Review

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Flavonoids as potential anti-hepatocellular carcinoma agents: Recent approaches using HepG2 cell line

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ABSTRACT: Numerous studies have documented that in cancer therapy flavonoids extracted from traditional Chinese medicine have anti-tumor activity or can enhance efficiency of chemotherapy in combination with chemotherapeutics. Thus, an awareness of flavonoids is needed by physicians and medical researchers. This review provides evidence about anti-hepatocellular carcinoma activity of flavonoids. First, as a common employed *in vitro* model, profile of HepG2 is shown. Second, the intracellular signaling pathways induced by flavonoids which inhibit the HepG2 cell line are summarized. Third, study situation of anti-HBV/HCV activity of flavonoids is shown. Our review is aimed at providing an understanding of anti-HBV/HCV activity and anti-HCC mechanisms of flavonoids, and an outlook on flavonoids application on cancer therapy.

Keywords: Flavonoids, herb medicine, hepatocellular carcinoma, HepG2

1. Introduction

Hepatocellular carcinoma (HCC) is one of the major health threats worldwide, especially in East Asia. Although chemotherapy is one of major conventional HCC therapies, the strong side effects and the emergence of drug resistance are serious problems. Meanwhile, hepatitis B virus (HBV) infection accounts for about 60% of the total liver cancer in developing countries and for about 23% of cancer in developed countries and the corresponding percentages for hepatitis C virus (HCV) infection are 33% in developing countries and 20% in developed countries (*1*). Therefore, in the development of anti-HCC agents, the anti-HBV and anti-HCV activities as well as the low side effects should be considered.

Over thousands of years, traditional Chinese medicine (TCM) and other herbal medicines have been used to treat cancer in China, Japan, and other Asian countries. They are still widely adopted because of the advantages of high efficiency, weak side effects, easy availability, and improvement of quality of life. Recently, in Europe and USA, herbal medicines are widely accepted as a form of complementary and alternative medicine (CAM) (*2*,*3*). However, on the other hand, some disadvantages of herbal medicines left several barriers for their clinical utility, such as uncertain effective constituents and unstable efficiency.

Recently, more and more effective components from herbal medicines have been identified and one of the most interesting chemicals is a flavonoid family. Flavonoids are a group of plant secondary metabolites with variable phenolic structures and are found in fruits, vegetables, roots, stems, flowers, wine, and tea (*4*). Flavonoids are usually divided into seven classes including flavonols, flavones, flavanones, flavononol, flavanols, isoflavones, and anthocyanidins (*5*) (Figure 1). Until now, over 5,000 naturally occurring flavonoids have been extracted from various herbal medicines and their chemical structures have been confirmed. Some of these flavonoids have been reported to have activities on treatment of various diseases such as heart disease, cancer, and virus infection (*6*) as well as potential protective activity against artificially induced-liver damage (*7*,*8*). In recent years, natural products have been increasingly recognized as new remedies for enhancing the efficacy and alleviating the adverse effects of tumor therapies (*9*). Accordingly, anti-HCC effects of flavonoids have been accumulated from *in vitro* and *in vivo* research evidences. This review overlooks the recent advances of research and development on flavonoids as anti-HCC agents.

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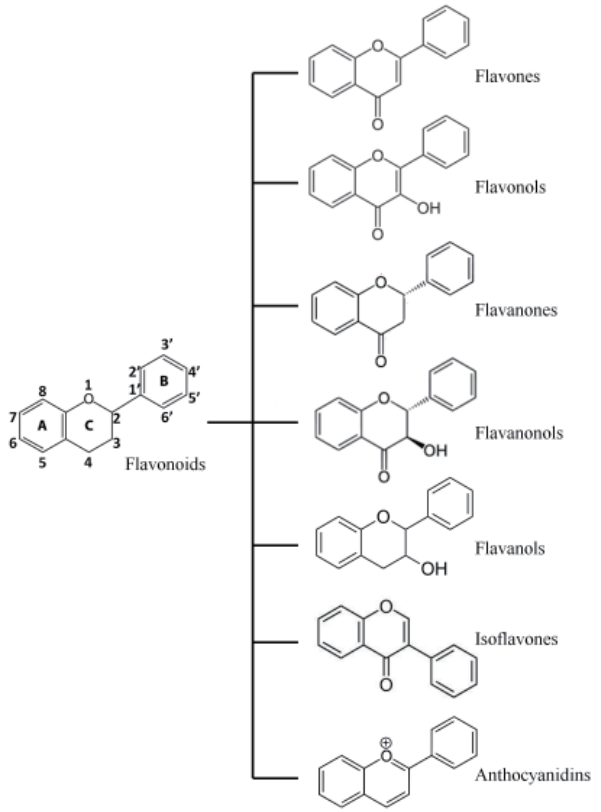

Figure 1. Molecular backbone structures of flavonoids and its subfamilies

2. HepG2 cell line, a model for investigation of flavonoids action

Since ancient times, it had been known that some TCM and other herbs could inhibit tumors, but the mechanisms were left in the dark for many centuries. Up to recent decades, molecular biological and cellular biological research gradually shed light on the mechanisms of cancer inhibition by medicines extracted from herbs. Especially very recent several years, more mechanisms of flavonoids action on HCC cell lines were illuminated and that gave a guide for selection of medicines and therapeutic methods. Among various HCC cell lines, HepG2 (ATCC No. HB-8065) is the one which has been employed most extensively in many experiments, since the cells persist a large part of cellular functions similar to those of normal hepatocytes such as expression of hepatocyte-specific cell surface receptors and synthesis and secretion of plasma proteins (*10*,*11*). Furethermore, because of the high degree of morphological and functional differentiation *in vitro*, HepG2 cell line is a suitable model to study intracellular trafficking, hepatocarcinogenesis, and drug targeting *in vitro* (*12*,*13*).

3. Signaling pathways targeted by flavonoids in HepG2 cell line

Until now, various flavonoids have been known to induce apoptosis and/or inhibit HCC cell proliferation (*14-19*). For example, flavones such as baicalein (*14*), casticin (*15*), apigenin (*16*), isoflavones such as tectorigenin (*17*), and flavonols such as galangin (*18*) and quercetin (*19*) have been reported the induction potency of apoptosis on various HCC cell lines. Various investigations using HepG2 cells have showed effects of flavonoids on signal pathways involving in apoptosis and cell proliferation. Typical mechanisms of flavonoids action on the signal pathways in HCC cells are reviewed below and the corresponding signal pathways and flavonoids are mapped in Figure 2 and summarized in Table 1, respectively.

3.1. *Unfolded protein response (UPR) pathway*

UPR pathway has been extensively implicated in proliferation, angiogenesis, and multidrug resistance of tumors (*20*). Oroxylin A, which is one of the major flavonoids produced by *Scutellaria baicalensis* Georgi (*21*), was demonstrated to depress the viability of HepG2 cells but not the normal hepatic cell line L02 (*22*). In HepG2 cells, oroxylin A treatment induced the emergence of intracellular H_2O_2 by transforming endogenous reactive oxygen species into H_2O_2, which triggered the subsequent activation of PERK-eIF2α-ATF4-CHOP branch of UPR pathway but not in normal L02 cells (*22*). PERK-eIF2α-ATF4-CHOP branch, which is a cellular stress-induced apoptosis pathway in endoplasmic reticulum (ER), includes a serial of molecules such as pancreatic ER kinase (PKR)-like ER kinase (PERK), eukaryotic initiation factor 2α (eIF2α), activating transcription factor 4 (ATF4), and CCAAT/enhancer binding protein homologous protein (CHOP). Then CHOP caused the activation of tribbles homolog 3 (TRB3) and the sequent decrease of p-Akt1/2/3 (Ser473) which is an activated form of Akt protein. Akt, an oncoprotein, is known to be frequently activated in tumor cells and positively related to poor prognosis of HCC (*23*). Since the inactivity of Akt by oroxylin A could stop boosting cancer progress and since the compound could target cancers, oroxylin A is expected as a candidate for HCC therapy (*24*). It is not a unique instance, wogonin, another *O*-methylated flavone also found in *S. baicalensis* Georgi (*25*), can also initiate UPR pathway to inhibit HepG2 cells (*26*). It is reported that wogonin touched off UPR pathway which in the next step blocked Akt phosphorylation (*27*). Overall, oroxylin A and wogonin can inhibit HepG2 cells proliferation through UPR pathway.

3.2. *Mitochondrial- and jun N-terminal kinases (JNK)-mediated apoptosis pathways*

Mitochondrial pathway of apoptosis begins with the permeabilization of the mitochondrial outer membrane (*27*). The permeabilization results in release

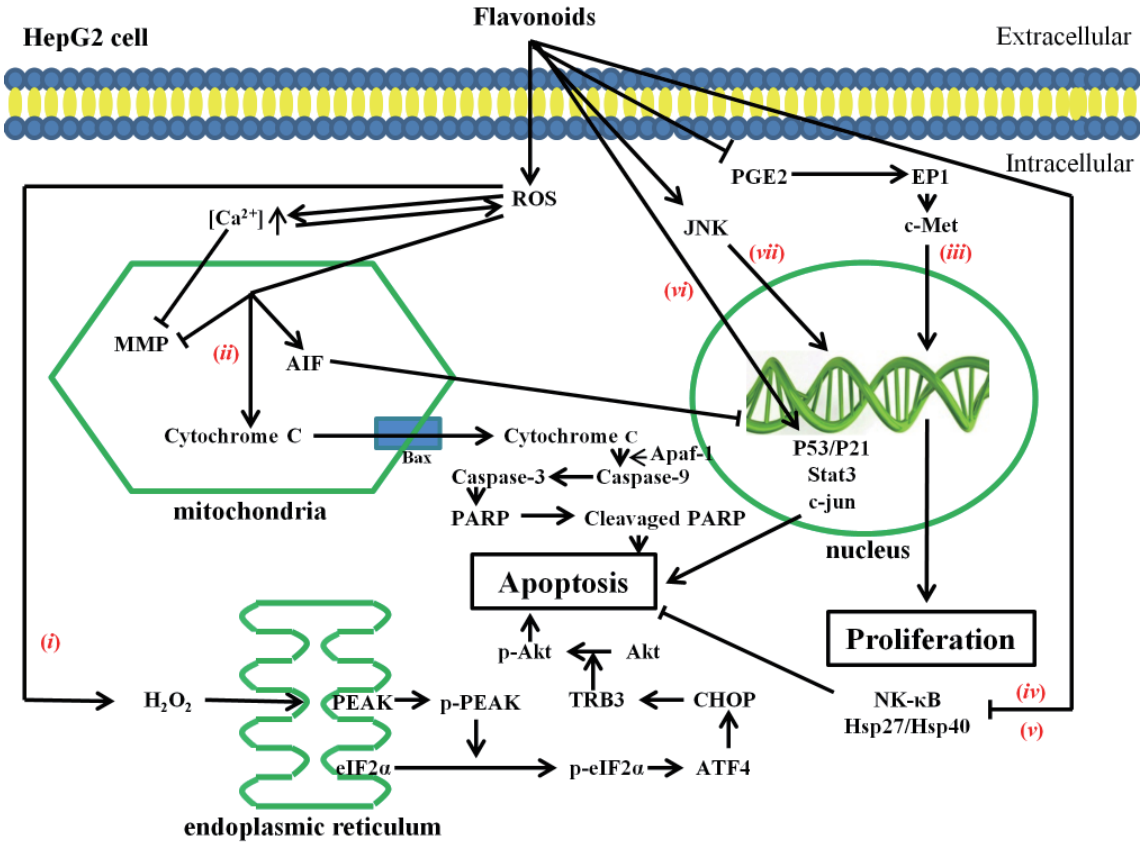

Figure 2. Signaling pathways affected by flavonoids in HepG2. Seven signaling pathways involving apoptosis and cell proliferation are mapped: (*i*) unfolded protein response pathway, (*ii*) mitochondrial-mediated apoptosis pathway, (*iii*) EGFR/c-Met signaling pathway, (*iv*) NF-κB-related pathway, (*v*) Heat shock protein-related pathway, (*vi*) tumor suppressor-related pathway, and (*vii*) JNK-mediated pathway. These pathways are reported to be affected by flavonoids and to involve in anti-HCC actions of flavonoids in HepG2 cell line. ROS, reactive oxygen species; PEAK, pancreatic ER kinase (PKR)-like ER kinase; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer binding protein homologous protein; TRB3, chop and tribble 3; MMP, matrix metalloproteinases; AIF, apoptosis inducing factor; PARP, poly-ADP-ribose-polymerase; PGE2, prostaglandin E2; EP1, prostaglandin E2 receptor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Hsp, heat shock protein.

Table1. Flavonoids discussed in this article

Subfamily	Flavonoids (Synonyms)	Typical origin	*Reference*
Flavone	Baicalein (5,6,7-Trihydroxyflavone)	*Scutellaria baicalensis* roots	*14*
	Casticin (3',5-Dihydroxy-3,4',6,7-tetramethoxyflavone)	*Vitex agnus-castus* leaves	*15,75*
	Apigenin (5,7,4'-trihydroxyflavone)	Orange, tea, chamomile, onion	*16*
	Oroxylin A (5,7-Dihydroxy-6-methoxyflavone)	*Scutellaria baicalensis* Georgi	*21,22,24*
	Wogonin (5,7-Dihydroxy-8-methoxyflavone)	*Scutellaria baicalensis* Georgi	*21,25,72*
	Diosmetin (5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chromen-4-one)	*Rosa agrestis* Savi	*68*
	Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone)	Artichoke (*Cynara scolymus*)	*68*
	5-Methoxy-(3,4"-dihydro-3",4"-diacetoxy)-2",2'-dimethylpyrano-(7,8:5",6")-flavone	*Solanum erianthum* D. Don	*71*
	Catechin (*trans*-(+)-3,3',4',5,7-Flavanpentol)	San-Huang-Xie-Xin -Tang	*73*
	Ladanein (5,6-Dihydroxy-7-methoxy-2-(4-methoxyphenyl)chromen-4-one)	*Marrubium peregrinum* L (Lamiaceae)	*74*
Isoflavone	Tectorigenin (*O*-Methylated isoflavone)	Leopard lily (*Belamcanda chinensis*)	*17,33*
	Genistein (4',5,7-Trihydroxyisoflavone)	*Genista tinctoria*	*35*
Flavonol	Galangin (3,5,7-Trihydroxyflavone)	*Alpinia officinarum*	*18*
	Quercetin (3,5,7,3',4'-Pentahydroxyflavone)	Fruits, vegetables, leaves and grains	*19,54,55*
	(−)-Epi-gallocatechin-3-gallate	Green tea	*47,48*
Flavanone	Xanthomol (1,2-Dihydropyrazolo[3,4-d]pyrimidin-4-one)	Hops	*53*
	8-Bromo-7-methoxychry (2-Bromo-α-ergocryptine)	*Oroxylum indicum* (L.)Vent.	*35*
Flavonolignans	Silymarin ((2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl]chroman-4-one)	*Scutellaria baicalensis* seeds	*67*

of apoptogenic proteins such as cytochrome *c* (*28*), apoptosis inducing factor (AIF) (*29*), and endonuclease G (*30*). In cytoplasm, cytochrome *c* initiates to activate various caspases such as caspases-2, -8, -9, and -10 (*31*) and the caspases in the next step cause cell death by cleaving a number of cellular proteins including DNA repair enzymes such as poly-ADP-ribose-polymerase (PARP) (*32*). A recent paper suggested that tectorigenin, one of the main components of the rhizome of *Iris tectorum* (*33*), induces apoptosis of HepG2 cells mainly *via* the mitochondrial-mediated pathway (*17*). The apoptosis of HepG2 cells was correlated with the production of reactive oxygen species (ROS), increased intracellular [Ca^{2+}], abnormal change of mitochondrial membrane potential, translocation of cytochrome *c*, activation of caspases-9, -8, and -3, and up-regulated transcription of endonuclease G and AIF-related genes in nuclear (*17*). Similar to this report, other studies also suggested the polyphenolic extract, galangin, genistein, and quercetin could induce apoptosis of HepG2 cells *via* changes of ROS and mitochondrial disruption (*18*,*34-36*). Moreover, JNK also play a critical role in a JNK-mediated apoptosis as well as mitochondrial-mediated (*37*). A study showed that 8-bromo-7-methoxychrysin (BrMC) promoted accumulation of intracellular ROS, initiation of caspase-3, and persistently activation of JNK in apoptosis of HepG2 and that, in JNK inhibitor-treated cells, BrMC-mediated apoptosis was partially attenuated (*38*). These suggest that the JNK pathway involves in BrMC-induced apoptosis of HepG2.

3.3 *Epidermal growth factor receptor (EGFR)/c-Met signaling pathway*

c-Met is frequently coexpressed with EGFR family members in human tumors, and it has been demonstrated that these receptor tyrosine kinases (RTKs) can crosstalk to each other and strengthen tumor cell invasion (*39-41*). In the next step, EGFR/c-Met signaling pathway can induce cancer cells proliferation, invasion, and angiogenesis through downstream molecules such as Ras, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and Akt and so on (*42-45*).

It was reported that exogenous prostaglandin E2 (PGE2) stimulates cancer cell invasion through an intricate signaling axis requiring EGFR ligand production and c-Met (*46*). On the other hand, (−)-epigallocatechin-3-gallate (EGCG), one of the amplest bioactive constituents in leaves of green tea, was shown to inhibit HepG2 cell invasion *via* suppressing expression of PGE2 receptor EP1 through activation of EGFR/c-Met signaling (*47*). Besides, treatment of HepG2 cells with EGCG initiated apoptosis and led to a decrease in the phosphorylated insulin-like growth factor (IGF)-1 receptor protein and its downstream signaling elements including the p-ERK (extracellular signal-regulated kinase), p-Akt, p-Stat-3, and p-GSK3B (glycogen synthase kinase 3β) proteins. EGCG also decreased the levels of both IGF-1 and IGF-2 proteins, but increased the levels of the IGFBP-3 (insulin-like growth factor binding protein 3) protein. So, EGCG was considered to be an inhibitor of RTKs (*48*).

3.4 *NF-κB-related pathway*

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA and incorrect regulation of NF-κB is known to be linked to cancers (*49*). Therefore, NF-κB is expected as a target molecule in cancer therapy (*49*). Tumor necrosis factor α (TNF) plays an important role in initiating and perpetuating NF-κB signaling. TNF causes the activation of inhibitor of κB (IκB) kinase (IKK), which in turn phosphorylates and degrades inhibitor kappa B protein α (IκBα) and leads to NF-κB translocation to the nucleus and binding to a specific DNA consensus sequence; all this results in the transcriptional activation of NF-κB regulated genes involved in inflammation, such as cyclooxygenase-2 (COX-2) (*50*). There are reports that HCC can evade apoptosis by a common strategy of NF-κB activation which plays a role of adaptive resistance to apoptosis (*51*) and activates the pro-inflammatory chemokine interleukin (IL)-8 that promotes the progression of HCC (*52*). Xanthohumol, the major prenylated chalcone found in hops, was reported to have anti-HCC activity in NF-κB inhibition (*53*). Thus xanthomol can inhibit HepG2 cell proliferation *via* blocking tumor necrosis factor (TNF)-induced NF-κB activity in HCC cells *in vitro* and not affect viability of normal cells even in ten-fold higher concentration in comparison to that inhibiting HepG2 cells (*53*). Quercetin, a dietary flavonoid, has been shown to have anti-inflammatory effects through the downregulation of the NF-κB pathway (*54*). In a study in HepG2 cells, quercetin was demonstrated to suppress TNF-induced inflammation through downregulation of NF-κB, ERK, JNK, COX-2, and ROS generation (*55*). There are also other flavonoids which were reported to have NF-κB inhibition activity, such as a synthesized flavonoid LYG-202 (*56*), luteolin (*57*), epicatechin (*58*), and hesperidin (*59*).

3.5. *Heat shock protein (Hsp)-related pathway*

Heat shock proteins (HSP) are a class of functionally related proteins involved in the folding and unfolding of other proteins (*60*). Heat shock proteins function as intracellular chaperones for other proteins and monitor cell situation so as to initiate repair mechanism in time (*61*,*62*). As HSP acts as survival factors in cells, targeting HSP will be a new strategy for

cancer treatment (*63*,*64*). A flavonol constituent, quercetin, picked a diverse way to suppress tumor cell proliferation. For example, increased expression of Hsp27 and Hsp40 has been implicated in development of resistance to chemotherapeutic drugs by increasing DNA repair capacity, whereas quercetin is shown to potentiate chemotherapeutics by inhibiting the expression of Hsp27 and Hsp40 (*36*). An another research using SILAC (stable isotope labeling by amino acids in cell culture)-MS (mass spectrometry) assay, which is a technique to quantify the changes of whole protein spectrum, showed that quercetin can significantly suppress HepG2 cell's proliferation *via* inhibiting the expression of HSP in the cells (*65*).

3.6 *Tumor suppressor-related pathway*

A tumor suppressor gene, or anti-oncogene, is a gene that protects a cell from transforming to cancer cell. Tumor-suppressor gene-coding proteins repressively regulate the cell cycle and/or promote apoptosis (*66*). Activation of tumor suppressors could be a significant strategy for inhibiting tumor. Baicalein and silymarin, extract of *Scutellaria baicalensis*, have been reported to have a synergetic anti-tumor effect. They suppressed HepG2 cell proliferation by increasing the ratio of cells in the G0/G1 phase and decreasing those in S-phase, which were associated with up-regulation of tumor suppressors such as Rb, p53, p21Cip1, and p27Kip1 and down-regulation of cyclin D1, cyclin E, CDK4, and phospho-Rb (*67*). A recent study showed that diosmetin and luteolin, extracted respectively from *Rosa agrestis* Savi and artichoke (*Cynara scolymus*), could caused G2/M phase arrest in HepG2 and up-regulation of p53 and p21 proteins *via* CYP1A-catalyzed metabolism which always play role in scavenging chemical carcinogens (*68*).

Tumor cells always try their best to survive through evading apoptosis or promoting proliferation, invasion, and metastasis. Thus, understanding mechanisms of cancer cells survival pathways and flavonoids anti-tumor pathways will benefit anti-HCC medicines selection and therapy design.

4. Anti-HBV/HCV flavonoids

HCC is one of the most common and pernicious malignancies. More than 80% of HCC patients in worldwide obtain chronic hepatitis B and C infections. In China, approximately 95% of HCC patients have chronic HBV infection (*69*). Thus, in another aspect of HCC treatment, anti-virus medicines are also in an emergent need. Recently, various compounds including flavonoids isolated from herbs have been identified to possess anti-HBV or anti-HCV activity (*6*). There was a research report that in HepG2.117, a HBV inducible HepG2 cell line, EGCG suppressed HBV replication by spoiling HBV replicative intermediates of DNA synthesis, resulting in a decreased production of HBV DNA (*70*). In a recent study, Wogonin and 5-methoxy-(3,4"-dihydro-3",4"-diacetoxy)-2",2'-dimethylpyrano-(7,8:5",6")-flavone decreased the expression level of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) proteins and replication level of HBV DNA (*71,72*). In regard to HCV inhibition, catechin, isolated from TCM San-Huang-Xie-Xin-Tang, was identified to cause suppression of HCV replication and lead to a concentration- dependent down-regulation of COX-2 and NF-κB which have particular relevance to HCV-related HCC (*73*). Ladanein was reported to suppress a post-attachment entry progression, rather than RNA replication or HCV assembly and effectively resist major HCV genotypes, including a variant which is resistant to an entry inhibitor (*74*).

As one of the most fatal cancers, especially in East Asia, more and more attention focuses on the treatment on HCC. Owing to the anti-tumor and anti-virus effect of some TCM and other herb medicines, the flavonoids extracted from TCM and other herbs were regarded as ideal candidates for HCC therapy. With further study, a many flavonoids showed anti-HCC or anti-HBV/HCV activity in experiments *in vitro* and/or *in vivo*. Some research data suggested the combination therapy of one flavonoid with other flavonoids or chemotherapeutics could greatly enhance efficiency. Furthermore, there will be more flavonoid medicines being developed and more therapies emerging.

5. Conclusion

In conclusion, flavonoids extracted from TCM and other herb medicine has shown interesting anti-tumor activity on various cancer cells including HCC. More and more flavonoids are continuing to be isolated from TCM and other herbs, which provides a tremendous pool for effective compound screening. Modern molecular biological technology and cell biological technology accelerate the screening. The accumulating effective flavonoids acting on diverse cellular signaling pathways make it possible to optimize the therapy by new medicine alteration and combination of two or more medicines. Based on former data, combination therapy has exhibited higher effectiveness than single drug therapy. Owning to a reality that some flavonoids-rich TCMs are fruits and vegetables, the combination of clinical therapy and planned dietetic therapy may obtain more satisfying results. At present, translating flavonoids into clinical medicines is a major mission for medical researchers.

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