



## OPEN ACCESS

## EDITED BY

Alaaeldin Ahmed Hamza,  
National Organization for Drug Control  
and Research (NODCAR), Egypt

## REVIEWED BY

Jin Fang Zhu,  
Xinjiang Agricultural University, China  
Qiong Zhang,  
Albert Einstein College of Medicine,  
United States

## \*CORRESPONDENCE

Chong Jin,  
✉ jinc@tzzxy.com  
Junmin Li,  
✉ lijim@tzc.edu.cn

## SPECIALTY SECTION

This article was submitted to Experimental  
Pharmacology and Drug Discovery,  
a section of the journal  
Frontiers in Pharmacology

RECEIVED 21 September 2022

ACCEPTED 25 January 2023

PUBLISHED 03 February 2023

## CITATION

Mo J, Tong Y, Ma J, Wang K, Feng Y,  
Wang L, Jiang H, Jin C and Li J (2023), The  
mechanism of flavonoids from *Cyclocarya  
paliurus* on inhibiting liver cancer based on  
*in vitro* experiments and  
network pharmacology.  
*Front. Pharmacol.* 14:1049953.  
doi: 10.3389/fphar.2023.1049953

## COPYRIGHT

© 2023 Mo, Tong, Ma, Wang, Feng, Wang,  
Jiang, Jin and Li. This is an open-access  
article distributed under the terms of the  
[Creative Commons Attribution License  
\(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or  
reproduction in other forums is permitted,  
provided the original author(s) and the  
copyright owner(s) are credited and that  
the original publication in this journal is  
cited, in accordance with accepted  
academic practice. No use, distribution or  
reproduction is permitted which does not  
comply with these terms.

# The mechanism of flavonoids from *Cyclocarya paliurus* on inhibiting liver cancer based on *in vitro* experiments and network pharmacology

Jinggang Mo<sup>1</sup>, Yingpeng Tong<sup>2</sup>, Junxia Ma<sup>3</sup>, Kunpeng Wang<sup>1</sup>,  
Yifu Feng<sup>1</sup>, Liezhi Wang<sup>1</sup>, Hao Jiang<sup>1</sup>, Chong Jin<sup>1\*</sup> and Junmin Li<sup>2,3\*</sup>

<sup>1</sup>Department of General Surgery, Taizhou Central Hospital (Taizhou University Hospital), Taizhou University, Taizhou, Zhejiang, China, <sup>2</sup>School of Advanced Study, Taizhou University, Taizhou, China, <sup>3</sup>Zhejiang Provincial Key Laboratory of Evolutionary Ecology and Conservation, Taizhou University, Taizhou, China

**Introduction:** *Cyclocarya paliurus* (Batal.) Iljinsk., a subtropical tree belonging to the family Juglandaceae, is rich in polysaccharides, flavonoids, and terpenoids. It has important pharmacological effects such as lowering blood lipids, blood sugar, and blood pressure. However, little has been discerned regarding anti tumor effects and their potential mechanisms.

**Method:** *In vitro* cell culture experiments were used to test the effect of *C. paliurus* total flavonoids (CTFs) extract on apoptosis mechanisms in HepG2 cells. Network pharmacology was applied to further explore the effects of CTFs on liver cancer as well as the mechanisms through which these effects might be achieved. Both 3-hydroxyflavone and luteolin were randomly selected to verify the effect on inducing apoptosis and inhibiting the proliferation of HepG2 cells.

**Results and Discussion:** Network pharmacological analysis was applied to these 62 compounds and their targets, and 13 flavonoids were further screened for their potential anti liver cancer activity. These 13 flavonoids included: tangeretin, baicalein, 7,3'-dihydroxyflavone, velutin, 3-hydroxyflavone, chrysin, kumatakenin, tricetin, luteolin, chrysoeriol, apigenin, pinocembrin, and butin. Together, these flavonoids were predicted to interact with AKT1, MAPK3, PIK3CA, EGFR, MAP2K1, SRC, IGF1R, IKBKB, MET, and MAPK14. It was predicted that the inhibitory effect on hepatocellular carcinoma would be accomplished by regulation of core proteins relating to such KEGG pathways as cancer, PI3K-Akt, proteoglycans in cancer, microRNAs in cancer, and endocrine resistance via core target proteins. Both 3-hydroxyflavone and luteolin were demonstrated to induce apoptosis and inhibit the proliferation of HepG2 cells. Our study provides scientific evidence supporting the use of CTFs for the treatment of liver cancer.

## KEYWORDS

*Cyclocarya paliurus*, flavonoids, active ingredients, *in vitro* experiments, network pharmacology, liver cancer, apoptosis

## 1 Introduction

Liver cancer represents a major public health concern, with the fifth highest incidence rate and the second highest fatality rate globally across all cancer types (Sun et al., 2017; Zhou et al., 2018; Liu et al., 2020). Traditional Chinese medicine (TCM) has been used to treat disease in China for over two thousand years (Tang et al., 2008), and clinical studies have provided evidence that TCM can provide positive therapeutic effects in the prevention and treatment of liver cancer (Chen et al., 2016; Tang et al., 2020). For example, the extract of *Brucea javanica* seeds was shown to eliminate sphere formation in cancer stem cells and inhibit tumor-initiating behavior in hepatocellular carcinoma cells (Ren et al., 2011; Chen et al., 2016). Identifying effective components from TCM to prevent and treat liver cancer is becoming an urgent issue (Liu et al., 2020).

Network pharmacology, a new branch of pharmacology based on systems and network biology, focuses on revealing the complex network of relationships between drug, target, and disease, systematically observing the intervention and influence of drugs on the disease network and providing insight into the molecular mechanism of drug therapies (Zhao and Iyengar, 2012; Kibble et al., 2015). Recently, network pharmacology has generated a great deal of interest for its potential in elucidating the mechanistic details behind myriad TCM treatments for complex diseases such as cancer, cardiovascular disorders, and diabetes (Duan et al., 2019; Wufuer et al., 2022). A recent study by Sun W. et al. (2021) predicated potential signaling pathways and active ingredients of the TCM root tuber *Drynariae Rhizoma* via network pharmacology, and found that flavonoids in *Rhizoma Drynariae* improved the outcomes of large bone defects in mice by activating the mitogen-activated protein kinase (MAPK) signaling pathway. Another study by Liu et al. (2020) used network pharmacology to explore the anti-tumor effect of total flavonoids extracted from the TCM herbal formulation Pien-Tze-Huang, on liver cancer, concluding that the mechanism underlying their effect on liver cancer involved a synergy of effects from multiple components on multiple targets and multiple pathways.

Flavonoids, a group of metabolites characterized by a diphenylpropane structure (C6-C3-C6), are widely distributed in plants, including fruits and vegetables (López-Lázaro, 2009). Flavonoids extracted from plants used in TCM have been shown to exert obvious *in vitro* and *in vivo* anti-tumor activities in such contexts as acute myeloid leukemia (Hirano et al., 1994), lung cancer (Yang et al., 2000), melanoma (Caltagirone et al., 2000), and others. A few studies showed that flavonoid monomers and their derivate flavonoids also have significant inhibitory effects on liver cancers (Eaton et al., 1996; Chen et al., 2009; Quan et al., 2013; Kasala et al., 2015). For example, the derivatives of chrysin extracted from *Passiflora caerulea* can inhibit the migration, invasion, proliferation, self-renewal, and stemness of liver cancer stem cells (Tang et al., 2020). Recently, several studies have even applied network pharmacology to predict the potential inhibitory effects of plant flavonoids on liver cancer (Liu et al., 2020; Sun W. et al., 2021).

*Cyclocarya paliurus* (Batal.) Iljinsk. is endemic to China. The leaves of *C. paliurus* have long been used in traditional Chinese medicine for their anti-diabetic, anti-bacterial, and anti-cancer properties (Xie et al., 2016; Wu et al., 2017). The polysaccharide from *C. paliurus* has been shown in modern studies to exert antiproliferative effects on various tumor cell lines (Luo et al., 2015; Shinbo et al., 2015; He et al., 2018). Flavonoids are the most

common compounds in the leaves of *C. paliurus* (Liu Y. et al., 2016), with a recent study identifying a total of 188 different flavonoids in the leaves of *C. paliurus* via LC-MS-MS (Sheng et al., 2021). *C. paliurus* total flavonoids (CTFs) have been shown to significantly lower blood sugar, blood fat, and blood pressure, as well as to improve immunity (Liu et al., 2018; Sun C. et al., 2021). Moreover, CTFs can effectively reduce oxidative stress and protect mice from acute liver injury (Xie et al., 2018). However, little has been known about the pharmacological effect of CTFs on liver cancer. In this study, we tested the effect of CTFs on apoptosis mechanisms in HepG2 cells. We then applied network pharmacology to further explore the effects of CTFs on liver cancer as well as the mechanisms through which these effects might be achieved.

## 2 Materials and methods

### 2.1 Plant samples

Leaves of *C. paliurus* were collected from Zhuzhang Village, Longquan City, Lishui City, Zhejiang Province, China (E118°48'28", N28°5'57"). A portion of leaves were frozen in liquid nitrogen immediately after harvest and transferred to a -80°C freezer until metabolomic analysis. The remaining leaves were dried at 70°C to a constant weight and used for the extraction of total flavonoids.

### 2.2 Preparation of CTFs and determination of the total flavonoids content

Dried leaves were ground and sieved through a 40-mesh sieve. Powders were ultrasonic soaked in 70% ethanol (1:5, w/v) for 30 min and filtered with filter paper. This process was repeated a total of three times, and supernatants were combined and concentrated in a vacuum rotary evaporator at 70°C. About 400 g extract was suspended in 1 L H<sub>2</sub>O and the solution was extracted with 250 mL ethyl acetate for six times. The ethyl acetate extracts were further subjected to polyamide macroporous adsorption resin and the CTFs were collected for downstream *in vitro* experiments. The total flavonoids content were determined by AlCl<sub>3</sub> colorimetry according to the method described by Sheng et al. (2021). Rutin was used as a standard and the determinations were carried out for three replicates.

### 2.3 *In vitro* experiments of CTFs

#### 2.3.1 Cell culture

HepG2 cells were purchased from the National Collection of Authenticated Cell Cultures, Shanghai, China and STR DNA typing (Dirks and Drexler, 2011) was used to identify this cell line (results were provided in (results were provided in Supplementary Material S1)). High-glucose DMEM medium supplemented with 10% fetal bovine serum (FBS) (Universal Biotech Co., Ltd., Shanghai, China) and 1% penicillin/streptomycin (Sangon Biotech Co., Ltd., Shanghai, China) was used to culture the cells in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C in a cell culture incubator (Mettler GmbH + Co. KG, Schwabach, Germany). Media was replaced at 2-day intervals.

### 2.3.2 CTFs inhibit the proliferation of HEPG2 cell

Cellular proliferation was assessed using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Universal Biotech Co., Ltd., Shanghai, China). HepG2 cells were cultured in 96-well plates for 24 h and treated with 0 mg/L, 1.51 mg/L, 3.02 mg/L, 7.55 mg/L, 15.1 mg/L, 30.2 mg/L, 45.3 mg/L, and 90.6 mg/L of CTFs (resolved in DMSO) and incubated for another 48 h. All treatment concentrations, including the control 0 mg/L, contained equal amounts of DMSO. Media was then replaced with fresh media that had been mixed with 10% (V/V) MTT (5 mg/mL). Wells were incubated with formazan solvent for an hour at 37°C before absorbance was detected at OD570 and OD690 using a Multimode Microplate Reader (TECAN Spark, Männedorf, Switzerland). Relative Cell Activity and the Inhibition Rate of CTFs were calculated as follows.  $\text{Relative Cell Activity} = (\text{OD570}_s - \text{OD690}_s) / (\text{OD570}_0 - \text{OD690}_0) \times 100\%$ , where  $\text{OD570}_s$  and  $\text{OD690}_s$  were absorbance of the tested sample at 570 nm and 690 nm, respectively, and  $\text{OD570}_0$  and  $\text{OD690}_0$  were absorbance of the negative control at 570 nm and 690 nm, respectively.  $\text{Inhibition Rate} = (1 - \text{Relative Cell Activity}) \times 100\%$ .

### 2.3.3 CTFs promote apoptosis in HEPG2 cells

Cellular apoptosis was assessed using the Apoptotic and Necrotic Detection Kit Triple Fluorescence Color (Sangon Biotech Co., Ltd., Shanghai, China). As for flow cytometric analysis (BD FACS Aria II, New Jersey, America), HepG2 cells were cultured in 12-well plates for 48 h and then incubated with 0 mg/L, 7.55 mg/L, 15.1 mg/L, and 30.2 mg/L of CTFs (resolved in DMSO) for 24 h. Next, cells were collected, centrifuged at 300 × g for 5 min, and washed twice with PBS at room temperature. Cells were resuspended with 400 μL 1 × assay buffer at the concentration of 1 × 10<sup>6</sup> cells/mL, and then incubated with Apoptin Green (4 μL), 7-AAD (2 μL), and CytoCalcein Violet 450 (2 μL) at room temperature for 20 min in the dark, according to the manufacture's instructions. Events in the upper left corner of the figures, marked Q1, represent dead cells and naked nuclei. Events in the upper right corner, marked Q2, represent late-stage apoptotic and necrotic cells. Events in the lower left corner, marked Q3, represent normal cells. Events in the lower right corner, marked Q4, represent early-stage apoptotic cells. Apoptotic rate was calculated by summing the percentages of Q2 + Q4. As for Confocal Laser Scanning Microscope (CLSM) analysis (IX83-FV3000, Olympus Corporation, Tokyo, Japan), HepG2 cells were cultured in 4-well glass bottom plates specially designed for CLSM applications (Xinyou Technology Co., Ltd., Hangzhou, China). Treatment with CTFs and cell staining with the Apoptotic and Necrotic Detection Kit, which was performed as described above.

## 2.4 Network pharmacology analysis of CTFs

### 2.4.1 Compound database construction

The flavonoids used for network pharmacology analysis in this study were collected from our previous work (Sheng et al., 2021). Briefly, the fresh leaves of *C. paliurus* were freeze-dried and extracted for secondary metabolite analysis by HPLC/MS/MS and its accompanying self-built database by Metware Biotechnology Co., Ltd. (Wuhan, China). Among 188 flavonoids in the leaves of *C. paliurus*, a total of 114 CTFs were verified to have clear chemical structures which were obtained from Pubchem databases (<https://pubchem.ncbi.nlm.nih.gov/>). If chemical structures could not be

found there, original research articles were reviewed and the structures were drawn by ChemDraw 16.0 software (<https://www.chemdraw.com.cn/xiazai.html>).

### 2.4.2 Protein target database construction

CTF structures were saved in SMILES format and searched in SwissADME database (<http://www.swissadme.ch/>) to screen compounds used in the present work based on Lipinski's Rule of Five. Targets of these filtered compounds were then predicted using SwissTargetPrediction (<http://www.swisstargetprediction.ch/>). Duplicates were deleted after combining the targets of each component, and potential targets were obtained through network topology analysis.

### 2.4.3 Anti-liver cancer target database construction

Relevant targets for the inhibition of liver cancer proliferation were identified by searching the MalaCards database (<https://www.malacards.org/>) with the following keywords: "Hepatocellular Carcinoma." The results were summarized and sorted according to the gene names. Common targets between the disease database and the compound database were indicated as potential targets of CTFs responsible for their therapeutic effects in the context of liver cancer.

### 2.4.4 GO and KEGG pathway enrichment analysis

Potential targets were imported into the DAVID 6.8 database (<https://david.ncifcrf.gov/>) to perform GO and KEGG pathway enrichment analysis. "OFFICE\_GENE\_SYMBOL" and "*Homo sapiens*" were selected. The top 10 enriched GO entries and top 30 enriched KEGG pathways that met the criteria of  $p < 0.01$  were selected and uploaded to the OmicShare (<http://www.omicshare.com/tools/>) cloud platform for data visualization, where the size of the bubble represents the number of genes in the pathway, and the color of the bubble represents the significance of enrichment.

### 2.4.5 Construction of core component-target-pathway network

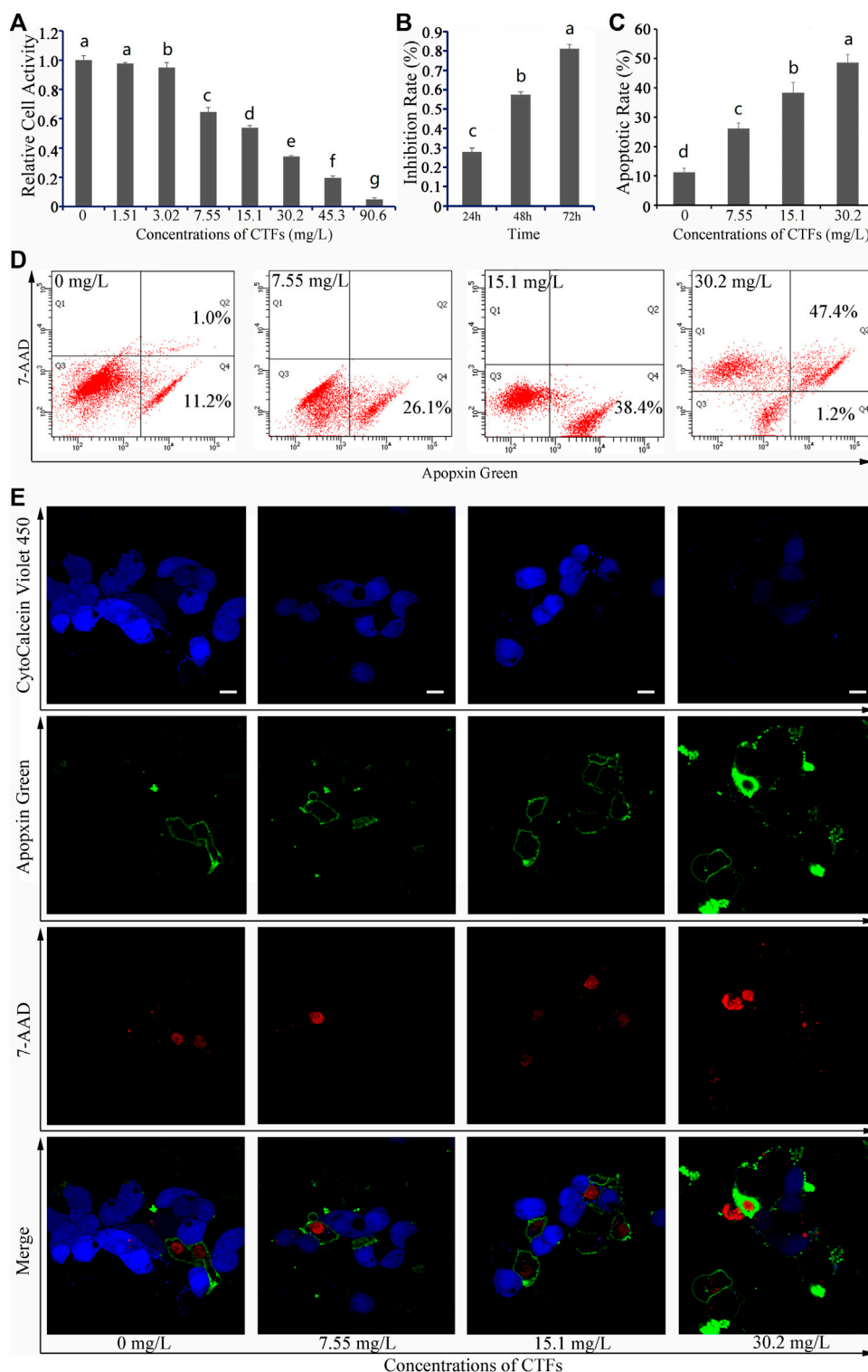
The integrated network of component-target-pathway was constructed using Cytoscape 3.9.0 to show the relationships among the active ingredient compounds, the target proteins, and the pathways. The topology characteristics of the network were evaluated.

## 2.5 Molecular docking of active ingredient-target

To further verify the reliability of the target prediction results, the top 10 scoring targets in the "component-target-pathway" network were selected for molecular docking analyses using Auto dock vina to confirm their expected binding capabilities with their cognate CTF ligands (Trott and Olson, 2010).

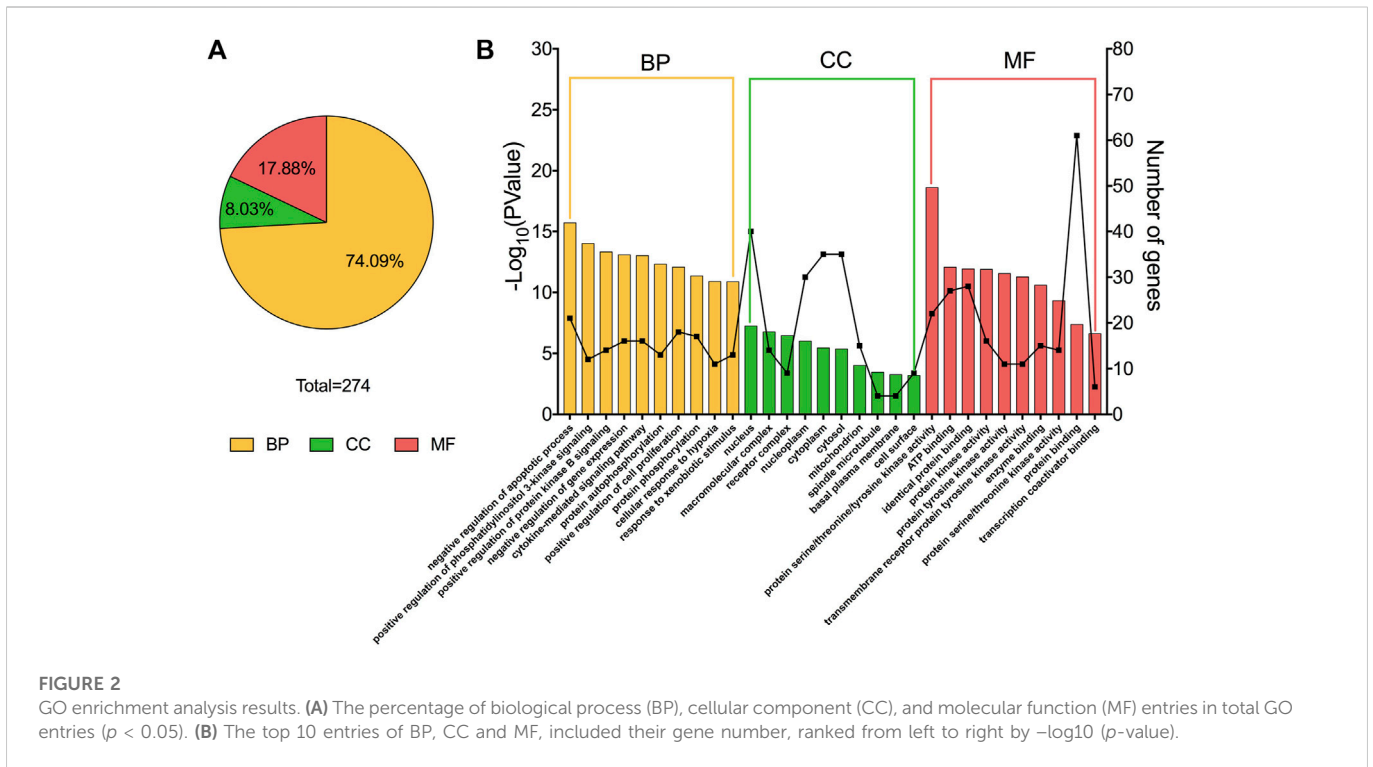
## 2.6 *In vitro* verification of predicted compounds

3-hydroxyflavone and luteolin were purchased from DESITE Biotechnology Co., Ltd., Chengdu City, Sichuan Province, China. 3-hydroxyflavone and luteolin were randomly selected and *in vitro* experiments to explore the specific effects were performed according to the methods described above for whole CTFs.



**FIGURE 1**

Effects of *Cyclocarya paliurus* total flavonoids (CTFs) on HepG2 cells. (A) Relative cell activity, (B) Inhibition rate, and (C) Apoptotic rate. Data are expressed as the mean  $\pm$  SEM ( $n = 3$ ). Different small letters indicate extremely significant differences between two treatments ( $p < 0.01$ ). (D) Representative FACS plots of cells treated with different concentrations of CTFs and stained with Apopxin Green and 7-AAD to identify apoptotic cells in the early and late stages, respectively. From left to right, the concentration of CTFs was 0  $\mu\text{g/mL}$ , 7.55  $\mu\text{g/mL}$ , 15.1  $\mu\text{g/mL}$ , and 30.2  $\mu\text{g/mL}$ . Data are representative of four independent experiments. (E) Representative CLSM images of HepG2 cells treated with different concentrations of CTFs and stained with CytoCalcein Violet 450 (in blue color, representing live cells), Apopxin Green (in green color), 7-AAD (in red color). From left to right, the concentration of CTFs was 0  $\mu\text{g/mL}$ , 7.55  $\mu\text{g/mL}$ , 15.1  $\mu\text{g/mL}$ , and 30.2  $\mu\text{g/mL}$ . Data are representative of four independent experiments.



## 2.7 Statistical analysis

Statistical analysis was carried out using SPSS 19.0. One-way analysis of variance (ANOVA) was applied to measure the significance of comparisons between groups after homogeneity of variance was confirmed. Tukey's *post hoc* HSD test was used in case of significance of the ANOVA test. Differences were considered statistically significant when the *p*-value was less than 0.05. All data are shown as mean  $\pm$  standard deviation.

## 3 Results

### 3.1 Effects of CTFs on HepG2 cells

The total flavonoids in CTFs was  $81.82\% \pm 0.36\%$ . The results of the MTT experiment showed that the cell activity of HepG2 cells decreased as CTFs concentration increased. When the concentration of CTFs reached 90.6 mg/L, the cell activity of HepG2 cells was only 4.94% relative to the DMSO-only control (Figure 1A). The IC<sub>50</sub> value of the inhibitory effect of CTFs on HepG2 cells was 10.79 mg/L, indicating an effective treatment concentration ranged from 0 to 30.2 mg/L in this context.

After 24, 48, and 72 h of treatment with CTFs, the inhibition rate of 15.1 mg/L CTFs on HepG2 cells was 28.03%, 57.46%, and 93.46%, respectively (Figure 1B), indicating that treatment with 48 h would give a moderate effect. Therefore 48 h was selected for further study.

After 48 h of treatment with 0 mg/L, 7.55 mg/L, 15.1 mg/L, and 30.2 mg/L of CTFs, the apoptosis rate of HepG2 cells was  $11.2\% \pm 1.4\%$ ,  $26.1\% \pm 1.9\%$ ,  $38.4\% \pm 3.5\%$ , and  $76.6\% \pm 2.8\%$ , respectively, indicating that CTFs could promote the apoptosis of HepG2 cells in a dose-dependent manner (Figures 1C, D). Based on the CLSM analysis, most of the cells in the 0  $\mu\text{M}$  group were live cells stained with blue,

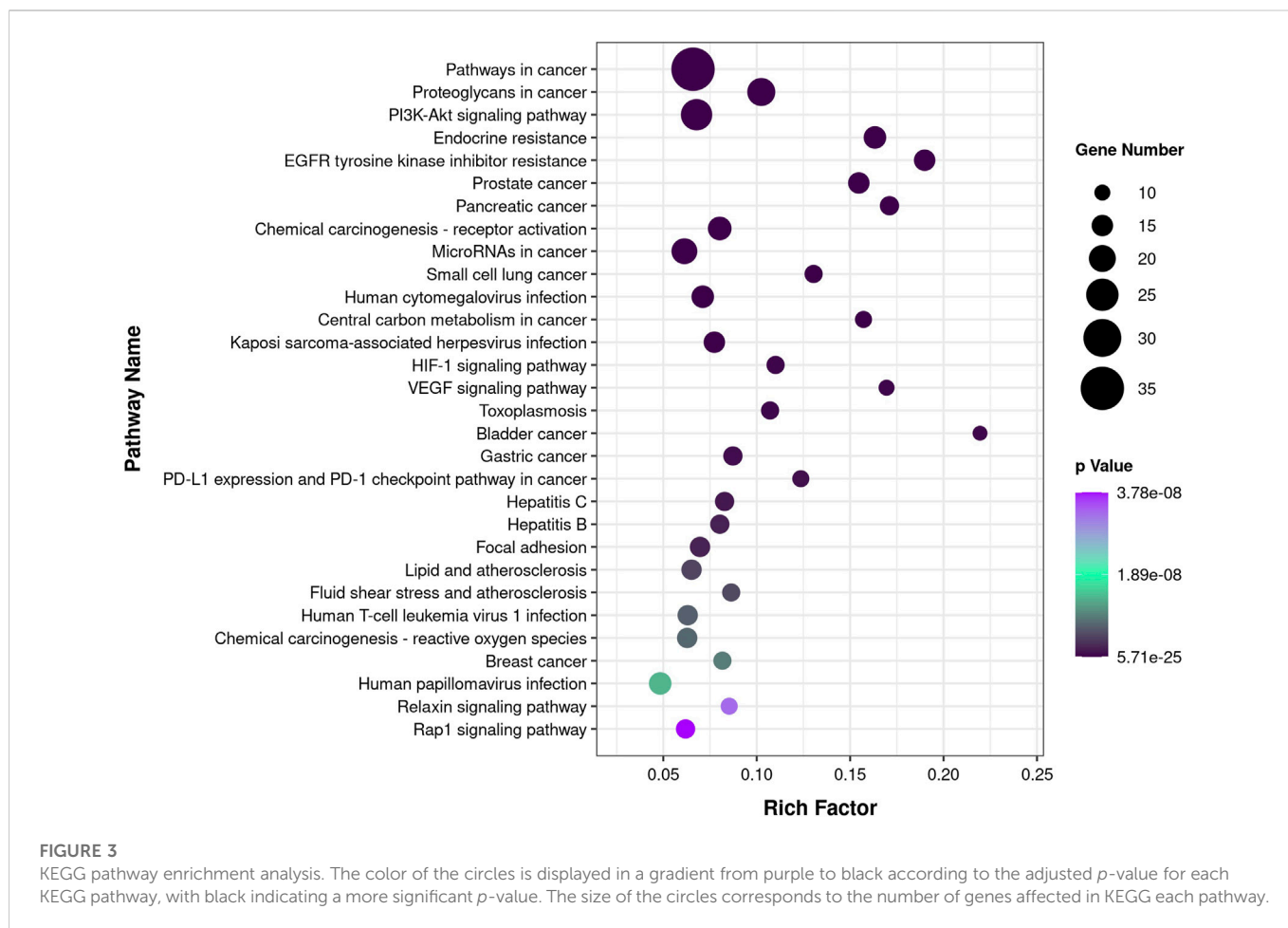
while only two or three cells were stained with green and/or red, indicating that the apoptotic cells in either the early or the late stage were very few (Figure 1E). In contrast, cells treated with 7.55 mg/L, 15.1 mg/L, and 30.2 mg/L CTFs were stained more and more weakly with blue and instead showed darker green and/or red, indicating that increased dosage of CTFs led to a corresponding increase of cells in the early and late stages of apoptosis, respectively (Figure 1E). Together, these results indicated that the inhibitory effect of CTFs on the growth of HepG2 cells was likely accomplished *via* induction of cellular apoptosis.

### 3.2 Screening of potential targets of C. paliurus flavonoids

After screening, a total of 62 compounds met the Lipinski rules (Supplementary Table S1), and a total of 354 target proteins were predicted through the SwissTargetPrediction Database. Interaction analyses of these two datasets yielded 64 common targets considered as potential anti-liver cancer targets of CTFs (Supplementary Table S2; Supplementary Figure S1).

### 3.3 GO and KEGG pathway enrichment of target proteins

GO enrichment analysis of target proteins indicate that the numbers of target proteins involved in the biological processes (BP), molecular functions (MF) and cellular components (CC) categories are 203 (74.09%), 49 (17.88%), and 22 (8.03%) (Figure 2A). The top 10 subcategories in BP, MF, and CC are shown in Figure 2B. In the BP category, target proteins were mainly involved in negative regulation of apoptotic processes. The



**TABLE 1** The top ten active ingredients, targets, and pathways that affect the entire network with high median values of betweenness centrality and closeness centrality.

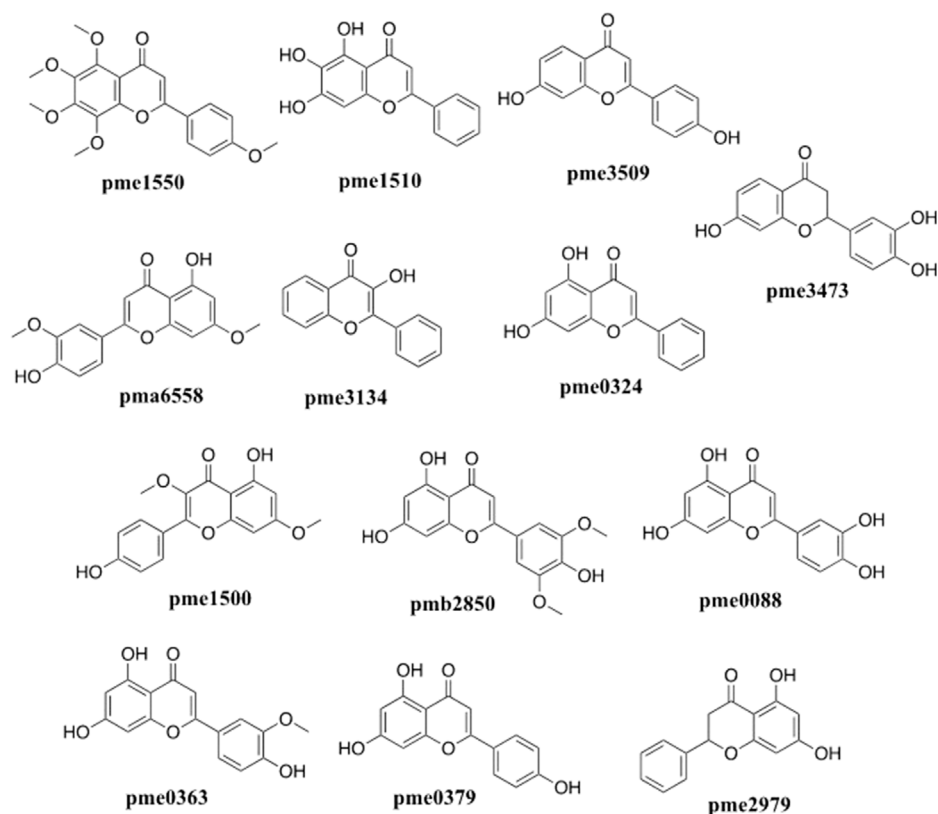
No.	Compound	Degree	Target	Degree	Pathway	Degree
1	Tangeretin (pme1550)	144	AKT1	113	hsa05200	89
2	Baicalein (pme1510)	141	MAPK3	96	hsa04151	78
3	7,4'-Dihydroxyflavone (pme3509)	140	PIK3CA	93	hsa05205	74
4	Velutin (pma6558)	139	EGFR	85	hsa05206	70
5	3-Hydroxyflavone (pme3134)	137	MAP2K1	77	hsa05163	69
6	Chrysin (pme0324)	136	SRC	68	hsa05165	69
7	Kumatakenin (pme1500)	135	IGF1R	62	hsa01522	68
8	Tricin (pmb2850), Luteolin (pme0088), Chrysoeriol (pme0363), Apigenin (pme0379), Pinocebrin (pme2979), Butin (pme3473)	133	IKBKB	60	hsa05207, hsa01521, hsa04010, hsa05166	68
9			MET	56		67
10			MAPK14	53		

CC category revealed that these proteins were most associated with the nucleus, and the MF category highlighted protein serine/threonine/tyrosine kinase activity as the most affected molecular function by CTFs. Together, these results provide a possible window into the pathways and processes through which CTFs

are likely to exert their inhibitory effects on hepatocellular carcinomas.

According to the results of enrichment analysis based on KEGG pathway analysis (Supplementary Table S3), the top 30 pathways related to those core targets are shown in Figure 3. The top five





**FIGURE 5**  
Chemical structures of the potential flavonoids compounds of *C. paliurus* with anti-liver cancer effects screened by network pharmacology analysis.

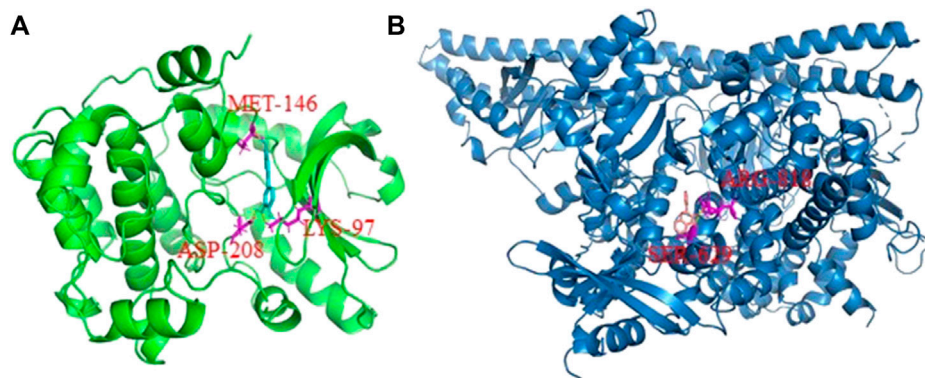
**TABLE 2** Molecular docking results of potential targets with the potential active compounds of *Cyclocarya paliurus*.

Compounds	Affinity (kcal/mol)								
	SRC	IGF1R	MET	AKT1	MAPK14	IKKBK	MAP2K1	PIK3CA	EGFR
Velutin (pma6558)	-6.5	-7.8	-8.4	-6.2	-8.5	-6.2	-8.7	-8.8	-9
Tricin (pmb2850)	-6.2	-8	-8.6	-6.4	-7.3	-6.5	-8.8	-9.1	-9.2
Luteolin (pme0088)	-6.8	-8.1	-8.5	-6.4	-8.4	-6.7	-9.1	-8.8	-9
Chrysin (pme0324)	-6.6	-8	-9.2	-6.7	-8.6	-6.6	-9	-9	-9.3
Chrysoeriol (pme0363)	-6.6	-7.8	-8.5	-6.1	-8.6	-6.5	-9.1	-8.8	-8.9
Apigenin (pme0379)	-6.6	-7.9	-8.7	-6.4	-8.3	-6.5	-8.9	-8.9	-8.8
Kumatakenin (pme1500)	-6.2	-7.7	-8.3	-6.1	-7.5	-6	-8.7	-8.8	-8.9
Baicalein (pme1510)	-6.6	-8.3	-8.6	-6.8	-8.4	-6.8	-8.8	-8.9	-8.8
Tangeretin (pme1550)	-6	-7.2	-7.7	-5.9	-8.1	-5.9	-8	-7.7	-7.8
Pinocembrin (pme2979)	-5.5	-7.3	-7	-6.5	-7.6	-6.4	-7.7	-7.7	-8.3
3-Hydroxyflavone (pme3134)	-6.3	-7.7	-8.2	-6.2	-7.9	-6.1	-8.3	-9.2	-9
Butin (pme3473)	-5.6	-7.6	-7.6	-6.2	-7.6	-6	-8.1	-8	-8.6
7,4'-Dihydroxyflavone (pme3509)	-6.7	-7.8	-8.6	-6.5	-8.4	-6.4	-9.1	-8.8	-9

modeled with MAP2K1 and 3-hydroxyflavone (pme3134) modeled with PIK3CA. Three amino acid residues—namely MET-146, ASP-208 and LYS-97—in MAP2K1 were modeled to form hydrogen

bonds with luteolin (Figure 6A). Similarly, ARG-818 and SER-629 of PIK3CA were modeled to form hydrogen bonds with 3-hydroxyflavone (Figure 6B).





**FIGURE 6**

The three-dimensional cartoon display of interactions between active compounds and target receptors. (A) MAP2K1 protein with luteolin; (B) PIK3CA protein with 3-hydroxyflavone. The amino acid residues which connected with the active compounds with hydron bond were shown in red.

### 3.6 *In vitro* verification of two predicted compounds

To validate these results, both 3-hydroxyflavone and luteolin were randomly selected from 13 flavonoids (Table 1; Figure 5) to inhibit proliferation and induce apoptosis of HepG2 cells *in vitro*. With increasing concentration of both 3-hydroxyflavone and luteolin, the inhibitory effect increased in a dose-dependent manner. When the concentration reached 100  $\mu\text{M}$ , the inhibition rate of 3-hydroxyflavone and luteolin reached 46.71% and 81.49%, respectively (Figures 7A, 8A).

After treatment with 0  $\mu\text{M}$ , 25  $\mu\text{M}$ , 50  $\mu\text{M}$ , and 100  $\mu\text{M}$  of 3-hydroxyflavone for 48 h, the apoptosis rate of HepG2 cells was  $5.6\% \pm 1.1\%$ ,  $7.7\% \pm 1.3\%$ ,  $55.7\% \pm 2.6\%$ , and  $63.5\% \pm 2.4\%$ , respectively (Figure 7B). After treatment with 0  $\mu\text{M}$ , 25  $\mu\text{M}$ , 50  $\mu\text{M}$ , and 100  $\mu\text{M}$  of luteolin for 48 h, the apoptosis rate of HepG2 cells was  $5.6\% \pm 1.1\%$ ,  $15.1\% \pm 1.3\%$ ,  $79.7\% \pm 2.3\%$ , and  $86\% \pm 4.9\%$ , respectively (Figure 8B). These indicated that both 3-hydroxyflavone and luteolin could promote the apoptosis of HepG2 cells in a dose-dependent manner (Figures 7C, 8C). Based on the CLSM analysis, cells treated with 25  $\mu\text{M}$ , 50  $\mu\text{M}$ , and 100  $\mu\text{M}$  3-hydroxyflavone or luteolin led to a corresponding increase of cells in the early and late stages of apoptosis, respectively (Figures 7D, 8D).

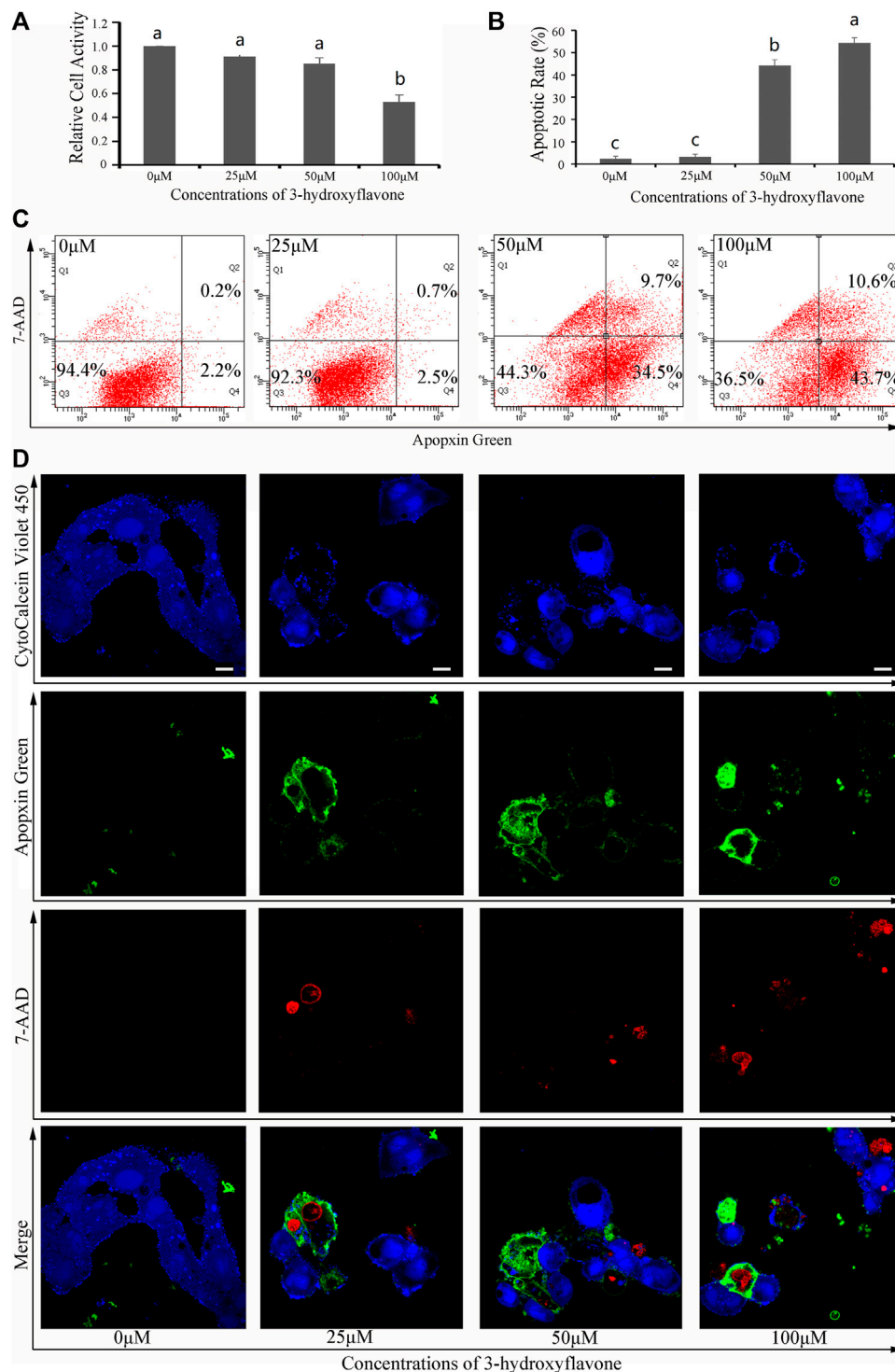
## 4 Discussion

In this study, we found that CTFs could significantly inhibit the proliferation of HepG2 cells *in vitro*, and further that the induction of apoptosis might be one of the main mechanisms for the antitumor effect of CTFs. Among the total CTFs, 13 specific flavonoids were identified by network pharmacology as the likely anti-liver cancer compounds, including tangeretin, baicalein, 7,4'-Dihydroxyflavone, velutin, 3-hydroxyflavone, chrysin, kumatakenin, tricrin, luteolin, chrysoeriol, apigenin, pinocembrin, and butin. The chemical structures of flavonoids are very similar, with a common diphenylpropane skeleton (C6-C3-C6). The degree of top 13 active flavonoids were ranged from 133 to 144 in present work. Except for 3-Hydroxyflavone (pme3134), the other top 12 active ingredients had hydroxyl or methoxy on their seven position of the diphenylpropane

skeleton, indicating the diphenylpropane skeleton was the main active group of flavonoids for anti-tumor effects were also influenced by substituent groups on the diphenylpropane skeleton. Similar studies focused on the anti-liver cancer effects of the flavonoids were performed in traditional Chinese medicines, such as *Peperomia dindygulensis* (Duan et al., 2019). He et al. (2018) also find that *C. paliurus* polysaccharide may show anti-thyroid cancer effect. To the best of our knowledge, this study is the first to report the potential anti-liver cancer effects of CTFs, and indicate likely active ingredients involved in these effects.

Three of these flavonoids—7,4'- dihydroxyflavone, velutin, and butin—were reported for the first time to have anti-cancer activity. Seven flavonoids of these flavonoids— tangeretin, kumatakenin, luteolin, tricrin, chrysoeriol, and pinocembrin—have been shown previously to have multiple anti-cancer activity (Table 3), but were shown for the first time here to exhibit specific anti-liver cancer activity. For example, tricrin was verified to have anti-tumor effect on prostate cancer PC3 cells (Ghasemi et al., 2018), colorectal tumor (Yue et al., 2020), and other cancer (Chen and Yao, 2013; Li et al., 2021). Four of these flavonoids, i.e., 3-hydroxyflavone, baicalein, chrysin, and apigenin, have been shown to inhibit the proliferation of hepatoma cells as well as those from many other cancers (Table 3). Baicalein, for example, has been shown to exert anti-cancer effects on multiple tumor cell lines, including ovarian cancer cells (Chen J. C. et al., 2013), colorectal cancer cells (Huang et al., 2012), bladder cancer cells (Wu et al., 2013), and breast cancer cells (Liu H. et al., 2016), as well as the liver cancer cell line used in the present study, HepG2 cells (Chen et al., 2009).

Our analysis identified 10 core target proteins for CTFs in the context of liver cancer, including AKT1, MAPK3, PIK3CA, EGFR, MAP2K1, SRC, IGF1R, IKBKB, MET, and MAPK14. These proteins can be divided into three groups. Proteins in the first group, including AKT1, PIK3CA, MAPK3, MAPK2K1, and MAPK14, have been verified to be involved in the apoptosis of hepatocellular carcinoma cells. In agreement with the existing literature, the present study also identified the PI3K/Akt (hsa04151) and MAPK (hsa04010) signaling pathways as key mechanisms for the potential therapeutic effect of CTFs on liver cancer. The PI3K/AKT/mTOR pathway, which has been previously implicated in hepatocellular carcinoma carcinogenesis (Sun E. J. et al., 2021). Inhibition of PI3K/Akt/mTOR signaling by apigenin

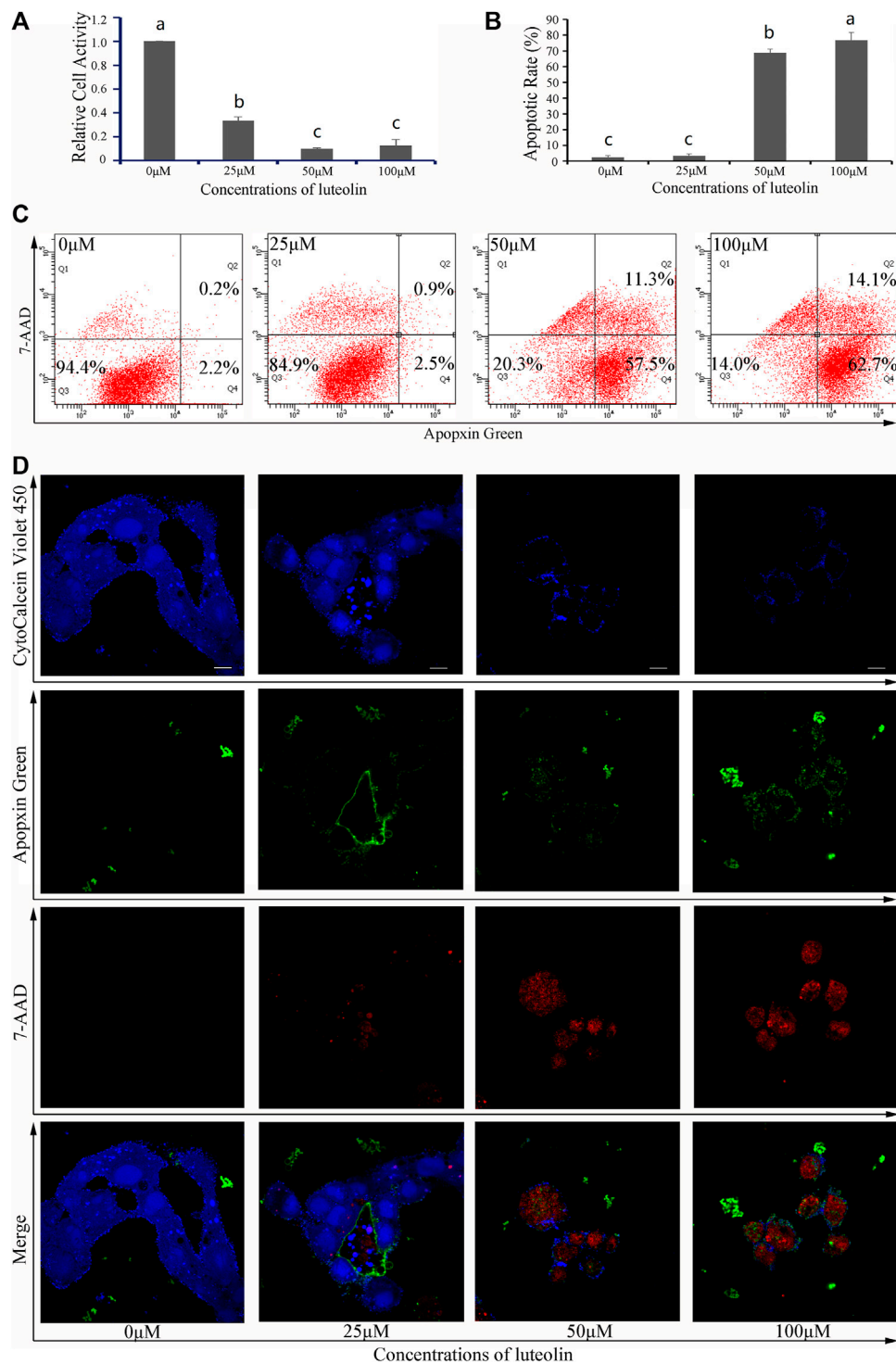


**FIGURE 7**

Effects of 3-hydroxyflavone on HepG2 cells. **(A)** Relative cell activity; **(B)** Apoptotic rate. Data are expressed as the mean  $\pm$  SEM ( $n = 3$ ). Different small letters indicate extremely significant differences between two treatments ( $p < 0.01$ ). **(C)** Representative FACS plots of cells treated with different concentrations of CTFs and stained with Apopxin Green and 7-AAD to identify apoptotic cells in the early and late stages, respectively. From left to right, the concentration of CTFs was 0  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M. Data are representative of four independent experiments. **(D)** Representative CLSM images of HepG2 cells treated with different concentrations of CTFs and stained with CytoCalcein Violet 450 (in blue color, representing live cells), Apopxin Green (in green color), 7-AAD (in red color). Representative CLSM images of apoptotic HepG2 cells treated with different concentrations of CTFs, CytoCalcein Violet 450 (in blue color, representing live cells), Apopxin Green (in green color), 7-AAD (in red color) and all of three fluorescence stains (merge). From left to right, the concentration of CTFs was 0  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M. Data are representative of four independent experiments.

and chrysoeriol induces apoptosis and autophagy in hepatocellular carcinoma cells (Yang et al., 2010; Yang et al., 2018). MAPKs also play a key role in intracellular communication, and their activating

pathways have been conserved throughout evolution, from plants, fungi, nematodes, and insects, to mammals. MAPK3 and MAPK14 are protein kinases related with cell growth and have been identified



**FIGURE 8**

Effects of luteolin on HepG2 cells. **(A)** Relative cell activity; **(B)** Apoptotic rate. Data are expressed as the mean  $\pm$  SEM ( $n = 3$ ). Different small letters indicate extremely significant differences between two treatments ( $p < 0.01$ ). **(C)** Representative FACS plots of cells treated with different concentrations of CTFs and stained with Apopxin Green and 7-AAD to identify apoptotic cells in the early and late stages, respectively. From left to right, the concentration of CTFs was 0  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M. Data are representative of four independent experiments. **(D)** Representative CLSM images of HepG2 cells treated with different concentrations of CTFs and stained with CytoCalcein Violet 450 (in blue color, representing live cells), Apopxin Green (in green color), 7-AAD (in red color). Representative CLSM images of apoptotic HepG2 cells treated with different concentrations of CTFs, CytoCalcein Violet 450 (in blue color, representing live cells), Apopxin Green (in green color), 7-AAD (in red color) and all of three fluorescence stains (merge). From left to right, the concentration of CTFs was 0  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M. Data are representative of four independent experiments.

previously in many carcinogenesis studies (Baba et al., 2010; Mesquita et al., 2020). There is a strong correlation between MAPK3 and liver cancer resistance (Gao et al., 2016).

The second group of these proteins, including EGFR, IGF1R and MET, represent membrane receptors of various growth factors. EGFR is a rational target for cancer therapy because it is commonly expressed

TABLE 3 Anti-cancer effect of 10 flavonoids.

Flavonoids	Anti-cancer effect	References
Tangeretin	Breast, colon, urinary bladder, lymphoblastoid leukaemia, ovarian, gastric, prostate cancer and leukaemia	Alhamad et al. (2021), Dong et al. (2014), Feng et al. (2016), Guo J. J. et al. (2015), Hirano et al. (1995), Ishii et al. (2010), Lin et al. (2019), Lust et al. (2010), Morley et al. (2007), Raza et al. (2020), Ting et al. (2015), and Zhu et al., 2018
Kumatakenin	Human ovarian cancer cells	Woo et al. (2017)
Luteolin	Prostate, gastric, lung, pancreatic, colon cancer and myeloid leukemia	Imran et al. (2019), Lin et al. (2008), Wu et al. (2008), and Zhou et al. (2009)
Tricin	Prostate, lung, rectal cancer, colorectal tumor	Chen and Yao (2013), Ghasemi et al. (2018), Li et al. (2021), and Yue et al. (2020)
Chrysoeriol	Multiple myeloma, breast cancer	Takemura et al. (2010) and Yang et al. (2010)
Pinocembrin	Prostate, colon cancer and melanoma	Chen Z. et al. (2013), Kumar et al. (2010), and Zheng et al. (2018)
3-Hydroxyflavone	Oral tumor, cervical cancer; adenocarcinoma, and hepatoma	Sakagami et al. (2020) and Lang et al. (2010)
Baicalein	Ovarian, cervical, colorectal, bladder, prostate, breast, ovarian, lung cancer and hepatoma	Chen et al. (2009), Chen J. C. et al. (2013), Guo Z. et al. (2015), Huang et al. (2012), Liu H. et al. (2016), Ma et al. (2016), Peng et al. (2015), Rui et al. (2016), Wu et al. (2013), Yu et al. (2020), and Zheng et al. (2014)
Chrysin	Cervical cancer, leukemia, esophageal squamous carcinoma, malignant glioma, breast carcinoma and prostate cancer, hepatoma	Eaton et al. (1996), Kasala et al. (2015), and Khoo et al. (2010)
Apigenin	Prostate colon, colorectal, gastric, breast, lung, bladder, pancreatic, ovarian, cervical cancer, hepatoma	Eaton et al. (1996), Farah et al. (2003), Kasala et al. (2015), Hu and Cao (2007), Meng et al. (2008), Pan et al. (2013), Ren et al. (2010), Shukla and Gupta (2008), Shukla and Gupta (2010), Wei et al. (2011), Yao (2010); Zhang et al. (2020), and Zhong et al. (2010)

at a high level in a variety of solid tumors and it has been implicated in the control of cell survival, proliferation, metastasis, and angiogenesis (Ciardiello and Tortora, 2003). A significant role for EGFR has been demonstrated in liver regeneration following acute and chronic liver damage, as well as in cirrhosis and hepatocellular carcinoma, highlighting its importance in liver regeneration (Chen et al., 2018). Therefore, blocking EGFR genes might be beneficial to liver cancer treatment. Insulin-like growth factor I receptor (IGF1R) has also been shown to play a critical role in cancer (Peiró et al., 2009), and the inhibition of IGF1R signaling could prevent prostate cancer cells (Fang et al., 2007). MET, also known as hepatocyte growth factor receptor, is related to biological functions as cell proliferation and progression, apoptosis, metastasis, and morphological changes (Cao et al., 2018). The mutation of MET genes was closely related with multiple cancer, such as hepatocellular carcinomas (Park et al., 1999), and lung cancer (Tsao et al., 1998).

The third group of these proteins, including SRC and IKBKB, represent proteins related with kinases. SRC tyrosine kinases regulate many important mechanisms in both normal and cancerous cells and are overexpressed in a broad range of cancers (Lee and Gautschi, 2006; Zhang and Wu, 2012). IKBKB is one of the most important catalytic subunits of IKK complexes and plays an important regulatory role in activation of NF- $\kappa$ B. The differential expression of IKBKB in human lung adenocarcinoma cells would affect the apoptosis rate (Qi et al., 2014).

In order to verify the accuracy of flavonoids predicted by network pharmacology, we randomly selected two flavonoids to validate *in vitro* with HepG2 cells. Both 3-hydroxyflavone and luteolin were verified to inhibit the proliferation of HepG2 cells in a dose-dependent manner. When the concentration reached 100  $\mu$ M, the inhibition rate of 3-hydroxyflavone and luteolin reached 46.71% and 81.49%,

respectively, while the apoptosis rate of HepG2 cells was  $63.5 \pm 2.4$  and  $86 \pm 4.9$ , respectively. For the first time, both of these compounds were shown here to possess anti-liver cancer activity. Little is currently known about 3-hydroxyflavone, while Luteolin (2-[3,4-fihydroxyphenyl]-5,7-dihydroxy-4- chromenone) is one of the most common flavonoids present in edible plants and TCMs (López-Lázaro, 2009). It has been shown to possess multitudinous antioxidant, anti-inflammatory, antimicrobial, and anti-allergic activities (López-Lázaro, 2009). It also possesses anti-cancer activities, such as chemopreventive activity through reduction of DNA damage, mutations, chromosomal aberrations, as well as chemotherapeutic activity through the induction of apoptosis (López-Lázaro, 2009). Fang et al. (2007) found that luteolin inhibits IGF1R signaling in prostate cancer cells. In this study, we found that the targets of 3-hydroxyflavone and luteolin were MAP2K1 and MET, respectively, while the pathways involved were hsa05163 and hsa01521, respectively. However, the mechanisms underlying the effects of 3-hydroxyflavone and luteolin remain unknown, and additional studies focusing on molecular biology, such as western blotting analysis and molecular interaction analysis, will be needed to more fully elucidate the complex, multifaceted mechanisms by which these compounds exert their anti-cancer effects.

## 5 Conclusion

CTFs have significant inhibitory effects on HepG2 *in vitro* cells by inducing apoptosis. We applied network pharmacology to identify potential key target proteins of CTFs in the context of liver cancer by constructing a target interaction network, and used molecular docking methods to validate the key findings. Our results

indicate that 13 flavonoid compounds from the leaves of *C. paliurus* have potential anti-liver cancer activity, including tangeretin, baicalein, 7,4'-dihydroxyflavone, velutin, 3-hydroxyflavone, chrysin, kumatakenin, tricrin, luteolin, chrysoeriol, apigenin, pinocembrin, and butin. These flavonoids were predicted to interact with AKT1, MAPK3, PIK3CA, EGFR, MAP2K1, SRC, IGF1R, IKKB, MET, and MAPK14. KEGG pathway enrichment analysis revealed that *C. paliurus* may exert its inhibitory effect on hepatocellular carcinoma by regulating pathways related to cancer (hsa05200), PI3K-Akt signaling pathway (hsa04151), proteoglycans in cancer pathway (hsa05205), microRNAs in cancer pathway (hsa05206), and endocrine resistance pathway (hsa01522) via core target proteins. Both 3-hydroxyflavone and luteolin were verified to inhibit the proliferation of HepG2 cells *in vitro* by inducing apoptosis. Although our results were obtained by network pharmacology and more experiments should be conducted to make it more confidence, our study provides scientific evidence supporting the use of CTFs for the treatment of liver cancer. Furthermore, we should realized that the low water-solubility of the flavonoids might limit the application of flavonoids in *in-vivo* cancer treatment, however, it may be overcome by different pharmaceutical methods, such as self-nanoemulsion drug delivery system and phospholipid complex.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

## Author contributions

JgM, CJ, and JL conceived the ideas and designed methodology; YT, KW, YF, LW, and HJ collected the data; YT analyzed the data; JxM did the cell experiment; JL led the writing of the manuscript. All

authors contributed critically to the drafts and gave final approval for publication.

## Funding

This work was supported by Jiebang Guashuai Program in Traditional Chinese Medicine Industry of Pan'an County, Zhejiang Province Public Welfare Technology Application Research Project (No. LGF21H160022), Natural Science Foundation of Zhejiang Province (No. LQ22H160055), Science and Technology Plan Project of Taizhou (No. 21ywb26, 21ywb29, 22ywa14 and 22ywb26), Medical Science and Technology Project of Zhejiang Province (No. 2017KY711).

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1049953/full#supplementary-material>

## References

- Alhamad, D. W., Elgendy, S. M., Al-Tel, T. H., and Omar, H. A. (2021). Tangeretin as an adjuvant and chemotherapeutic sensitizer against various types of cancers: A comparative overview. *J. Pharm. Pharmacol.* 73, 601–610.
- Baba, Y., Noshio, K., Shima, K., Meyerhardt, J. A., Chan, A. T., Engelman, J. A., et al. (2010). Prognostic significance of AMP-activated protein kinase expression and modifying effect of MAPK3/1 in colorectal cancer. *Br. J. Cancer* 103 (7), 1025–1033.
- Caltagirone, S., Rossi, C., Poggi, A., Ranelletti, F. O., Natali, P. G., Brunetti, M., et al. (2000). Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *Intern. J. Cancer* 87, 595–600. doi:10.1002/1097-0215(20000815)87:4<595::aid-ijc21>3.0.co;2-5
- Cao, J. J., Wu, Z. H., Tong, T. T., Zhu, Q. Z., Zhao, E. H., and Cui, H. J. (2018). Advances in mesenchymal to epithelial transition factor signaling pathway and inhibitors. *Chin. J. Biotechnol.* 34 (3), 334–351. doi:10.13345/j.cb.170265
- Chen, C. H., Huang, T. S., Wong, C. H., Hong, C. L., Tsai, Y. H., Liang, C. C., et al. (2009). Synergistic anti-cancer effect of baicalein and silymarin on human hepatoma HepG2 Cells. *Food Chem. Toxicol.* 47 (3), 638–644. doi:10.1016/j.fct.2008.12.024
- Chen, H. J., Hu, M. H., Xu, F. G., Xu, H. J., She, J. J., and Xia, H. P. (2018). Understanding the inflammation-cancer transformation in the development of primary liver cancer. *Hepatoma Res.* 4, 29. doi:10.20517/2394-5079.2018.18
- Chen, J. C., Li, Z. L., Chen, A. Y., Ye, X. Q., Luo, H. T., Rankin, G. O., et al. (2013). Inhibitory effect of baicalin and baicalein on ovarian cancer cells. *Intern. J. Mol. Sci.* 14 (3), 6012–6025. doi:10.3390/ijms14036012
- Chen, J. H., Kim, S. H., Fan, P. W., Liu, C. Y., Hsieh, C. H., and Fang, K. (2016). The aqueous extract of Chinese medicinal herb *Brucea javanica* suppresses the growth of human liver cancer and the derived stem-like cells by apoptosis. *Drug Des. devel. Ther.* 10, 2003–2013. doi:10.2147/DDDT.S107909
- Chen, Y., and Yao, H. (2013). Effects of tricrin on proliferation and apoptosis of human rectal cancer SW1116 cells. *Nat. Prod. Res. Dev.* 25, 31–35.
- Chen, Z., Rasul, A., Zhao, C., Millimouno, F. M., Tsuji, I., Yamamura, T., et al. (2013). Expression analysis of abscisic acid (ABA) and metabolic signalling factors in developing endosperm and embryo of barley. *Bangladesh Pharm. Soc.* 8 (3), 255–262. doi:10.1016/j.jcs.2013.06.009
- Ciardello, F., and Tortora, G. (2003). Epidermal growth factor receptor (EGFR) as a target in cancer therapy: Understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs. *Eur. J. Cancer* 39 (10), 1348–1354. doi:10.1016/s0959-8049(03)00235-1
- Dirks, W. G., and Drexler, H. G. (2011). Online verification of human cell line identity by STR DNA typing. *Methods Mol. Biol.* 731, 45–55. doi:10.1007/978-1-61779-080-5\_5
- Dong, Y., Cao, A., Shi, J., Yin, P., Wang, L., Ji, G., et al. (2014). Tangeretin, a citrus polymethoxyflavonoid, induces apoptosis of human gastric cancer AGS cells through extrinsic and intrinsic signaling pathways. *Oncol. Rep.* 31, 1788–1794. doi:10.3892/or.2014.3034
- Duan, Z. K., Zhang, H., Zhao, P., Yan, Z. Y., and Huang, X. X. (2019). Network pharmacology-based virtual screening of natural products from *Peperomia dindygulensis* for the application to treat liver cancer. *Asian J. Tradit. Med.* 14 (6), 253–259.

- Eaton, E. A., Walle, U. K., Lewis, A. J., Hudson, T., Wilson, A. A., and Walle, T. (1996). Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase. Potential role in drug metabolism and chemoprevention. *Drug Metab. Dispos.* 24, 232–237.
- Fang, J., Zhou, Q., Shi, X. L., and Jiang, B. H. (2007). Luteolin inhibits insulinlike growth factor 1 receptor signaling in prostate cancer cells. *Carcinogenesis* 28, 713–723. doi:10.1093/carcin/bgl189
- Farah, M., Parhar, K., Moussavi, M., Eivemark, S., and Salh, B. (2003). 5,6-Dichlororibifuranosylbenzimidazole- and apigenin-induced sensitization of colon cancer cells to TNF- $\alpha$ -mediated apoptosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 285 (5), G919–G928. doi:10.1152/ajpgi.00205.2003
- Feng, S. L., Yuan, Z. W., Yao, X. J., Ma, W. Z., Liu, L., Liu, Z. Q., et al. (2016). Tangeretin, a citrus pentamethoxyflavone, antagonizes ABCB1-mediated multidrug resistance by inhibiting its transport function. *Pharmacol. Res.* 110, 193–204. doi:10.1016/j.phrs.2016.04.003
- Gao, L., Wang, X. D., Niu, Y. Y., Duan, D. D., Yang, X., Hao, J., et al. (2016). Molecular targets of Chinese herbs: A clinical study of hepatoma based on network pharmacology. *Sci. Rep.* 6, 24944. doi:10.1038/srep24944
- Ghasemi, S., Lorigooini, Z., Dehkordi, N. A., and Motamedi, M. (2018). Effect of isolated tricin from arial part of *Allium atroviolaceum* Boiss. on proliferation and apoptosis in PC3 cell line. *J. Babol Univ. Med. Sci.* 20 (6), 36–40.
- Guo, J. J., Li, Y. J., and Xin, L. L. (2015). Tangeretin prevents prostate cancer cell proliferation and induces apoptosis via activation of notch signalling and regulating the androgen receptor (AR) pathway and the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathways. *Bangladesh J. Pharmacol.* 10, 937. doi:10.3329/bjpv.v10i4.23699
- Guo, Z., Hu, X., Xing, Z., Xing, R., Lv, R., Cheng, X., et al. (2015). Baicalein inhibits prostate cancer cell growth and metastasis via the caveolin-1/AKT/mTOR pathway. *Mol. Cell. Biochem.* 406, 111–119. doi:10.1007/s11010-015-2429-8
- He, Z., Lv, F., Gan, Y., Gu, J., and Que, T. (2018). Anticancer effects of *Cyclocarya paliurus* polysaccharide (CPP) on thyroid carcinoma *in vitro* and *in vivo*. *Intern. J. Polym. Sci.* 2018, 2768120.
- Hirano, T., Abe, K., Gotoh, M., and Oka, K. (1995). Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. *Br. J. Cancer* 72, 1380–1388. doi:10.1038/bjc.1995.518
- Hirano, T., Gotoh, M., and Oka, K. (1994). Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. *Life Sci.* 55, 1061–1069. doi:10.1016/0024-3205(94)00641-5
- Hu, T. P., and Cao, J. G. (2007). Study on pro-apoptotic effect of apigenin on human gastric cancer cells and its underlying mechanisms. *Inter. J. Pathol. Clin. medic.* 27 (1), 6–10.
- Huang, W. S., Kuo, Y. H., Chin, C. C., Wang, J. Y., Yu, H. R., Sheen, J. M., et al. (2012). Proteomic analysis of the effects of baicalein on colorectal cancer cells. *Proteomics* 12 (6), 810–819. doi:10.1002/pmic.2011100270
- Imran, M., Rauf, A., Abu-Izneid, T., Nadeem, M., Shariati, M. A., Khan, I. A., et al. (2019). Luteolin, a flavonoid, as an anticancer agent: A review. *Biomed. Pharmacother.* 112, 108612. doi:10.1016/j.biopha.2019.108612
- Ishii, K., Tanaka, S., Kagami, K., Henmi, K., Toyoda, H., Kaise, T., et al. (2010). Effects of naturally occurring polymethoxyflavonoids on cell growth, p-glycoprotein function, cell cycle, and apoptosis of daunorubicin-resistant T lymphoblastoid leukemia cells. *Cancer Invest.* 28, 220–229. doi:10.3109/07357900902744486
- Kasala, E. R., Bodduluru, L. N., Madana, R. M., Gogoi, A. K. V. R., and Barua, C. C. (2015). Chemopreventive and therapeutic potential of chrysin in cancer: Mechanistic perspectives. *Toxicol. Lett.* 233 (2), 214–225. doi:10.1016/j.toxlet.2015.01.008
- Khoo, B. Y., Chua, S. L., and Balaram, P. (2010). Apoptotic effects of chrysin in human cancer cell lines. *Intern. J. Mol. Sci.* 11, 2188–2199. doi:10.3390/ijms11052188
- Kibble, M., Saarinen, N., Tang, J., Wennerberg, K., Mäkelä, S., and Aittokallio, T. (2015). Network pharmacology applications to map the unexplored target space and therapeutic potential of natural products. *Nat. Product. Rep.* 32 (8), 1249–1266. doi:10.1039/c5np00005j
- Kumar, M., Nair, M., Hema, P. S., Mohan, J., and Santhoshkumar, T. R. (2010). Pinocembrin triggers Bax-dependent mitochondrial apoptosis in colon cancer cells. *Mol. Carcinog.* 46 (3), 231–241. doi:10.1002/mc.20272
- Lang, Q. Y., Zhang, H. X., Li, J., Xie, F., Zhang, Y. F., Wan, B., et al. (2010). 3-hydroxyflavone inhibits endogenous Aurora B and induces growth inhibition of cancer cell line. *Mol. Biol. Rep.* 37, 1577–1583. doi:10.1007/s11033-009-9562-y
- Lee, D., and Gautschi, O. (2006). Clinical development of SRC tyrosine kinase inhibitors in lung cancer. *Clin. Lung Cancer* 7 (6), 381–384. doi:10.3816/clc.2006.n.020
- Li, J. X., Li, R. Z., Sun, A., Zhou, H., Neher, E., Yang, J. S., et al. (2021). Metabolomics and integrated network pharmacology analysis reveal tricin as the active anti-cancer component of weijing decoction by suppression of PRKCA and sphingolipid signaling. *Pharmacol. Res.* 171, 105574. doi:10.1016/j.phrs.2021.105574
- Lin, J. J., Huang, C. C., Su, Y. L., Luo, H. L., Lee, N. L., Sung, M. T., et al. (2019). Proteomics analysis of tangeretin-induced apoptosis through mitochondrial dysfunction in bladder cancer cells. *Int. J. Mol. Sci.* 20 (5), 1017. doi:10.3390/ijms20051017
- Lin, Y., Shi, R. X., Wang, X., and Shen, H. M. (2008). Luteolin, a flavonoid with potential for cancer prevention and therapy. *Curr. Cancer Drug Targets* 8 (7), 634–646. doi:10.2174/15680908786241050
- Liu, H., Dong, Y. H., Gao, Y. T., Du, Z. P., Wang, Y. T., Cheng, P., et al. (2016). The fascinating effects of baicalein on cancer: A review. *Int. J. Mol. Sci.* 17 (10), 1681–1699. doi:10.3390/ijms17101681
- Liu, S. H., Wang, R., Lou, Y., and Liu, J. (2020). Uncovering the mechanism of the effects of Pien-Tze-Huang on liver cancer using network pharmacology and molecular docking. *Evidence-based Compl. Alt. Med.* 2020, 4863015. doi:10.1155/2020/4863015
- Liu, Y., Cao, Y., Fang, S., Wang, T., Yin, Z., Shang, X., et al. (2018). Antidiabetic effect of *Cyclocarya paliurus* leaves depends on the contents of antihyperglycemic flavonoids and antihyperlipidemic triterpenoids. *Molecules* 23, 1042. doi:10.3390/molecules23051042
- Liu, Y., Qian, C., Ding, S., Shang, X., Yang, W., and Fang, S. (2016). Effect of light regime and provenance on leaf characteristics, growth and flavonoid accumulation in *Cyclocarya paliurus* (Batal) Iljinskaja coppices. *Bot. Stud.* 57, 28. doi:10.1186/s40529-016-0145-7
- López-Lázaro, M. (2009). Distribution and biological activities of the flavonoid luteolin. *Mini-Rev. Med. Chem.* 9 (1), 31–59. doi:10.2174/138955709787001712
- Luo, Z., Zeng, H., Ye, Y., Liu, L., Li, S., Zhang, J., et al. (2015). Safflower polysaccharide inhibits the proliferation and metastasis of MCF-7 breast cancer cell. *Mol. Med. Rep.* 11 (6), 4611–4616. doi:10.3892/mmr.2015.3310
- Lust, S., Vanhoecke, B., Van Gele, M., Philippe, J., Bracke, M., and Offner, F. (2010). The flavonoid tangeretin activates the unfolded protein response and synergizes with imatinib in the erythroleukemia cell line K562. *Mol. Nutr. Food Res.* 54, 823–832. doi:10.1002/mnfr.200900186
- Ma, X., Yan, W., Da, Z., Gao, X., Ma, Y., Xu, Q., et al. (2016). Baicalein suppresses metastasis of breast cancer cells by inhibiting EMT via downregulation of SATB1 and Wnt/ $\beta$ -catenin pathway. *Drug Des. Dev. Ther.* 10, 1419–1441. doi:10.2147/DDDT.S102541
- Meng, Y., Qing-Yong, M. A., and Tao, M. A. (2008). The *in vitro* research on the effect of apigenin in human colon cancer cell SW480. *J. Mod. Oncol.* 16 (5), 701–703.
- Mesquita, F. P., Moreira-Nunes, C. A., Silva, E., Lima, L. B., Montenegro, R. C., Zuerker, W. J., et al. (2020). MAPK14 (p38 $\alpha$ ) inhibition effects against metastatic gastric cancer cells: A potential biomarker and pharmacological target. *Toxicol. Vitro* 66, 104839. doi:10.1016/j.tiv.2020.104839
- Morley, K. L., Ferguson, P. J., and Koropatnick, J. (2007). Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. *Cancer Lett.* 251 (1), 168–178. doi:10.1016/j.canlet.2006.11.016
- Pan, X. D., Yang, Z. P., Zang, L. Q., Yang, Z. Y., and Zhou, S. G. (2013). The growth inhibition and apoptosis-inducing effects of apigenin on human lung cancer NCI-H1299 cells. *Guangdong Med. J.* 34 (15), 2281–2284.
- Park, W. S., Dong, S. M., Kim, S. Y., Na, E. Y., Shin, M. S., Pi, J. H., et al. (1999). Somatic mutations in the kinase domain of the met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res.* 59 (2), 307–310.
- Peiró, G., Benlloch, S., Sánchez-Tejada, L., Adrover, E., Lerma, E., Peiró, F. M., et al. (2009). Low activation of insulin-like growth factor 1-receptor (IGF1R) is associated with local recurrence in early breast carcinoma. *Breast Cancer Res. Treat.* 117, 433–441. doi:10.1007/s10549-008-0139-6
- Peng, Y., Guo, C., Yang, Y., Li, F., Zhang, Y., Jiang, B., et al. (2015). Baicalein induces apoptosis of human cervical cancer HeLa cells *in vitro*. *Mol. Med. Rep.* 11, 2129–2134. doi:10.3892/mmr.2014.2885
- Qi, K., Li, Y., Li, X. B., Zhang, F., Shao, Y., and Zhou, Q. H. (2014). Expression and significance of IKKB in pulmonary adenocarcinoma A549 cells and its cisplatin-resistant variant A549/DDP. *Chin. J. Lung Cancer* 17 (5), 363–368. doi:10.3779/j.issn.1009-3419.2014.05.01
- Quan, M. F., Xiao, L. H., Liu, Z. H., Guo, H., Ren, K. Q., Liu, F., et al. (2013). 8-bromo-7-methoxychrysin inhibits properties of liver cancer stem cells via downregulation of  $\beta$ -catenin. *World J. Gastroenterol.* 19 (43), 7680–7695. doi:10.3748/wjg.v19.i43.7680
- Raza, W., Luqman, S., and Meena, A. (2020). Prospects of tangeretin as a modulator of cancer targets/pathways. *Pharmacol. Res.* 161, 105202. doi:10.1016/j.phrs.2020.105202
- Ren, D. M., Villeneuve, N. F., Jiang, T., Wu, T. D., Lau, A., Toppin, H. A., et al. (2011). Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proc. Natl. Acad. Sci.* 108 (4), 1433–1438. doi:10.1073/pnas.1014275108
- Ren, X., Zhang, H., and Li, Y. (2010). Study on pro-apoptotic effect of apigenin on human ovarian cancer cells and its underlying mechanisms. *Mod. J. Integ. Tradit. Chin. West. Med.* 19 (15), 1821–1826.
- Rui, X., Yan, X. I., and Zhang, K. (2016). Baicalein inhibits the migration and invasion of colorectal cancer cells via suppression of the AKT signaling pathway. *Oncol. Lett.* 11, 685–688. doi:10.3892/ol.2015.3935
- Sakagami, H., Jiang, Y. L., KusAMA, K., Atsumi, T., Ueha, T., Toguchi, M., et al. (2020). Induction of apoptosis by flavones, flavonols (3-hydroxyflavones) and isoprenoid-substituted flavonoids in human oral tumor cell lines. *Anticancer Res.* 20 (1A), 271–277.
- Sheng, X. L., Chen, H. W., Wang, J. M., Zheng, Y. L., Li, Y. L., Jin, Z. X., et al. (2021). Joint transcriptomic and metabolic analysis of flavonoids in *Cyclocarya paliurus* leaves. *ACS Omega* 6, 9028–9038. doi:10.1021/acsomega.1c00059
- Shinbo, T., Fushida, S., Tsukada, T., Harada, S., Kinoshita, J., Oyama, K., et al. (2015). Protein-bound polysaccharide K suppresses tumor fibrosis in gastric cancer

- by inhibiting the TGF $\beta$  signaling pathway. *Oncol. Rep.* 33 (2), 553–558. doi:10.3892/or.2014.3636
- Shukla, S., and Gupta, S. (2010). "Apigenin and cancer chemoprevention," in *Bioact. Foods promot. Health*, 663–689.
- Shukla, S., and Gupta, S. (2008). Apigenin-induced prostate cancer cell death is initiated by reactive oxygen species and p53 activation. *Free Radic. Biol. Medic.* 44 (10), 1833–1845. doi:10.1016/j.freeradbiomed.2008.02.007
- Sun, C., Shang, X., Ding, H., Cao, Y., and Fang, S. (2021). Natural variations in flavonoids and triterpenoids of *Cyclocarya paliurus* leaves. *J. For. Res.* 32, 805–814. doi:10.1007/s11676-020-01139-1
- Sun, E. J., Wankell, M., Palamuthusingam, P., McFarlane, C., and Hebbard, L. (2021). Targeting the PI3K/Akt/mTOR pathway in hepatocellular carcinoma. *Biomedicines* 9 (11), 1639. doi:10.3390/biomedicines9111639
- Sun W., W., Li, M., Xie, L., Mai, Z., Zhang, Y., Luo, L., et al. (2021). Exploring the mechanism of total flavonoids of *Drynariae Rhizoma* to improve large bone defects by network pharmacology and experimental assessment. *Front. Pharmacol.* 12, 603734. doi:10.3389/fphar.2021.603734
- Sun, X., Li, L., Ma, H. G., Sun, P., Wang, Q. L., Zhang, T. T., et al. (2017). Bisindolylmaleimide alkaloid BMA-155Cl induces autophagy and apoptosis in human hepatocarcinoma HepG-2 cells through the NF- $\kappa$ B p65 pathway. *Acta Pharmacol. Sin.* 38 (4), 524–538. doi:10.1038/aps.2016.171
- Takemura, H., Uchiyama, H., Ohura, T., Sakakibara, H., Kuruto, R., Amagai, T., et al. (2010). A methoxyflavonoid, chrysoeriol, selectively inhibits the formation of a carcinogenic estrogen metabolite in MCF-7 breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 118 (1–2), 70–76. doi:10.1016/j.jsbmb.2009.10.002
- Tang, J. L., Liu, B. Y., and Ma, K. W. (2008). Traditional Chinese medicine. *Lancet* 372, 1938–1940. doi:10.1016/S0140-6736(08)61354-9
- Tang, K. Y., Du, S. L., Wang, Q. L., Zhang, Y. F., and Song, H. Y. (2020). Traditional Chinese medicine targeting cancer stem cells as an alternative treatment for hepatocellular carcinoma. *J. Integ. Med.* 18 (3), 196–202. doi:10.1016/j.joim.2020.02.002
- Ting, Y. W., Chiou, Y. S., Pan, M. H., Ho, C. T., and Huang, Q. Q. (2015). *In vitro* and *in vivo* anti-cancer activity of tangeretin against colorectal cancer was enhanced by emulsion-based delivery system. *J. Funct. Food* 15, 264–273. doi:10.1016/j.jff.2015.03.034
- Trott, O., and Olson, A. J. (2010). AutoDock vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461. doi:10.1002/jcc.21334
- Tsao, M. S., Ni, L., Chen, J. R., Pappas, J., Ho, J., To, C., et al. (1998). Differential expression of Met/hepatocyte growth factor receptor in subtypes of non-small cell lung cancers. *Lung Cancer* 20 (1), 1–16. doi:10.1016/S0169-5002(98)00007-5
- Wei, S., Quan, M., and Huang, Q. (2011). Relationships between glutathione depletion and apigenin-induced apoptosis of pancreatic cancer PANC-1 cell line cultured *in vitro*. *Anti-tumor Pharm.* 1 (3), 197–199.
- Woo, J. H., Ahn, J. H., Jang, D. S., Lee, K. T., and Choi, J. H. (2017). Effect of kumatakenin isolated from cloves on the apoptosis of cancer cells and the alternative activation of tumor-associated macrophages. *J. Agricul. Food Chem.* 65 (36), 7893–7899. doi:10.1021/acs.jafc.7b01543
- Wu, B., Zhang, Q., Shen, W. M., and Zhu, J. (2008). Anti-proliferative and chemosensitizing effects of luteolin on human gastric cancer AGS cell line. *Mol. Cell. Biochem.* 313, 125–132. doi:10.1007/s11010-008-9749-x
- Wu, J. Y., Tsai, K. W., Li, Y. Z., Chang, Y. S., Lai, Y. C., Laio, Y. H., et al. (2013). Anti-bladder-tumor effect of baicalin from *Scutellaria baicalensis georgi* and its application *in vivo*. *Evid. Based Complement. Altern. Med.* 2013, 579751. doi:10.1155/2013/579751
- Wu, Z. F., Gao, T. H., Zhong, R. L., Lin, Z., Jiang, C. H., Ouyang, S., et al. (2017). Antihyperlipidaemic effect of triterpenic acid-enriched fraction from *Cyclocarya paliurus* leaves in hyperlipidaemic rats. *Pharm. Biol.* 55, 712–721. doi:10.1080/13880209.2016.1267231
- Wufuer, Y., Yang, X., Guo, L. Y., Aximujiang, K., Zhong, L., Yunusi, K., et al. (2022). The antitumor effect and mechanism of total flavonoids from *Coreopsis tinctoria* Nutt (snow chrysanthemum) on lung cancer using network pharmacology and molecular docking. *Front. Pharm.* 13, 761785. doi:10.3389/fphar.2022.761785
- Xie, J., Wang, W., Dong, C., Huang, L., Wang, H., Li, C., et al. (2018). Protective effect of flavonoids from *Cyclocarya paliurus* leaves against carbon tetrachloride-induced acute liver injury in mice. *Food Chem. Toxicol.* 119, 392–399. doi:10.1016/j.fct.2018.01.016
- Xie, J. H., Wang, Z. J., Shen, M. Y., Nie, S. P., Gong, B., Li, H. S., et al. (2016). Sulfated modification, characterization and antioxidant activities of polysaccharide from *Cyclocarya paliurus*. *Food Hydrocoll.* 53, 7–15. doi:10.1016/j.foodhyd.2015.02.018
- Yang, C. S., Chung, J. Y., Yang, G. Y., Chhabra, S. K., and Lee, M. J. (2000). Tea and tea polyphenols in cancer prevention. *J. Nut.* 130 (2), 472S–478S. doi:10.1093/jn/130.2.472S
- Yang, J. L., Pi, C. C., and Wang, G. H. (2018). Inhibition of PI3K/Akt/MTOR pathway by apigenin induces apoptosis and autophagy in hepatocellular carcinoma cells. *Biomed. Pharmacother.* 103, 699–707. doi:10.1016/j.biopha.2018.04.072
- Yang, Y., Zhou, X., Xiao, M., Zhenya, H., Gong, Q., Jiang, L. J., et al. (2010). Discovery of chrysoeriol, a PI3K-AKT-mTOR pathway inhibitor with potent antitumor activity against human multiple myeloma cells *in vitro*. *J. Huazhong Univ. Sci. Technol.* 30 (6), 734–740. doi:10.1007/s11596-010-0649-4
- Yao, S. H. (2010). Apigenin-induced apoptosis of bladder cancer cell BIU-87 and its mechanism. *J. Chongqing Med. Uni.* 35 (8), 1194–1197.
- Yu, M., Zeng, M., Pan, Z., Wu, F., Guo, L., and He, G. (2020). Discovery of novel akt1 inhibitor induces autophagy associated death in hepatocellular carcinoma cells. *Eur. J. Med. Chem.* 189, 112076. doi:10.1016/j.ejmech.2020.112076
- Yue, G. G. L., Gao, S., Lee, K. M., Chan, Y. Y., Wong, E. C. W., Zheng, T., et al. (2020). A natural flavone tricrin from grains can alleviate tumor growth and lung metastasis in colorectal tumor mice. *Molecules* 25 (16), 3730. doi:10.3390/molecules25163730
- Zhang, E., Zhang, Y., Fan, Z., Cheng, L., Han, S., and Che, H. (2020). Apigenin inhibits histamine-induced cervical cancer tumor growth by regulating estrogen receptor expression. *Molecules* 25 (8), 1960. doi:10.3390/molecules25081960
- Zhang, H., and Wu, X. L. (2012). The role of SRC in gastric cancer. *Prog. Mod. Biomed.* 12 (13), 2592–2595.
- Zhao, S., and Iyengar, R. (2012). Systems pharmacology: Network analysis to identify multiscale mechanisms of drug action. *Ann. Rev. Pharm. Toxicol.* 52 (1), 505–521. doi:10.1146/annurev-pharmtox-010611-134520
- Zheng, Y. F., Wang, K., Wu, Y. Q., Chen, Y. F., Chen, X., Hu, C. W., et al. (2018). Pinocembrin induces ER stress mediated apoptosis and suppresses autophagy in melanoma cells. *Cancer Lett.* 431, 31–42. doi:10.1016/j.canlet.2018.05.026
- Zheng, Y. H., Yin, L. H., Grahm, T. H., Ye, A. F., Zhao, Y. R., and Zhang, Q. Y. (2014). Anticancer effects of baicalin on hepatocellular carcinoma cells. *Phytother. Res.* 28, 1342–1348. doi:10.1002/ptr.5135
- Zhong, Y., Krisanapun, C., Lee, S. H., Nuansanit, T., Sams, C., Peungvicha, P., et al. (2010). Molecular targets of apigenin in colorectal cancer cells: Involvement of p21, NAG-1 and p53. *Eur. J. Cancer* 46 (18), 3365–3374. doi:10.1016/j.ejca.2010.07.007
- Zhou, J., Sun, H., Wang, Z., Cong, W. M., Wang, J. H., Zeng, M. S., et al. (2018). Guidelines for diagnosis and treatment of primary liver cancer in China (2017 edition). *Liver Cancer* 7, 235–260. doi:10.1159/000488035
- Zhou, Q., Yan, B., Hu, X. W., Li, X. B., Zhang, J., and Fang, J. (2009). Luteolin inhibits invasion of prostate cancer PC3 cells through E-cadherin. *Mol. Cancer Ther.* 8 (6), 1684–1691. doi:10.1158/1535-7163.MCT-09-0191
- Zhu, W. B., Xiao, N., and Liu, X. J. (2018). Dietary flavonoid tangeretin induces reprogramming of epithelial to mesenchymal transition in prostate cancer cells by targeting the PI3K/Akt/mTOR signaling pathway. *Oncol. Lett.* 15, 433–440. doi:10.3892/ol.2017.7307