ORIGINAL RESEARCH

Integrating Network Pharmacology and In Vitro Experiments for Assessing the Anti-Tumor Effects of *Phyllanthus Urinaria L* Anti-neoplastic Decoction in Hepatocellular Carcinoma

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ABSTRACT

Background • In China, traditional Chinese medicine (TCM) is an important part of the comprehensive treatment of hepatocellular carcinoma (HCC), and Chinese herb formulas with the effect of "yiqi jianpi jiedu huayu" (replenishing qi, strengthening spleen, and removing toxicity and blood stasis) are the common and efficient treatments for HCC. However, the mechanism of these formulas in treating HCC remain unclear.

Objective • In this paper, our goal is to explore the potential mechanism of Phyllanthus urinaria L anti-neoplastic decoction (PAD), the representative formula of "yiqi jianpi jiedu huayu", in treating HCC.

Design • The research team performed the network pharmacology and *in vitro* experiment (preparation of PAD aqueous extract, cell cultures and MTT assay, cell apoptosis assay, wound healing assay, transwell assays, western blot).

Setting • The study took place in the Department of Hepatology, the Fourth Clinical Medical College of Guangzhou University of Chinese Medicine (Shenzhen Traditional Chinese Medicine Hospital), China.

Outcome Measures • The active components and targets of PAD and HCC targets were screened by five Chinese herbs and two disease databases respectively. The network pharmacology was utilized to construct the relationship network between PAD and HCC, and the mechanism was predicted by pathway enrichment analysis. The experiment was performed to verify the intervention effect of PAD on HCC and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway.

Results • The relationship network between PAD and HCC suggested that PAD mainly regulated the potential therapeutic targets of HCC by key active components such as quercetin, luteolin, calycosin, wogonin, and pinocembrin. Pathway analysis demonstrated PAD could play an anti-HCC effect via multiple pathways (e.g., PI3K/Akt). Results of the experiment showed that PAD could effectively inhibit the proliferation and migration of HCC cells, and promote HCC cells apoptosis in a concentration-dependent behavior. Additionally, PAD could decrease the protein expression of phosphorylated PI3K/Akt.

Conclusion • PAD mainly exerts an anti-HCC effect through multiple active components represented by quercetin and multiple pathways represented by the PI3K/Akt pathway. This study provided an experimental basis for the clinical application of PAD. (*Altern Ther Health Med.* 2025;31(3):114-121).

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INTRODUCTION

Hepatocellular carcinoma (HCC), the main type of primary liver cancer, is one of the most common malignant tumors globally. HCC is easily prone to relapse and drug resistance, and the survival rate of patients with HCC is still poor according to 2020 global cancer data statistics.[1] Limited therapeutic options are available for patients with intermediate- to advanced-stage HCC, and exploring the underlying mechanisms of HCC and developing drugs to treat HCC are of paramount importance. In China, traditional Chinese medicine (TCM) is an important part of the comprehensive treatment of cancer. Previous studies have shown that Chinese herb formulas, as one of the main intervention measures of TCM, can improve the survival quality of HCC patients, prolong the survival time, and

reduce the side effects of modern medicine treatment.[2,3] However, the mechanism of Chinese herb formulas in the treatment of HCC is not clear. Thus, the study of the mechanism of TCM in treating HCC is conducive to the promotion of TCM and benefits more patients.

Chinese herbs have great potential to be developed into pharmaceuticals.[4] Based on TCM theory, Chinese herb formulas with the effect of "Jianpi Jiedu" (strengthening spleen and removing toxicity) could promote the quality of life of HCC patients,[5] "Fuzheng" (strengthens healthy qi) could remarkably prolong the survival time of patients.[6] Nevertheless, the mechanisms of action of these formulas also need to be further explained by modern medical research. Phyllanthus urinaria L anti-neoplastic decoction (PAD), the representative Chinese herbs prescription of "yiqi jianpi jiedu huayu" (replenishing qi, strengthening spleen, and removing toxicity and blood stasis), is currently under investigation in our laboratory, and PAD contains the following 7 Chinese herbs: *Phyllanthus urinaria L.* (Yexiazhu in Chinese, YXZ); *Scutellaria barbata D. Don.* (Banzhilian in Chinese, BZL); *Cremastra appendiculata (D. Don) Makino* (Shancigu in Chinese, SCG); *Curcuma zedoaria (Christm.) Rosc* (Ezhu in Chinese, EZ); *Carthamus tinctorius L.* (Honghua in Chinese, HH); *Astragalus membranaceus (Fisch.) Bunge* (Huangqi in Chinese, HQ); *Polygonatum sibiricum Red.* (Huangjing in Chinese, HJ). *Phyllanthus urinaria L.* the sovereign drug in PAD, is a common Chinese herb used by our TCM team in treating HCC, which demonstrated good anti-HCC effects in both clinical and experimental studies.[7,8] The remaining 6 Chinese herbs also have good anti-cancer effects. For instance, preceding studies showed that *Astragalus membranaceus* could promote vascular normalization in tumor-derived endothelial cells of HCC by reduced expression of HIF1a,[9] and *Scutellaria barbata* suppressed HCC tumorigenesis *in vivo* by inducing ferroptosis of HCC cells.[10] Modern pharmacological research has proved that *Cremastra appendiculata (D. Don) Makino* has a good therapeutic effect on liver cancer and other malignant tumors.[11] It was reported *Carthamus tinctorius L.* has the activities of suppressing the development of HCC and anti-hepatic fibrosis.[12] However, the potential role of PAD in cancer treatment has not been studied. Recently, the emergence of network pharmacology has innovated the traditional mode of drug research and provided new technical support for the systematic research and innovative drug development of TCM.[13] In this study, a combination of network pharmacology and experimental verification was utilized to clarify the mechanism of PAD in the treatment of HCC.

METHODS

Prediction of active components and targets of PAD

The active components of PAD were collected through the TCM System Pharmacology Database (TCMSP, https://tcmsp-e.com/tcmsp.php),[14] SymMap (http://www.symmap.org),[15] Integrative Pharmacology-based Research Platform of TCM (TCMIP, http://www.tcmip.cn/),[16] Bioinformatics Analysis Tool for Molecular mechANism of TCM (BATMAN-TCM, http://bionet.ncpsb.org/batman-tcm/),[17] and HERB (http://herb.ac.cn/).[18] Moreover, the components of PAD were selected via drug-likeness (DL) ≥ 30% and oral bioavailability (OB) ≥ 18% for further study,[14] and the targets of the active components were also acquired from the same databases. Besides, UniProt (https://www.uniprot.org/uniprot/) was used to transform the target names into corresponding gene names.

Screening the common targets of PAD and HCC

HCC-associated target genes were obtained from TCMIP, HERB, SymMap, MalaCard (https://www.malacards.org/), and OMIM database (https://omim.org/) using "hepatocellular carcinoma" as the keyword. After excluding the duplicate target genes, potential target genes of HCC were screened. Then, common targets of PAD and HCC were found by using the Venn diagram, and the network of "PAD active components-common targets" was built through Cytoscape software (version 3.7.2).

GO and KEGG pathway enrichment analyses for common targets

To predict the mechanism of PAD in treating HCC, GO and KEGG pathway enrichment analyses on common targets were performed using R software (version 3.6.3) loaded with packages of "ggplot2" and "clusterProfiler."[19] The GO analysis of common targets was classified into 3 functional groups, containing biological process (BP), cellular component (CC), and molecular function (MF). The top 10 significant items in each functional group were ranked according to adjusted p-value and the result was visualized by barplot, and the 20 key KEGG pathways were ranked based on target count and the result was displayed by a bubble chart.

In vitro validation experiments

Preparation of PAD aqueous extract. In order to prepare the aqueous extract of PAD [30g YXZ (*Phyllanthus urinaria L.*), 30g BZL, (*Scutellaria barbata D. Don.*), 10g SCG (*Cremastra appendiculata (D. Don) Makino*), 15g EZ (*Curcuma zedoaria (Christm.) Rosc*), 20g HH (*Carthamus tinctorius L.*), 30g HQ (*Astragalus membranaceus (Fisch.) Bunge*), 30g HJ (*Polygonatum sibiricum Red.*)], dried raw Chinese herbs of PAD purchased from Shenzhen Traditional Chinese Medicine Hospital were soaked in 1000 ml distilled water for 30min, and then were decocted for 30min. The extraction procedure was repeated two times. The extracts were pooled and the supernatant was collected by filtration. Furthermore, the mixtures were centrifuged at 5000 rpm for 10 min and then the supernatant was lyophilized in a vacuum freeze-drying machine (ALPHA2-4/LSC, Martin Christ, Germany). In this study, the dried powder of PAD was redissolved in DMEM (Gibco, USA) complete culture medium to 100 mg/ml, followed by centrifugation at 4000 rpm for 10 min, filtered with a 0.22 μm pore-size filter, and stored at -20°C for further use.

Cell cultures and MTT assay. HCC cell lines, HepG2 and Hep3B, were gifted by Prof. George G. Chen (The

Chinese University of Hong Kong, Hong Kong, China). These two cell lines were cultured in DMEM complete culture medium with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). Hep3B or HepG2 cell lines plated in 96-well plates (4000 cells/well) were treated with different concentrations (0, 0.5, 1, 1.5, 2, 2.5, and 3mg/ml) of PAD for 24, 48, and 72 hours, respectively. Moreover, 10μl of MTT (5mg/ml; Solarbio, China) was added to each well and incubated at 37°C for another 4 h. Then, the supernatants in each well were discarded and 100μl of dimethyl-sulfoxide (DMSO; Sigma, USA) was added to each well. A microplate reader (168-1130A, Bio-Rad, USA) was utilized to measure the absorbance at 490 nm.

Cell apoptosis assay (flow cytometry). The dosages (1, 1.5, and 2mg/ml) of PAD were selected for the following experiments *in vitro* based on the result of the MTT assay. In cell apoptosis assay, Hep3B or HepG2 cell lines seeded into 6-well plates at a density of 1×10^6 cells/well were treated with 3 concentration gradients (0, 1, 2, and 3mg/ml) of PAD for 48h. Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, China) was used to evaluate apoptosis of HepG2 and Hep3B cells according to the manufacturer's protocol. Briefly, HepG2 or Hep3B cells were collected by centrifugation (4°C, 1000rpm, 3min), washed with precooled PBS (Gibco, USA), and stained with Annexin V-FITC and propidium iodide, and quantified by flow cytometry (Beckman Coulter, USA).

Wound healing assay

Scratch wound healing assay was adapted to assess the migration ability of Hep3B and HepG2 cells. Cells were cultured overnight in a 6-well plate at a density of 5×10^5 cells/well. Subsequently, a 200μl sterile pipette tip was utilized to make linear scratch wounds for each well. The cells were then treated with various concentrations (0, 1, 2, and 3mg/ml) of PAD, and photomicrographs (100×) were taken using the inverted fluorescence microscope (DMi8, Leica, Germany) at times of 0h and 48h. The area of each scratch wound was calculated by open-source ImageJ 1.51 (NIH, USA).

Transwell assays

The migration capacities of Hep3B and HepG2 cells were also evaluated with a 24-well transwell plate (Corning, USA). In the transwell migration assay, cells were seeded in the upper chamber (5×10^4/250ul/well) and were treated with various concentrations (0, 1, 2, and 3mg/ml) of PAD, and the lower chamber was added with 750μl DMEM medium containing 20% FBS. Afterward, cells were incubated for 48h (37°C, 5% CO_2), and then cells were fixed with 75% ethanol for 30min and stained with 0.1% crystal violet (Aladdin, China) for 15min. The migrated cells were photographed in 3 randomly selected visual fields under Evos XL Core microscope (Life Technologies), and cell number was counted with ImageJ 1.51 (NIH, USA).

Western blot analysis

Western blot was performed according to the experimental protocols in the previous research.[8] Total protein in cells was extracted with the radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor (Zhonghuihecai, China). Protein lysates were then resolved in SDS-PAGE (Beyotime, China) gel and transferred to polyvinylidene fluoride (PVDF) membrane (Merck Millipore). The PVDF membranes were probed with primary antibodies: phosphoinositide 3-kinase (PI3K), phosphorylated-PI3K (p-PI3K), protein kinase B (Akt), and phosphorylated-Akt (p-Akt) (Cell Signaling Technology, USA), and β-actin (Santa Cruz, USA). After that, a 1:2000 dilution of the m-IgGκ BP-HRP and mouse anti-rabbit IgG-HRP (Santa Cruz, USA) were used as the secondary antibody. The results were recorded by the ChemiDoc touch (Bio-Rad, USA), and ImageJ was used for protein level quantification. Expression levels of the proteins were normalized to β-actin then the treatment group was normalized with the control group.

Table 1. The number of active components in each Chinese herb of PAD.

Herb name	TCMSP	TCMIP	BATMAN-TCM	SymMap	HERB	Total
YXZ	-	-	2	-	3	3
BZL	29	5	5	27	35	35
SCG	3	2	-	6	6	6
EZ	3	2	-	5	5	6
HH	22	2	7	27	27	27
HQ	20	5	9	24	29	29
HJ	12	1	-	12	12	13

Abbreviations: YXZ, *Phyllanthus urinaria L.*; BZL, *Scutellaria barbata D. Don.*; SCG, *Cremastra appendiculata (D. Don) Makino*; EZ, *Curcuma zedoaria (Christm.) Rosc*; HH, *Carthamus tinctorius L.*; HQ, *Astragalus membranaceus (Fisch.) Bunge*; HJ, *Polygonatum sibiricum Red.*

Statistical Analysis.

Experimental data in this study were depicted as mean ± standard deviation (SD), and statistical analyses were completed using SPSS version 22.0. When the total variance was the same, the one-way analysis of variance (ANOVA) test and Bonferroni analysis were applied, and Welch's ANOVA and Dunnett's T3 tests were performed when the variances were irregular. In this research, $P < .05$ was considered statistically significant for the difference, and statistical graphs were plotted by Graphpad Prism version 8 (GraphPad, USA).

RESULTS

Active components and targets of PAD

The numbers of active components of each Chinese herb in the five databases are displayed in Table 1. After removing duplicate components, a total of 96 active components were acquired, and 1403 corresponding targets were identified. In addition, we obtained 936, 578, 74, 115, and 188 HCC-related target genes from MalaCard, SymMap, TCMIP, HERB, and OMIM databases, respectively, and a total of 1130 potential therapeutic targets for HCC were found after excluding duplicate targets.

Network of PAD active components and common targets

A total of 218 common targets (overlapped genes) were identified via the Venn diagram, and results are presented in

Figure 1. Venn diagram of PAD targets and HCC targets.

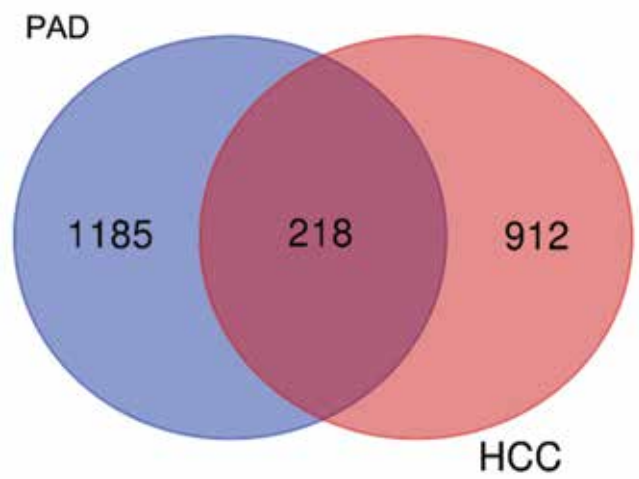


Figure 2. Network of PAD active components and common targets. The yellow, green, and purple nodes represent Chinese herbs for "benefiting qi and invigorating spleen", "clearing heat and removing toxicity", and "promoting blood circulation for removing blood stasis", respectively. The purple rectangle nodes stand for the common targets of PAD and HCC; In the yellow nodes, diamond and circle represent the active components of HQ (*Astragalus membranaceus (Fisch.) Bunge*)and HJ (*Polygonatum sibiricum Red.*); In the green nodes, triangle, and diamond represent the active components of SCG (*Cremastra appendiculata (D. Don) Makino*) and BZL (*Scutellaria barbata D. Don.*), respectively; In the red nodes, triangle and circle represent the active components of HH (*Carthamus tinctorius L.*) and EZ (*Curcuma zedoaria (Christm.) Rosc*), respectively. The blue hexagon represents the common components of multiple Chinese herbs (including *Phyllanthus urinaria L.*).

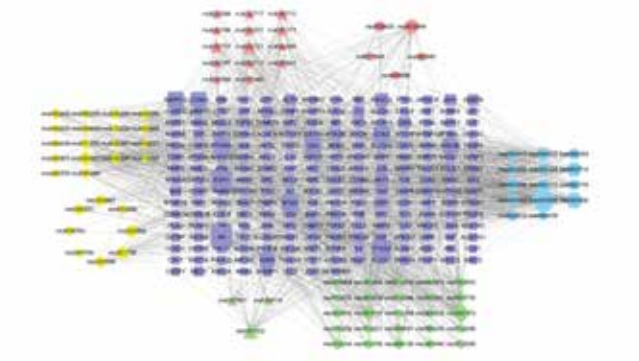


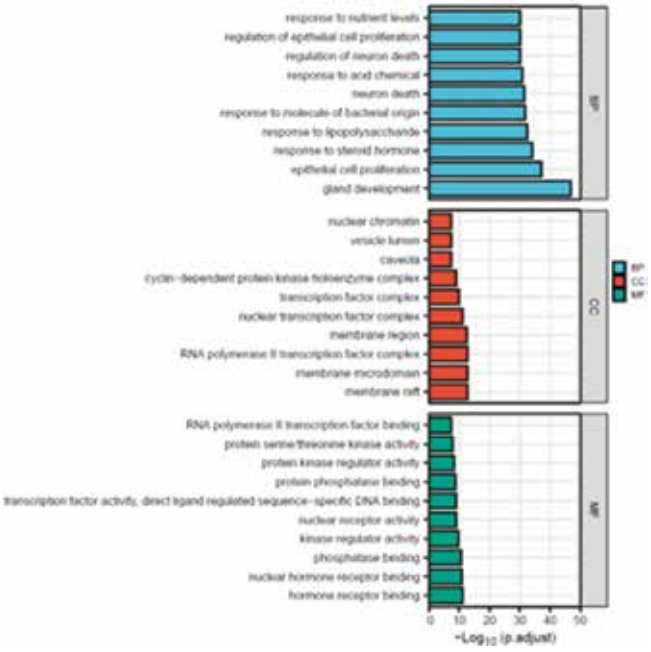
Figure 1. In network pharmacology, the 218 common targets were thought to be the targets for PAD to exert its anti-HCC effect. In order to further predict the action mechanism, a network of active components of PAD and common targets was constructed through Cytoscape (Figure 2). As illustrated in Figure 2, the network of PAD active components and common targets included 305 nodes (87 active components and 218 target genes) and 1478 edges, and the 7 Chinese herbs were divided into three types ("benefiting qi and invigorating spleen", "clearing heat and removing toxicity", and "promoting blood circulation for removing blood stasis"). Additionally, the active components connected with the most common targets (top 5 of each type) in the network were demonstrated in Table 2, and quercetin, luteolin, calycosin, pinocembrin, etc. might be the critical anti-HCC components of PAD.

Table 2. Data of active components of PAD (top 5 of each type)

Type	component	count	Herb
common components	Quercetin	92	YXZ, HQ, BZL, HH
	Luteolin	55	BZL, HH
	Baicalein	47	HJ,BZL,HH
	Kaempferol	41	YXZ, HQ, HH
	Beta-Sitosterol	36	HQ, HJ, BZL, SCG, HH
benefiting qi and invigorating spleen	Calycosin	43	HQ
	isorhamnetin	25	HQ
	(+)-Medicarpin	21	HQ
	Medicarpin	21	HQ
	DFV	21	HJ
clearing heat and removing toxicity	Wogonin	72	BZL
	24-Ethylcholest-4-En-3-One	45	BZL
	Ellagic Acid	39	SCG
	campesterol	26	BZL
	Eriodictyol	23	BZL
promoting blood circulation for removing blood stasis	Pinocembrin	36	EZ
	Quercetagetin	31	HH
	7,8-Dimethyl-1H-Pyrimido[5,6-G] Quinoxaline-2,4-Dione	25	HH
	Pyrethrin II	18	HH
	Poriferast-5-En-3Beta-Ol	18	HH

Abbreviations: Count, target gene cont; YXZ, *Phyllanthus urinaria L.*; BZL, *Scutellaria barbata D. Don.*; SCG, *Cremastra appendiculata (D. Don) Makino*; EZ, *Curcuma zedoaria (Christm.) Rosc*; HH, *Carthamus tinctorius L.*; HQ, *Astragalus membranaceus (Fisch.) Bunge*; HJ, *Polygonatum sibiricum Red.*

Figure 3. Top 10 most significant items in each category in GO analysis.

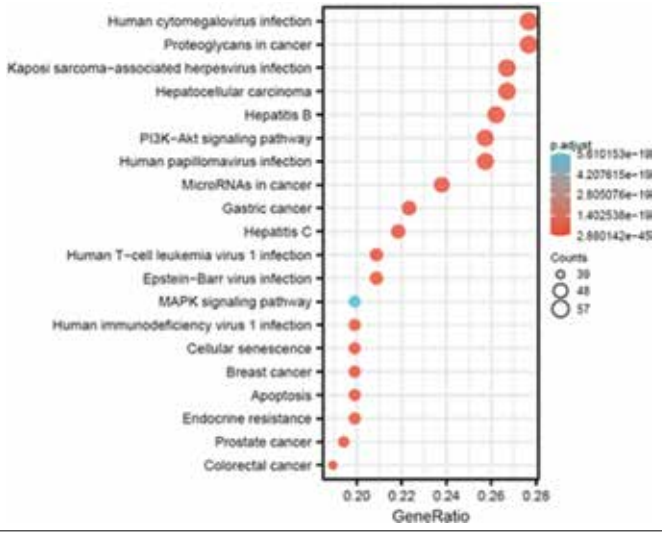


Abbreviations: BP: biological process, CC: cellular component, MF: molecular function.

The results of KEGG and GO enrichment analyses

The results of GO enrichment analysis showed that the common targets of PAD and HCC were mostly enriched in BP items such as epithelial cell proliferation, response to steroid hormone, regulation of epithelial cell proliferation, response to nutrient levels, etc.; CC items such as membrane

Figure 4. Top 20 KEGG pathways of common targets.



raft, membrane microdomain, RNA polymerase II transcription factor complex, membrane region, etc.; MF items such as hormone receptor binding, nuclear hormone receptor binding, phosphatase binding, kinase regulator activity, etc (Figure 3). Furthermore, KEGG analysis demonstrated that common targets were correlated with proteoglycans in cancer, hepatocellular carcinoma, hepatitis B, PI3K/Akt, mitogen-activated protein kinase (MAPK), apoptosis pathway, etc., and the result of the first 20 signaling pathways based on gene count was displayed in a bubble chart (Figure 4).

The effect of PAD on proliferation and apoptosis in Hep3B and HepG2 cells

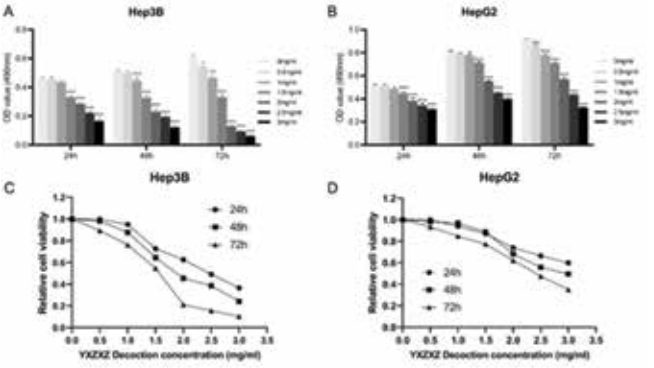
The effect of PAD on the proliferation and growth of HepG2 and Hep3B cells was detected by MTT assay (24h, 48h, and 72h). The MTT result showed that different concentrations of PAD had an inhibitory effect on Hep3B and HepG2 cells, and in a certain concentration range, the cell viability of Hep3B and HepG2 cells decreased gradually with the increase of drug concentration and exposure time (Figure 5).

Based on the experimental results of the MTT assay, PAD with the concentration of 0mg/ml (control group), 1mg/ml, 2mg/ml, and 3mg/ml and the 48h time point were selected for further experiments. As demonstrated in Figure 6, the total apoptotic rate of Hep3B and HepG2 cells increased at 48h with the increase in the concentration of PAD, and the difference was statistically significant (1.5mg/ml and 2mg/ml).

The effect of PAD on the migration of Hep3B and HepG2 cells

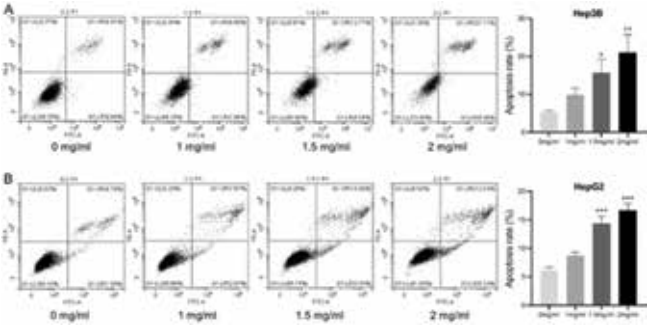
Firstly, the migration activity of Hep3B and HepG2 cells was measured by the scratch wound healing assay. The result showed that wound healing areas in HepG2 and Hep3B cells increased following treatment with PAD, and the wound healing rate decreased with the increase of PAD concentration in a dose-dependent manner (Figure 7). Then, cell migration

Figure 5. Effect of PAD on the proliferation of HCC cells assessed by the MTT assay (A-D). Treatment with PAD resulted in decreased cell OD value (A)/viability (C) of Hep3B (n=4); Treatment with PAD resulted in decreased cell OD value (B)/viability (D) of HepG2 (n=4);



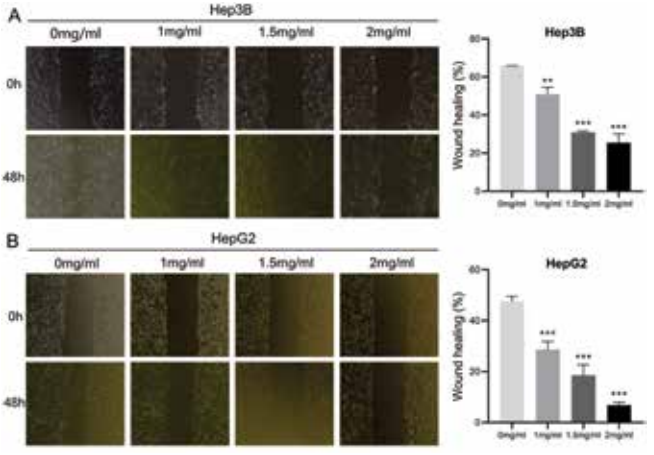
$^{**}P < .01$, $^{***}P < .001$ *vs* 0mg/ml (control)

Figure 6. Effect of PAD on the apoptosis in HepG2 and Hep3B cells. The total apoptosis rate (early apoptosis percentage + late apoptosis percentage) of Hep3B (A) and HepG2 (B) tended to increase in the 1.5mg/ml and 2mg/ml groups (n=3). In bar graphs, values are presented as mean ± SD



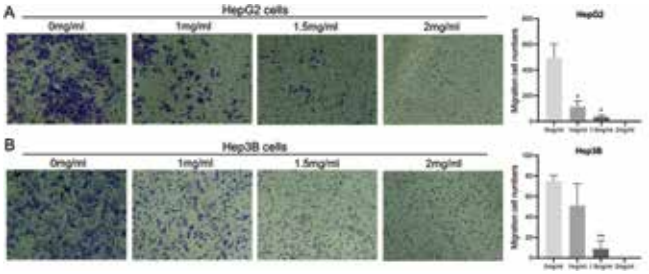
$^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ *vs* 0mg/ml (control).

Figure 7. PAD could inhibit the migration of HepG2 and Hep3B cells (48h). The treatment with PAD (1, 1.5, 2mg/ml) reduced the wound healing rate compared with the control (0mg/ml), suggesting that it could suppress the migration ability of Hep3B (A) and HepG2 (B) cells. In bar graphs, values are presented as mean ± SD



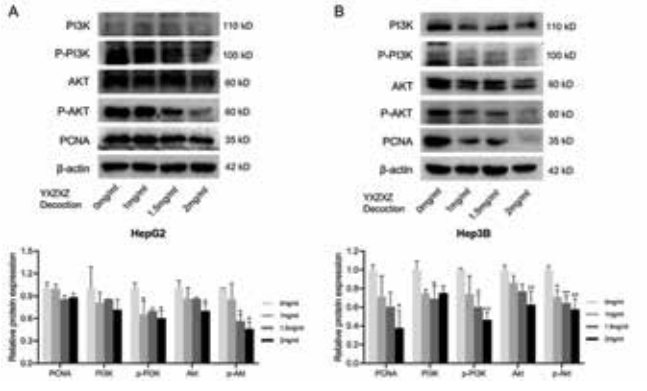
$^{**}P < .01$, $^{***}P < .001$ *vs* 0mg/ml (control).

Figure 8. PAD inhibited cell migration of HepG2 and Hep3B cells, which were determined by transwell assay (48h). (A) PAD could remarkably suppress cell migration in HepG2 and Hep3B (B) in a concentration-dependent manner. In bar graphs, values are presented as mean ± SD



*$P < .05$, **$P < .01$ *vs* 0mg/ml (control).

Figure 9. Effect of PAD on PI3K, p-PI3K, AKT, p-Akt protein expression levels in HepG2 and Hep3B (48h). (A) PAD treatment notably inhibited p-PI3K, Akt, and p-Akt protein expression of HepG2 compared with the control (0mg/ml), and no significant differences in PI3K and PCNA were observed. Values presented as mean ± SD; *$P < .05$, **$P < .01$ vs 0mg/ml (control). (B) PAD treatment significantly inhibited PCNA, PI3K, p-PI3K, Akt, and p-Akt protein expression levels of Hep3B compared with the control (0mg/ml)



was also determined by transwell migration assay, and PAD with the concentration of 1.5mg/ml and 2mg/ml could inhibit the migration of Hep3B cells, while PAD with the concentration of 1mg/ml, 1.5 mg/ml, and 2mg/ml can inhibit the migration of HepG2 cells (Figure 8).

Effect of PAD on the expression of PI3K/Akt pathway-related proteins

Based on the result of network pharmacology, the changes in protein expression of total PI3K, total Akt, p-PI3K, and p-Akt were detected by western blot method to clarify the regulatory effect of PAD on PI3K/Akt pathway. As shown in Figure 9, protein expression levels of proliferating cell nuclear antigen (PCNA), PI3K, p-PI3K, Akt, and p-Akt were downregulated in Hep3B after treatment with PAD compared with the control group. In particular, PAD has inhibitory effects on PCNA, PI3K, Akt, and p-Akt at a high concentration. Furthermore, the protein expression levels of Akt, p-PI3K, and p-Akt also decreased in HepG2 after treatment with the high concentration of PAD compared with the control group. Thus, PAD could inactivate the phosphorylation of both p-PI3K, Akt, and p-Akt in HCC cells.

DISCUSSION

Based on several years of clinical and scientific research experience, our team selected Phyllanthus Urinaria L which exists widely in Asian countries as the core Chinese herb for intervention in HCC,[20] and developed compound Phyllanthus urinaria L for patients with HCC. It is traditionally believed that Phyllanthus Urinaria L can reduce heat, protect the liver, and detoxify body from poison, and it has long been used in TCM for hepatitis B, jaundice, dropsy, and so on. Our preliminary clinical studies have shown that the compound Phyllanthus Urinaria L, a TCM prescription for the treatment of HCC, is useful in preventing or delaying the development of hepatitis B virus-related cirrhosis to HCC.[7] According to the theories of TCM, it is believed that "deficiency, toxin, stasis" are the core pathogenesis in the process of hepatitis B virus-associated HCC, and the corresponding TCM treatment methods are suggested to be "Yiqi Jianpi Jiedu Huayu" (replenishing qi, strengthening spleen, and removing toxicity and blood stasis). Based on TCM theories, the previous basis and clinical experience, we optimized the compound Phyllanthus Urinaria L and set up PAD. PAD is composed of 7 kinds of Chinese herbs such as Phyllanthus urinaria L. According to the theory of TCM, PAD has the effect of "yiqi jianpi jiedu huayu". In this study, a variety of valuable active components and corresponding targets in PAD were found through the study of network pharmacology, which has therapeutic potential for the treatment of HCC. The network of PAD active components and common targets summarized several critical components (e.g., quercetin, luteolin, calycosin, wogonin, and pinocembrin) that may serve a potential therapeutic role. Quercetin, one of the flavonols, could suppress the growth factor-induced migration of HCC cells by inhibiting the AKT pathway.[21] Luteolin synergizes the antitumor effects of 5-fluorouracil against HCC cells (HepG2) through apoptosis induction. Recent research indicated that baicalein could decrease signal transducer and activator of transcription 3 (STAT3) activity, further downregulate programmed death-ligand 1 (PD-L1) expression, and then restore T cell sensitivity to kill HCC cells.[22] Calycosin resisted HCC by activating MAPK, STAT3, and nuclear factor kappa-B (NF-κB) pathways.[23] An experiment study demonstrated that wogonin and 5-fluorouracil could increase the sensitivity of chemotherapy drugs by regulating the PI3K/Akt pathway.[24] Also, pinocembrin was reported to inhibit the migration and invasion abilities of breast cancer cells via suppression of the PI3K/AKT pathway.[25] Our previous study has proved that quercetin, luteolin, and kaempferol are present in Phyllanthus urinaria L., the monarch drug in PAD, by using HPLC-Q-TOF-MS/MS.[26] In addition, the anti-HCC effects of several components (such as uercetagetin, 24-Ethylcholest-4-En-3-One, etc.) have not been reported, which sets implications for future research.

GO enrichment analysis displayed that common targets of PAD and HCC were significantly involved in the CC and BP items related to the regulation of epithelial cell proliferation, phosphatase binding, and kinase regulator activity, which were involved in the process of tumorigenesis, metastasis, and invasion. KEGG analysis exhibited that PAD might exert its anti-HCC effects through the pathway associated with inflammation and malignancies. According to previous research, the mechanisms of liver cancer caused by hepatitis B virus and hepatitis C virus include persistent liver inflammation with impaired antiviral immune response, immune and viral protein-mediated oxidative stress, and loss of control of cellular signaling pathways by viral proteins.[27] Increasing evidence has shown the importance of the PI3K/Akt pathway in HCC progression, and the PI3K/Akt pathway was overexpressed in nearly 50% of HCCs and the abnormal activation of this pathway could affect cell proliferation, metabolism, tumor cell differentiation, autophagy, and epithelial mesenchymal transition (EMT).[28] Furthermore, miRNAs signatures associated with increased risk of HCC, tumor development, advanced stages, and vascular invasion, were identified in human HCC, and some of them have been confirmed in animal models and tested as therapeutic targets.[29] The aberrant activation of MAPK pathway is correlated with cellular transformation and tumorigenesis, and this pathway also upregulates the expression of EMT-related genes, contributing to the induction and maintenance of the mesenchymal state in cancer cells.[30] It is speculated that PAD could regulate inflammatory and immune response-, apoptosis-, migration- and invasion-related pathways, and other related pathways to inhibit HCC.

To verify this result of network pharmacology, experimentation was carried out. The MTT assay demonstrated that PAD could inhibit the proliferation of HepG2 and Hep3B cells in a dose- and time-dependent manner, and PAD has a stronger inhibitory effect on Hep3B cells. Compared with the control group, PAD could remarkably enhance the apoptosis of both Hep3B and HepG2 cells, and the apoptotic rate increased with increasing drug concentrations. Moreover, the scratch wound healing and transwell assay suggested that PAD could suppress the migration of HCC cells. Hence, our experimental results showed that PAD, the representative prescription of "yiqi jianpi jiedu huayu" could inhibit the proliferation and migration as well as promote apoptosis in HCC cells. Preceding literature has also revealed that "strengthening spleen" (jianpi in Chinese), "removing toxicity" (jiedu in Chinese), or "removing toxicity and blood stasis" (jiedu huayu in Chinese) prescriptions could inhibit the proliferation, migration, and invasion of HCC cells, and regulate the signal transduction pathway.[31-33] To further explore the possible mechanism of PAD in the treatment of HCC, PI3K/Akt pathways predicted in network pharmacology were validated. The results of western blot displayed that a higher concentration of PAD could remarkably reduce the protein expression levels of p-PI3K and p-Akt in HCC cells, indicating that PAD may influence the proliferation, apoptosis, and migration of HCC cells by inhibiting the activation of PI3K/Akt pathway.

In this study, the associated mechanism of PAD in the treatment of HCC was preliminarily revealed by an integrative strategy combining network pharmacology analysis and experimental validation. However, there are several limitations of the present study should be mentioned. Firstly, due to the massive information of each database being constantly updated, the results of network pharmacology may not be perfectly reflective of all the key target genes of PAD in treating HCC, which should be further studied in the future. Moreover, our study lacks the verification of other pathways or targets. Besides, the present study lacks *in vivo* experiments, and further *in vivo* studies are required. In the future, screening for active components with clinical application value and in vivo/in vitro evaluation of these components could be interesting for drug discovery.

CONCLUSIONS

Overall, PAD, a representative prescription for "yiqi jianpi jiedu huayu", has the characteristics of multi-component, multi-target, and multi-pathway for the treatment of HCC. Besides, PAD may inhibit the proliferation and migration as well as promote the apoptosis of HCC cells by suppressing PI3K/Akt signaling pathway.

AUTHORS' DISCLOSURE STATEMENT

The authors declare there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

Chunshan Wei and Zhulin Wu contributed equally to this paper and are regarded as co-first authors.

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