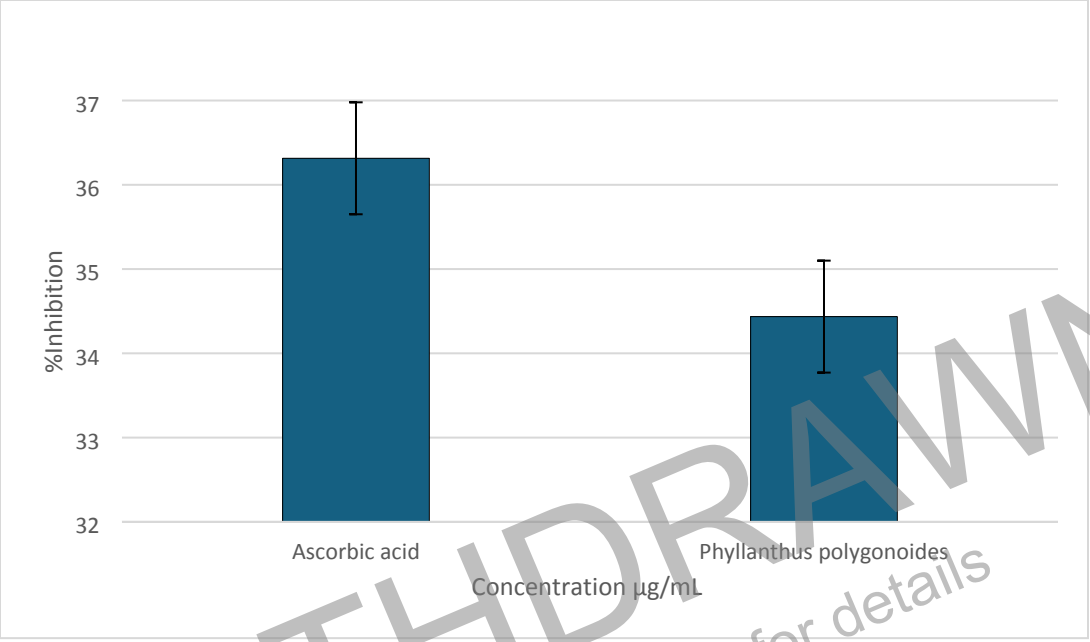
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Figure.3 Hemolytic effect of *P. polygonoides* on Human blood

DPPH Assay

Eight different concentrations 7.81 µg/mL, 15.6 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL of *P. polygonoides* showed different percentages of inhibition (Table 1). The scavenging activity of each dose was increased in a concentration dependent manner. The 1000 µg/mL extract showed the highest antioxidant activity, where *P.polygonoides* extract showed 34.43% inhibition compared with Ascorbic Acid, which showed 36.31% inhibition. The observed IC50 value of *P. polygonoides* extract was 58.99 µg/mL and Ascorbic Acid was 49.44 µg/mL The radical scavenging activity of those three extracts, at their highest concentration 1000 µg/mL, were higher than the antioxidant capacity of ascorbic acid at the same concentration. DPPH assay results have been shown in Figure 5.

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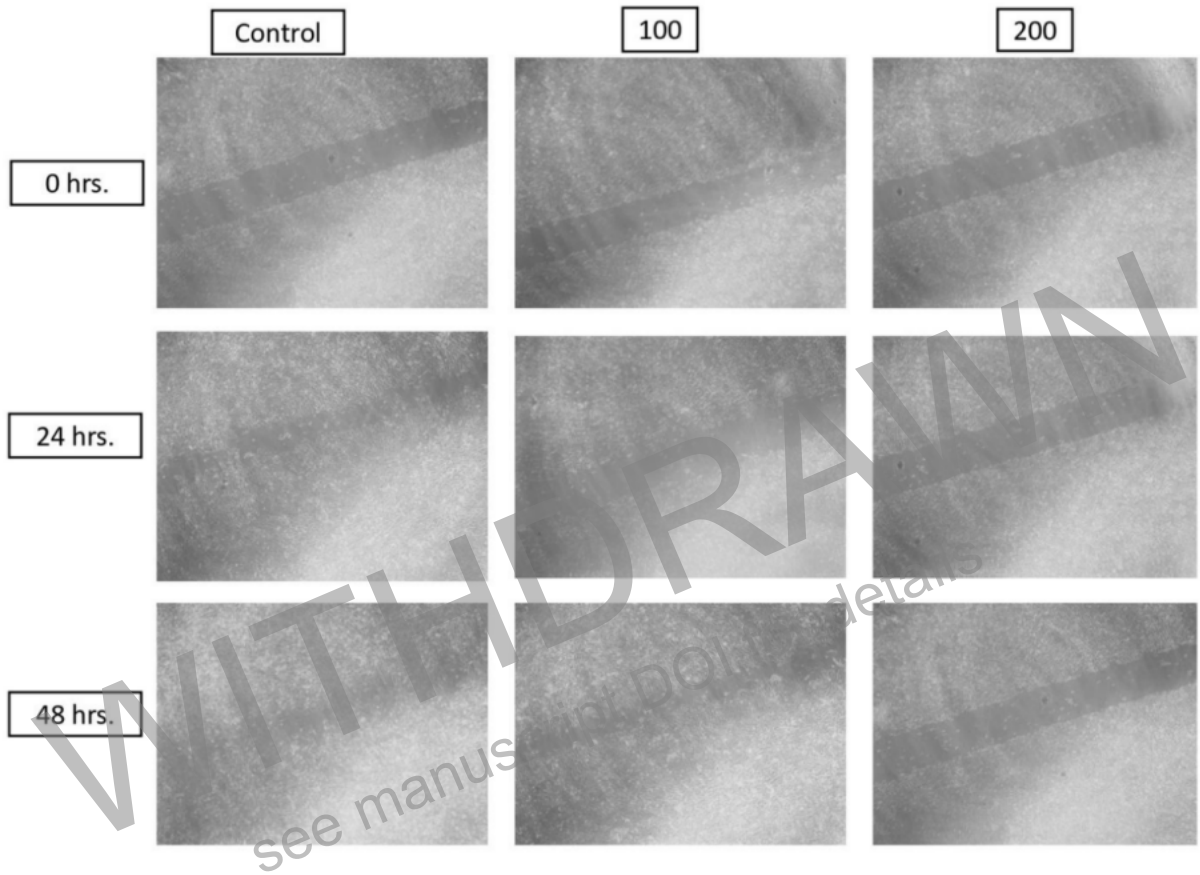
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FIGURE 4. The radical scavenging activity, represented by percentage of inhibition, of solvent extract compared to ascorbic acid.

Wound Healing assay

A wound healing assay was conducted to evaluate the growth potential of the HepG2 cell line. Plant extracts at concentrations of 100 µg/ml and 200 µg/ml were used. The treated groups were compared to controls at 24 and 48 hours. Minimal healing was observed at 24 hours for both doses compared to the control. By 48 hours, cell migration was seen in the 100 µg/ml group, while the 200 µg/ml group showed no change in the wound. The control group displayed significant wound healing Figure 5.

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Figure 5 Effect of *P.polygonoides* plant extract on wound healing at 10X magnification

GCMS

The GC-MS analysis of the methanolic plant extract of *P. polygonoides* are mentioned in Table 1. The GC-MS study detected a total of 25 compounds in the methanolic extract. A total of 25 compounds were identified by GC-MS analysis of the methanolic plant extract of P. polygonoides, as shown in the supplied chromatogram Figure 6. The proportion of these compounds is shown by the Total Ion Chromatogram (TIC), where each peak in the extract represents a different chemical entity. The retention time, which shows how long it takes for each chemical to pass through the gas chromatography column, is shown by the x-axis of the chromatogram. The abundance is shown by the y-axis, which displays the concentration of each component found. The compounds with the greatest peaks, which were detected at retention durations of 8.502, 9.809, and 11.152 minutes, indicate that their concentrations are notably greater than those of the other compounds. Significant abundance of the corresponding compounds are also shown by further peaks at 6.832, 7.766, 13.332, 14.266, and 16.624 minutes. The majority of chemicals that elute at early retention periods tend to be less polar

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and more volatile than those that appear subsequently. Understanding the complicated chemistry of the methanolic extract is crucial for comprehending its possible biological actions and uses, and this in-depth investigation offers insights into that composition. The extract has a variety of chemicals at different quantities, which highlights its potential for a wide range of pharmacological effects.

Table 1. Chemical composition of *P. polygonoides* leaves (methanolic extract)

Peak #	R. Time (min)	Area %	Molecular Formula	Name
1	6.577	4.98	C24H22N2O2	2,3,11,12-Dibenzo-1,13-dioxa-4,10-diazacyclooctadeca-2,11-diene
2	6.631	9.39	C24H22N2O2	2,3,11,12-Dibenzo-1,13-dioxa-4,10-diazacyclooctadeca-2,11-diene
3	7.764	3.71	C8H18O2	Ethanol, 2-[(2-ethylhexyl)oxy]-
4	8.299	14.47	C18H32O2	Estra-1,3,5(10),15-tetraen-17-one, 3-hydroxy-, TMS derivative
5	9.358	1.91	C16H12N2O2	2-Benzylaniline, N-trimethylsilyl-
6	9.808	8.97	C17H23NO6	Pyridoxine, 3TMS derivative
7	11.150	5.37	C24H22N2O	2,3,11,12-Dibenzo-1,13-dioxa-4,10-diazacyclooctadeca-2,11-diene
8	12.306	4.52	C23H40O5PS	Propyl gallate, 3TMS derivative
9	13.332	3.84	C24H52O4Si4	D-Xylulose, tetrakis(trimethylsilyl) ether, pentafluorobenzyloxime
10	13.423	1.33	C18H36O2	Hexadecanoic acid, methyl ester
11	14.268	3.37	C24H22N2O2	2,3,11,12-Dibenzo-1,13-dioxa-4,10-diazacyclooctadeca-2,11-diene
12	14.584	1.72	C18H32O2	9,12,15-Octadecatrienoic acid, methyl ester
13	14.643	1.67	C20H40O	Phytol
14	15.114	3.48	C24H22N2O2	2,3,11,12-Dibenzo-1,13-dioxa-4,10-diazacyclooctadeca-2,11-diene

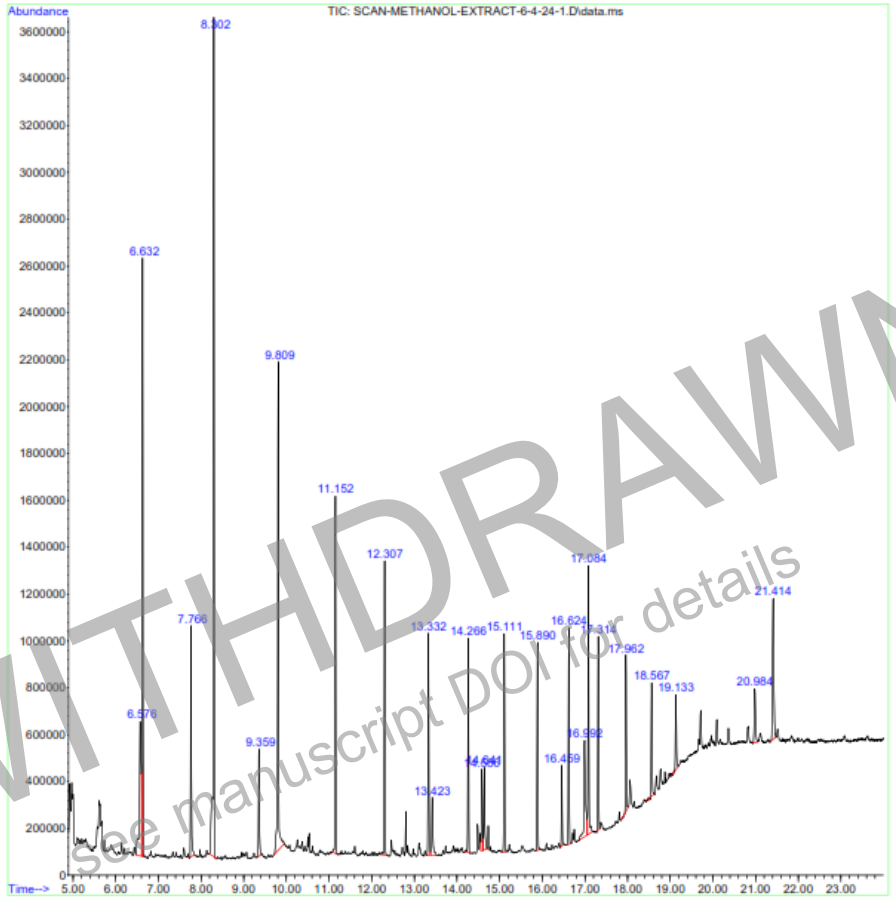
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Peak #	R. Time (min)	Area %	Molecular Formula	Name
15	15.889	3.44	C19H24N2O7P	Sethoxydim, trimethylsilyl ether
16	16.456	1.26	C30H40O2	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-]
17	16.622	3.52	C26H42O3	1,4-Androstadien-3,17-dione, di-trimethylsilyl
18	16.991	3.25	C18H36O4	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
19	17.082	4.78	C24H38O4	Bis(2-ethylhexyl) phthalate
20	17.312	3.27	C26H42O3	1,4-Androstadien-3,17-dione, di-trimethylsilyl
21	17.964	2.75	C16H20Osi	1,4-Androstadien-3,17-dione, di-trimethylsilyl
22	18.569	1.99	C15H18Osi	1,4-Androstadien-17β-ol-3-one, di-trimethylsilyl
23	19.136	1.41	C8H9NO2	Paracetamol, 2TMS derivative
24	20.981	1.45	C14H15NO	4-tert-Butylaniline, N-trimethylsilyl-
25	21.414	4.14	C13H15F3NO2S	Acetamide, N-[4-[3-[4-[(dimethylamino)sulfonyl]-1-piperazinyl]-2-hydroxypropoxy]phenyl]-

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Figure 6: GC-MS chromatogram of *P. polygonoides* plant extract

FTIR

The chemical bonds or functional groups in the dried leaf powder and leaf extract of *P. polygonoides* were identified using FTIR. The bonds were identified by analyzing the infrared absorption spectra. The peak at 1026.34 cm−1 corresponds to the C-C skeletal vibration present in the extract. The peak observed at 2848.28 cm-1 is due to the symmetric stretching of saturated (sp3) carbon. The peak at 2916.41 cm-1 can be attributed to CH3 asymmetry stretching. The peak at 1894.84 cm-1 represents the C=O of esters. The peak at 2635.14 cm-1 is characteristic of N-D stretching vibration. These absorption peaks provide useful information regarding the occurrence of hydroxyl, carbonyl, aromatic and other functional groups in the phytoconstituents present in the *P. polygonoides* extract [39]. FTIR Results have been shown in Table 2 and Figure 7

Table 2. FTIR spectral peak values and functional groups of dried leaf powder of *P. polygonoides*

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Wave numbers (cm-1)	Functional Group	Predicted compounds
2916.41	CH3 asymmetry stretching	Lipid
2848.28	C-H stretching vibration	Alkane
2635.14	N-D stretching vibration	Imine
1894.84	C=O of esters	Membrane lipid, fatty acid
1026.34	CH3 rocking vibration	P-O-C2H5; Organic phosphorus compound

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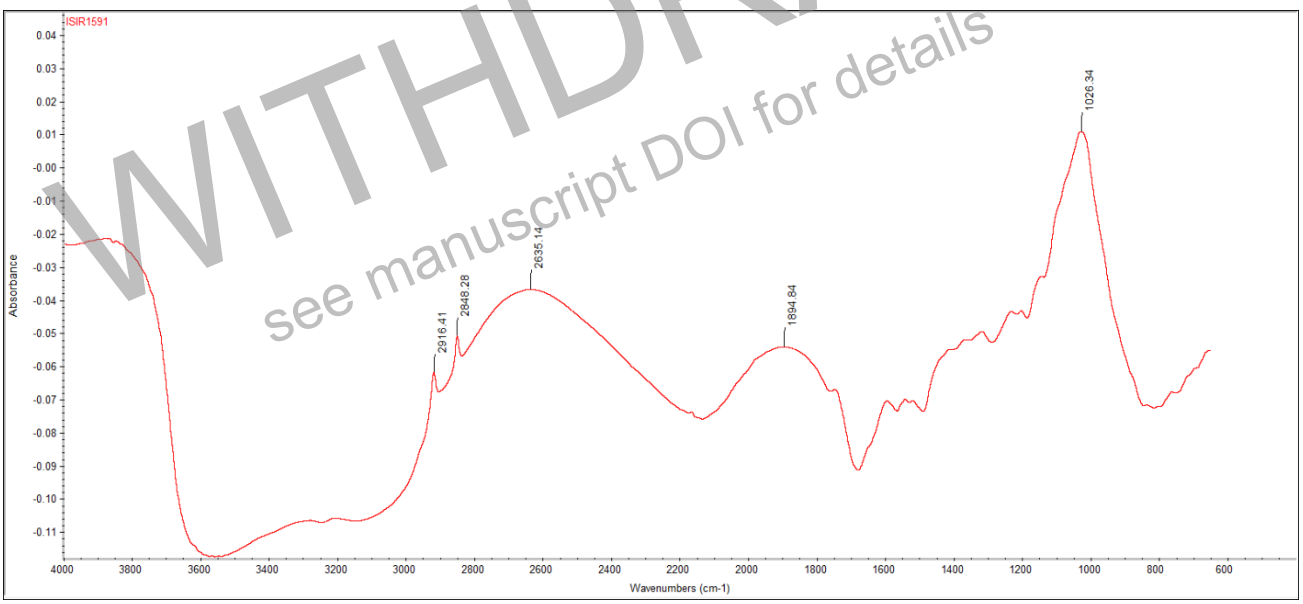


Figure 7: FTIR spectrum of dried leaf powder of *P. polygonoides*

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Networkpharmacology

Screening of chemical compounds of *Phyllanthus polygonoides*

Seventy-one compounds were obtained after removing duplications from *Phyllanthus polygonoides* compounds library. After performing ADMET analysis on 71 chemical constituents, 9 phytochemicals were selected according to the Lipinski rule of five as shown in Table 3

Table 3 showing the top 9 selected compounds after ADMET analysis

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Sr.No	Compounds	MW(g/mol)	HBA	HBD	LogP
1	2,3,11,12-Dibenzo-1,13-dioxa-4,10-diazacyclooctadeca-2,11-diene	354.49	2	2	4.01
2	2-(Piperidin-4-yl)acetic acid	143.18	3	2	0.05
3	2,4-Diamino-5-[3,4-[p-chlorobenzylidine]dioxybenzyl]pyrimidine	354.79	4	2	2.98
4	3-Hydroxyanthranilic Acid	153.14	3	3	0.29
5	Ethanol, 2-[(2-ethylhexyl)oxy]-	174.28	2	1	2.38
6	Estra-1,3,5(10),15-tetraen-17-one, 3-hydroxy-	268.35	2	1	3.27
7	2-Benzylaniline	183.25	0	1	2.86

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8	N-Benzhydryl-1-(tert-butyl)-1,1-dimethylsilanamine	297.51	1	1	4.5
	4-Piperidineacitic acid				

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Analysis of Network Pharmacology

Identification of potential targets

A total of 993 target genes of ten active compounds were screened out through the SwissTargetPrediction. Similarly, 8753 disease genes were retrieved using GeneCards ,118 using Ensembl, and 1395 using DisGNet databases. Fifty-eight common targets of HCC were selected through Venn diagram as shown in Figure 8. Top 9 compounds were selected from the 58 compounds using the degree score revealed by Table 4.

Table 4. The degree of 10 active compounds that were analyzed in Cytoscape.

Sr. No.	Compounds	Class Categories	Degree
1	2,3,11,12-Dibenzo-1,13-dioxa-4,10-diazacyclooctadeca-2,11-diene	Polycyclic compound	17
2	2-(Piperidin-4-yl)acetic acid	Acid derivative	5
3	2,4-Diamino-5-[3,4-[p-chlorobenzylidine]dioxybenzyl]pyrimidine	Heterocyclic compound	12
4	3-Hydroxyanthranilic Acid	Acid derivative	29
5	Ethanol, 2-[(2-ethylhexyl)oxy]-	Ether	16
6	Estra-1,3,5(10),15-tetraen-17-one, 3-hydroxy-	Steroid	17

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7	2-Benzylaniline	Aromatic amine	14
8	N-Benzhydryl-1-(tert-butyl)-1,1-dimethylsilanamine	Amine derivative	6
9	4-Piperidineacitic acid	amino acid derivative	15

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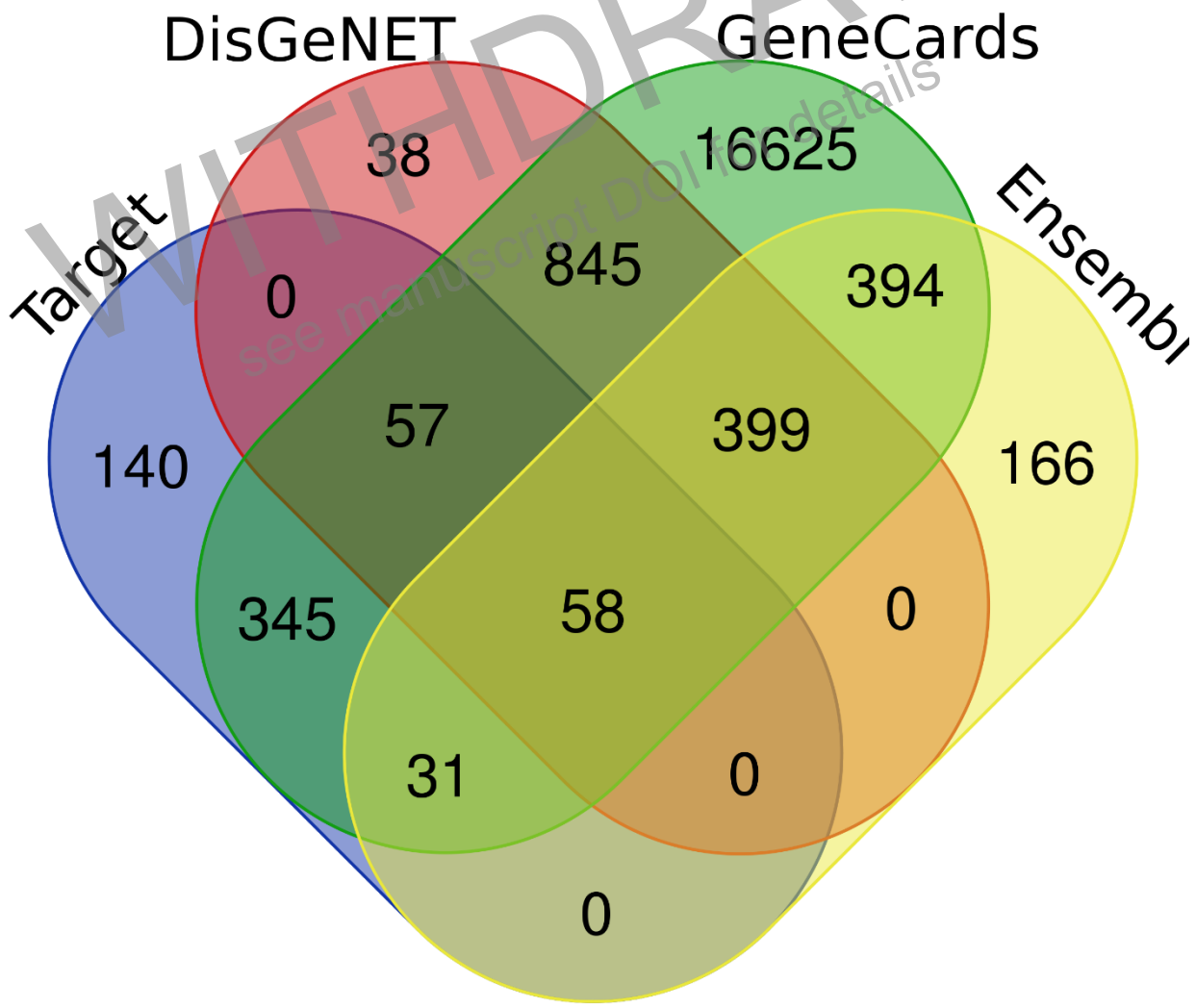


Figure 8. Venn diagram showing the common targets..

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Compound Target network

A network containing 58 target genes and 9 compounds was generated. Utilizing a "network analyzer" for analysis revealed that the compound-target network consists of 999 edges and 67 nodes as shown in Figure 9

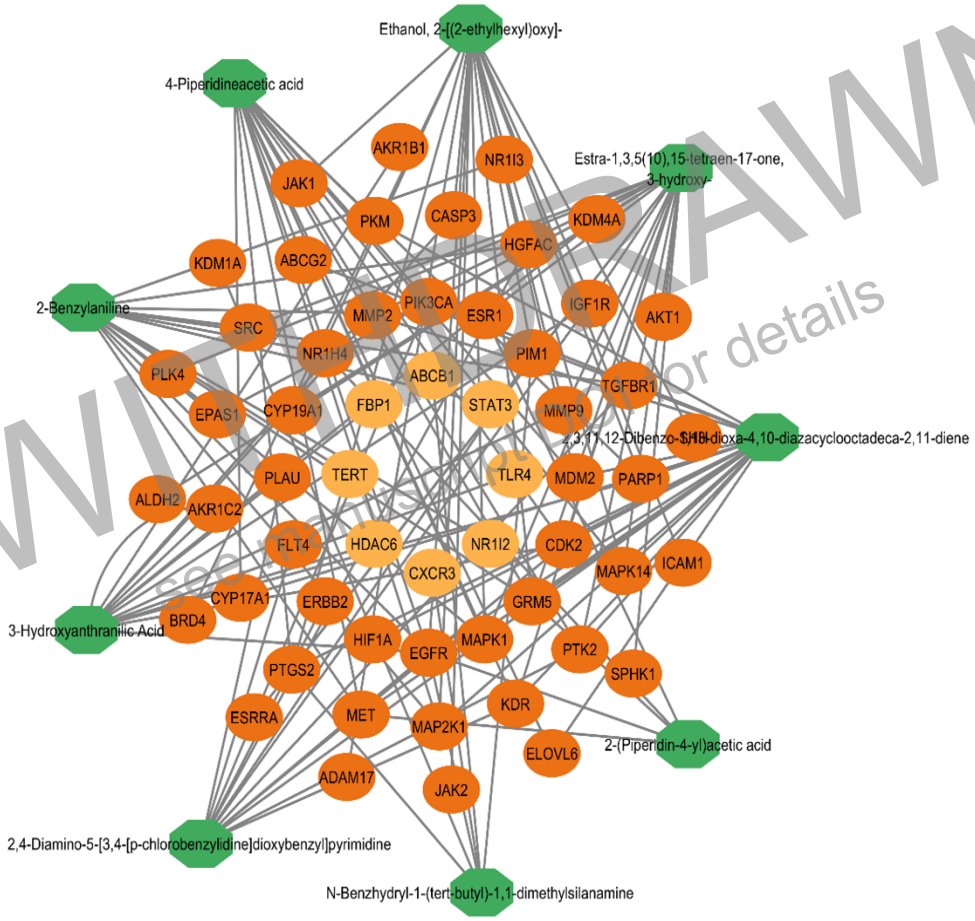


Figure 9. Compound-target network (green color indicates active compounds; yellow orange indicates targets) connecting common targets of disease and chemicals

Analysis of PPI Network

The PPI network contained fifty-eight nodes and 586 edges. The green circles within the network represent the top 10 hub genes, characterized by high degree values. These hub genes include

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AKT1, STAT3, HIF1A, EGFR, ESR1, CASP3, SRC, ERBB2, MMP9, KDR revealed by Figure 10.

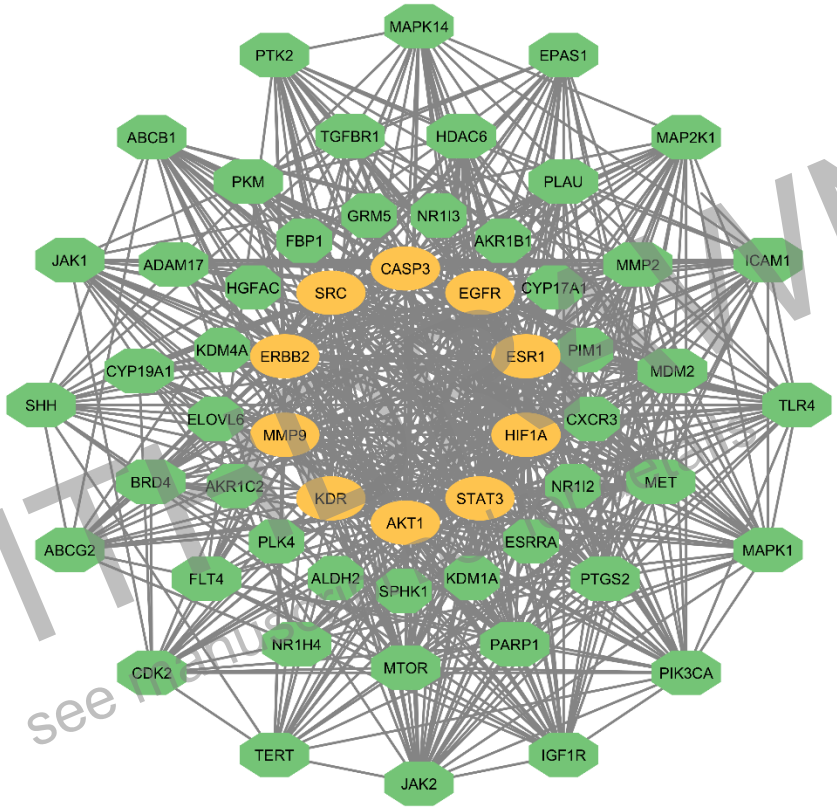
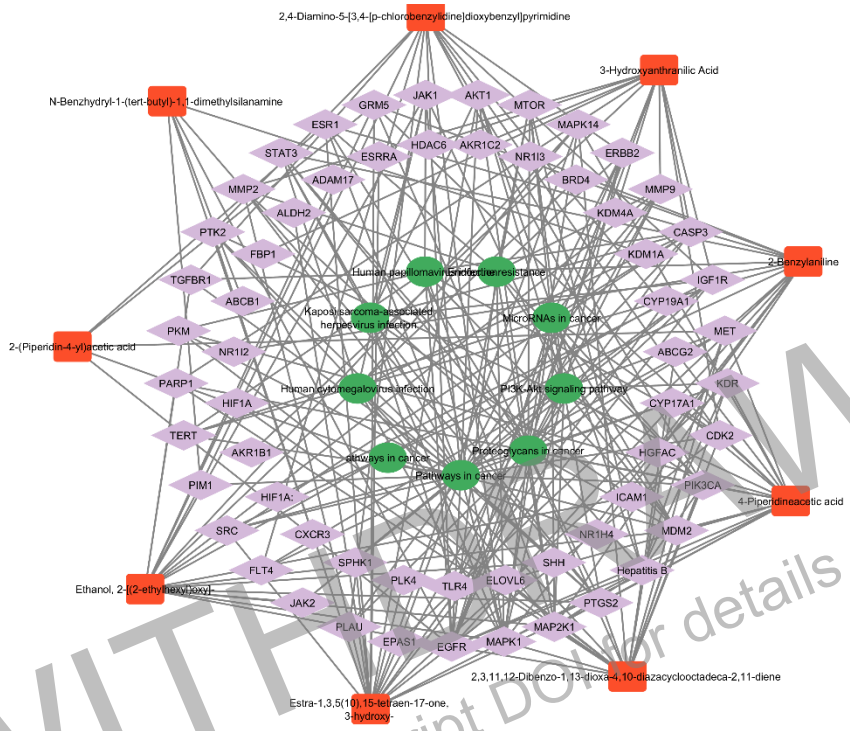


Figure 10. PPI network of 58 interconnected targets of HCC and *P. pollygonoides*

Compound-Target-Pathway network

A target–pathway–compound network was constructed using compounds as common genes and the top 10 enriched pathways in Cytoscape as shown in Figure 11. The network comprised 79 nodes and 247 edges, encompassing eight active components, 58 targets, and 10 pathways.

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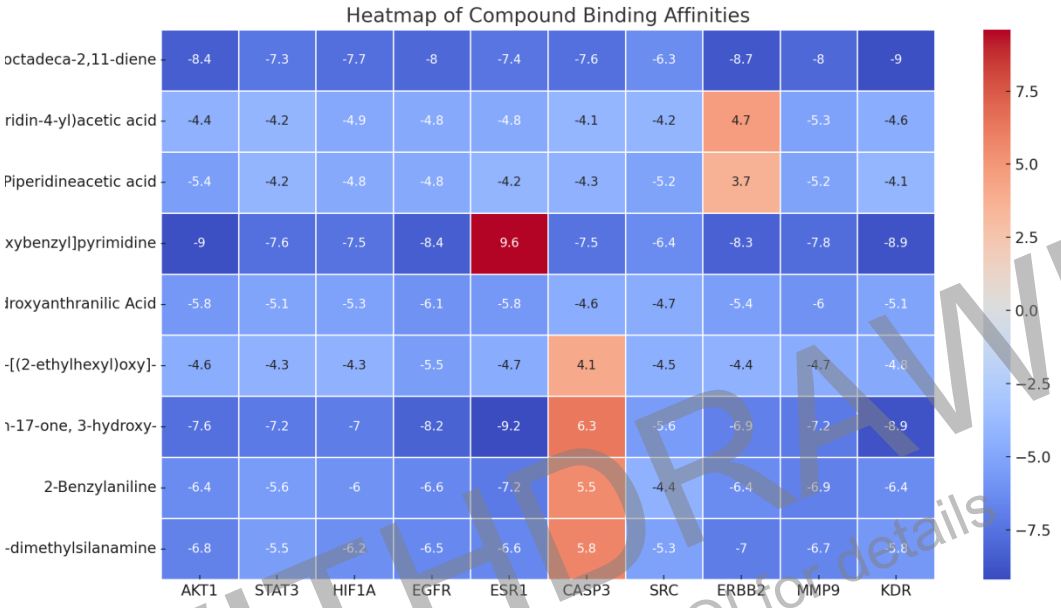
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Figure 11. The network shows the relationship between active chemicals, target genes, and enrichment pathways

Molecular docking

Selected compounds from *P.pollygonoides* were docked against HCC targets. The docking score ranged from −4.2 to −9.2 kcal/mole. Docking of these nine targets with 9 active ingredients of *P. pollygonoides*, suggested triterpenoid saponin, flavonoids, anthocyanidin, and phytosterols possessed lowest binding scores with selected targets. Docking Results have been shown as in Figure 12 for Binding affinities and Figure 13 showing the 3D interactions of genes-targets.

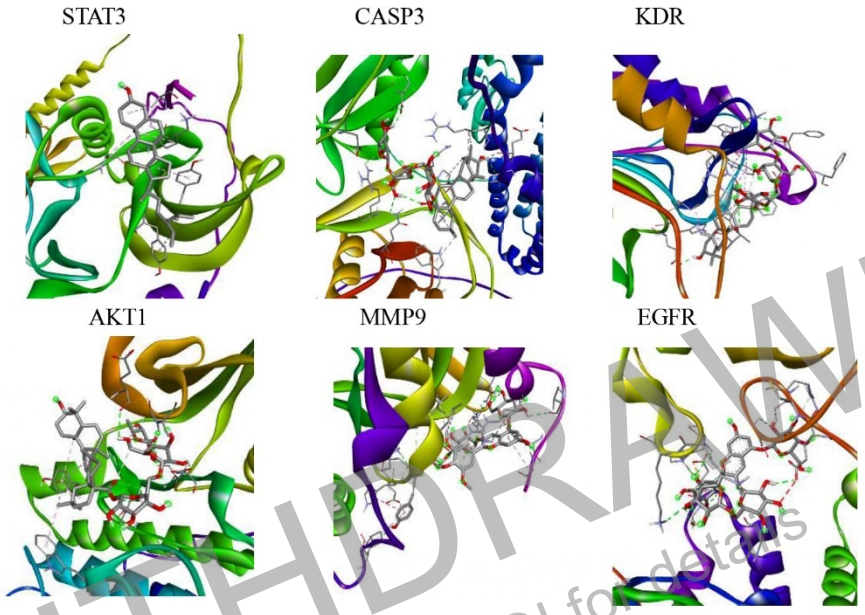
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Figure 12 representing the compounds-targets binding affinities.

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Figure 12. The docking complex of five targets with their best binding components shows the binding site residues with the protein. (**A**) STAT3; (**B**) CASP3; (**C**) KDR; (**D**) AKT1; (**E**) MMP9; (**F**)EGFR

Discussion

Phyllanthus polygonoides is a medicinal plant that has been used in traditional medicine to treat various ailments [1]. Previous studies have shown that it possesses important biological activities such as antioxidant, anti-inflammatory, antidiabetic and anticancer properties [40]. The current study evaluated the antitumor effects of *P. polygonoides* extract on HepG2 cells [41]. *P. polygonoides* contains several polyphenols and flavonoids that contribute to its free radical scavenging and antioxidant properties. These phytochemicals are known to possess anti-cancer properties [42]. The MTT assay results of this study revealed that *P. polygonoides* extract exhibited cytotoxicity against HepG2 cells in a dose-dependent **(Figure.12)** [43]. A significant decrease in viability was seen at the 200 µg/ml dose, resulting in 29% viability, and at the 100 µg/ml dose, with 35% viability, compared to the control. This shows that *P. polygonoides* has potential anti-proliferative effects against liver cancer cells [44].

The hemolysis assay results demonstrated that *P. polygonoides* extract did not cause significant hemolysis of RBCs. Less than 10% hemolysis is considered non-toxic to RBCs [45]. This suggests

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that the extract is safe and does not damage cell membranes at effective antiproliferative doses. *P. polygonoides* extract, similar to the four essential oils tested from other plants like *Syzygiumjambolanum* (myrtle), *Lippiagracilis* (gracefulfalse buttonweed), *Myracrodruonurundeuva* (Brazilian copaiba) and *Bixaorellana* (annatto or achiote), did not show significant hemolysis above 10% even at the highest concentration of 1000 µg/ml [45]. This suggests the extract, like the oils, is non-toxic and safe for use as it does not damage red blood cell membranes at effective concentrations. Some plants such as *Saponaria officinalis* (soapwort) and *Spinacia oleracea* (spinach) are reported to cause hemolysis due to compounds such as saponins, this assay reliably indicated a lack of such activity for both P. polygonoides and the other oils [46].

In the DPPH radical scavenging assay, *P. polygonoides* extract showed antioxidant activity in a concentration-dependent manner, with an IC50 value of 58.99 µg/mL. According to the scale of Phongpaichit et al., this IC50 value indicates intermediate antioxidant activity [47]. The phytochemicals in *P. polygonoides* likely contribute to its free radical scavenging ability. Many medicinal plant extracts have shown higher DPPH scavenging activity than *P. polygonoides,* with IC50 values <50 µg/mL, indicating strong antioxidant potential. For example *Mangifera indica* leaf extract IC50 = 17.6 µg/mL [48] *Cissus quadrangularis* stem extract IC50 65 µg/ml [49] Some studies have reported similar or lower antioxidant activity compared to *P. polygonoides*. Pongamia pinnata leaf extract IC_{50} 11.67 µg/Ml [50]. The phytochemicals present in different plant extracts, such as polyphenols, flavonoids, tannins etc. influence their free radical scavenging potential and DPPH IC50 values. The constituents in P. polygonoides likely contribute to its intermediate antioxidant activity. The wound healing assay results showed that *P. polygonoides* extract significantly inhibited the migration of HepG2 cells in a dose-dependent manner. At 100 µg/mL some healing was observed but the wound remained persistent at 200 µg/mL even at 48 hours. This indicates that the extract suppresses the metastatic potential of liver cancer cells . Similar wound healing inhibitory effects have been seen with other plant extracts. For example, anthocyanin-rich mulberry extract inhibited hepatoma cell migration in a concentration-dependent manner, with complete inhibition at 100 µg/mL. The wound gap was still visible even after 48 hours of treatment. Ardisia japonica extract showed potent inhibition of mouse melanoma cell migration up to 80% at 200 µg/mL concentration. A persistent wound gap was observed at 48 hours [51]. Hydroxychavicol isolated from Piper betle significantly inhibited prostate cancer cell

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migration in a dose-dependent way, with near complete suppression at 100 µg/mL. Some studies have reported comparable or stronger inhibitory effects at lower concentrations [52]. For instance, curcumin suppressed breast cancer cell migration by over 90% even at 25 µg/mL [52]. Thus, P. polygonoides extract exhibits promising anti-metastatic activity against hepatocellular carcinoma cells comparable to various plant extracts tested on different cancer cell lines.

GC-MS analysis identified 25 major phytochemical components in the *P. polygonoides* extract including flavonoids, terpenoids and phenolic compounds. These plant secondary metabolites are known to possess anti-cancer properties and likely contribute to the anticancer activity observed [53]. GC-MS analysis of *Sarcocephalus latifolius* root extract identified 33 compounds including flavonoids, terpenes, fatty acids esters that have been reported for their antioxidant and cytotoxic effects [54]. GC-MS of *Moringa oleifera* leaf extract revealed compounds like quercetin, kaempferol and their glycosides, which are powerful antioxidants [55]. FTIR spectral analysis provided functional group information about hydroxyl, carbonyl, aromatic and other groups present in the extract. This helps in understanding the phytoconstituents responsible for bioactivity

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.Hepatocellular carcinoma (HCC) is a primary malignancy of the liver that ranks as the second leading cause of cancer-related deaths worldwide. Some of the key genes and pathways involved in HCC pathogenesis include AKT1, STAT3, HIF1A, EGFR, ESR1, CASP3, SRC, ERBB2, MMP9 and KDR. Abnormal activation and overexpression of these genes promote tumor growth, angiogenesis, invasion and metastasis in HCC.

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver that involves dysregulation of several key genes including AKT1, STAT3, HIF1A, EGFR, ESR1, CASP3, SRC, ERBB2, MMP9 and KDR. Phyllanthus polygonoides is a traditional medicinal herb used to treat liver diseases. Previous studies have identified several compounds from P. polygonoides that may exert anticancer effects against HCC by modulating these gene targets. Docking studies revealed that compounds like 2,3,11,12-Dibenzo-1 ,13-dioxa-4,10-diazacyclooctadeca-2,11-diene, 2-(Piperidin-4-yl)acetic acid, 2,4-Diamino-5-[3,4-[p-chlorobenzylidine]dioxybenzyl]pyrimidine and 3-Hydroxyanthranilic Acid from P. polygonoides interact strongly with AKT1, STAT3, HIF1A, EGFR, ESR1, CASP3, SRC, ERBB2, MMP9 and KDR, with binding energies ranging from -4.1 to -9.0 kcal/mol. Specifically, 2,3,11,12-Dibenzo-1 ,13-dioxa-4,10-diazacyclooctadeca-2,11-diene showed high affinity for AKT1, STAT3, HIF1A, EGFR and KDR (-8.0 to -9.0

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kcal/mol), while 3-Hydroxyanthranilic Acid bound potently to AKT1, STAT3, EGFR, ERBB2 and MMP9 (-5.1 to -6.0 kcal/mol). Estra-1,3,5(10),15-tetraen-17-one, 3-hydroxy- and 2-Benzylaniline also strongly interacted with AKT1, STAT3, EGFR, ESR1 and CASP3.

These compounds may exert anti-HCC activities by inhibiting the expression and downstream signaling of these oncogenic targets, thereby suppressing processes such as proliferation, survival, angiogenesis and metastasis in liver cancer. Further studies are required to validate the effects of these compounds on HCC tumor growth and progression in vivo and their potential as adjuvant therapeutic agents.

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Conclusion

In conclusion, the present study provides compelling evidence that Phyllanthus polygonoides has potential anti-hepatocellular carcinoma properties. The methanolic extract demonstrated significant cytotoxicity against HepG2 liver cancer cells in vitro. It also exhibited antioxidant activity, suggesting chemopreventive effects against oxidative stress-induced liver damage and carcinogenesis. *P. polygonoides* suppressed the migratory ability of HepG2 cells, indicating anti-metastatic effects. GC-MS and FTIR analyses characterized various phytochemicals present in the extract, including flavonoids, terpenoids and fatty acid derivatives known to possess antitumor activities. Network pharmacology identified prominent gene targets of HCC like AKT1, STAT3, HIF1A, EGFR, ESR1, CASP3, SRC, ERBB2 and MMP9 that are potentially modulated by bioactive compounds from *P. polygonoides*. The compound-target interaction network provided insights into mechanisms of multitarget regulation. Notable hub genes in the PPI network were predicted to play key roles in coordinated HCC pathways. Molecular docking investigations demonstrated strong binding interactions between compounds and important oncogenic targets. The compound-target-pathway network elucidated relationships between components, targets and significantly enriched metabolic pathways. The current study provides a framework for future work exploring P. polygonoides as a supplemental treatment approach for liver cancer

Author Contributions

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Conflicts of Interest

The authors declare no conflict of interest.

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