Chemoprevention of liver cancer by plant polyphenols

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A R T I C L E   I N F O

Article history:
Received 14 December 2011
Accepted 2 April 2012
Available online 11 April 2012

Keywords:
Liver cancer
Chemoprevention
Plant polyphenols

A B S T R A C T

Primary liver cancer or hepatocellular carcinoma (HCC) is one of the most frequent tumors representing the fifth commonest malignancy worldwide and the third cause of mortality from cancer. Currently, the treatments for HCC are not so effective and new strategies are needed for its fight. Chemoprevention, the use of natural or synthetic chemical agents to reverse, suppress or prevent carcinogenesis is considered an important way for confronting HCC. Many of the chemopreventive agents are phytochemicals, namely non-nutritive plant chemicals with protective or disease preventive properties. In this review, we focus on plant polyphenols, one of the most important classes of phytochemicals, their chemopreventive properties against HCC and discuss the molecular mechanisms accounting for this activity.

Abstract

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1. Introduction

Primary liver cancer or hepatocellular carcinoma (HCC) is one of the most frequent tumors representing the fifth commonest malignancy worldwide and the third cause of mortality from cancer (Parkin et al., 2001). Most important factors leading to HCC are chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV), prolonged exposure to some dietary carcinogens especially to aflatoxin (Bosch et al., 1999; Pang et al., 2006), alcoholism, and possibly obesity (Neuschwander-Tetri and Caldwell, 2003) and diabetes (El-Serag et al., 2004). Other factors include cirrhosis of various etiologies such as alcoholic cirrhosis and cirrhosis associated with genetic liver diseases, and primary hemochromatosis (Pang et al., 2006; El-Serag, 2001). So, HCC manifests high incidence in areas with high prevalence in HBV (e.g. South-East Asia and sub-Saharan Africa) and HCV (e.g. Southern Europe) (Nordenstedt et al., 2010). In Northern Europe and the United States the primary cause of HCC is alcoholic liver disease and HCV (El-Serag, 2001; Bosch et al., 2005; Michielsen et al., 2005). Treatment options for patients with HCC include surgical resection (Takayama et al., 1998) and liver transplantation, which are only applicable to a small proportion of patients with early tumors (Poon and Fan, 2004). Other treatments for HCC are percutaneous ethanol injection (Ebara et al., 1990), percutaneous radiofrequency ablation (Curley and Izzo, 2002) and transcatheter arterial embolization (Ikeda et al., 1991), but they are used basically for palliation (Poon et al., 2002). As regards chemotherapy, so far, the only drug that is used for HCC is sorafenib but the gain in survival is modest (Horgan et al., 2010). One of the main reasons for which the patients with HCC have poor prognosis is the multicentric occurrence of HCC in the liver of patients with chronic hepatitis or cirrhosis caused especially by HBV or HCV infection (Yamamoto et al., 1999). Thus, even if the first cancer is diagnosed and removed, another malignant cell clone may arise to form a second primary cancer (Moriwaki, 2002). Moreover, there may be recurrence in patients with HCC due to an intrahepatic metastasis, namely the appearance of a cell clone which is derived from the initial transformed cell clone (Chen et al., 2000). The frequency of this recurrent liver cancer is estimated at 20–25% per year (Moriwaki, 2002). In addition, another reason accounting for dismal prognosis of HCC is the deterioration of liver function during HCC treatment (Nagasue et al., 1999; Eguchi et al., 2000). Therefore, due to the fact that the current treatments for HCC are not so effective, new strategies are needed not only to prevent the development or post-therapeutic recurrence of HCC but also not to contribute to any deterioration of liver function.

So far, some strategies have been developed to prevent the development of HCC. One of the most effective strategies of prevention from liver cancer in the general population is vaccination against hepatitis virus infection. For example, HBV vaccination reduced significantly the incidence of HCC in children in Taiwan (Chang et al., 1997). However, vaccination against HCV has not been developed yet, and so other methods, such as screening for HCV in donated blood, are used to prevent HCV transmission via blood transfusion. Moreover, interferon has also been used to reduce the risk of liver cancer by eradicating HCV from patients with chronic liver diseases (Kasahara et al., 1998; Cammà et al., 2001). Other prevention strategies against HCC aim at reducing aflatoxin exposure. Aflatoxins are mycotoxins produced by some Aspergillus species, and found in food such as corn and nuts, particularly under high moisture conditions. In regions, where consumption of aflatoxin-contaminated food is common, the incidence rates of HCC tend to be high (Nordenstedt et al., 2010). Aflatoxin exposure can be reduced by using better methods to harvest, store and process susceptible foods. However, the complete elimination of aflatoxin contamination is not considered possible.

The above prevention strategies belong to primary prevention aiming at the general population or subjects with increased risk for HCC (Moriwaki, 2002). The secondary prevention against HCC aims mainly at patients with either pre-malignant lesions or with recurrent tumor but who had received anti-cancer treatment for the initial tumor. Another important strategy suitable for both primary and secondary prevention is chemoprevention. Chemoprevention is defined as the use of natural or synthetic chemical agents to reverse, suppress or prevent carcinogenic progression to invasive cancer (Sporn and Liby, 2005). Many chemopreventive agents are phytochemicals, namely non-nutritive plant chemicals that have protective or disease preventive properties. Some very informative and extensive reviews regarding the chemopreventive effects of phytochemicals against hepatocarcinogenesis have been published recently (Glaouert et al., 2010; Seren et al., 2008; Mann et al., 2009). In this review, we focus on plant polyphenols, one of the major classes of phytochemicals, their chemopreventive properties against HCC and discuss the molecular mechanisms accounting for this activity.

2. Plant polyphenols

The major structural characteristic of these compounds is one or more hydroxyl groups binding to one or more aromatic rings. Several thousand polyphenolic molecules have been identified in higher plants and several hundred are found in edible plants (Manach et al., 2004). Plant polyphenols are divided into two major groups, flavonoids and non-flavonoids (Manach et al., 2004). Flavonoids share a common flavan core formed with 15 carbon atoms and this class can be divided into flavans (e.g. catechins), flavones (e.g. quercetin, myricetin, kaempherol), anthocyanidins (e.g. cyaniding, delphinidin), flavones (e.g. apigenin, diosmin), flavanones (e.g. naringenin, hesperitin) and chalcones (e.g. phloretin). The non-flavonoids contain an aromatic ring with one or more hydroxyl group. Non-flavonoids include stilbene (e.g. trans-resveratrol), phenolic acids (e.g. caffeic acid, gallic acid), saponin (e.g. ginsenoside), and other polyphenols such as curcumin and tannins. Polyphenols are synthesized by plants for defense against infection and provide protective effects against stress such as ultraviolet light, pathogens and physical damage (Robbins, 2003). Among the interesting biological properties exhibited by plant polyphenols, in recent years, there has been increasing interest regarding cancer prevention. Indeed, a number of in vitro and in vivo studies have shown that plant polyphenols could be used as chemopreventive agents against different cancer types including HCC (Korkina et al., 2009; Ramos, 2008).

2.1. Flavonoids

2.1.1. Epigallocatechin gallate

Epigallocatechin gallate (EGCG), also known as epigallocatechin 3-gallate, is the ester of epigallocatechin and gallic acid. EGCG is
the most abundant polyphenol in tea but is also found in other plants. It is one of the most well studied polyphenolics in relation to HCC.

2.1.1. In vitro studies. EGCG has been shown to inhibit the growth of different human hepatoma cell lines as HepG2, HuH-7, Hep3B and HLE (Huang et al., 2009; Nishikawa et al., 2006). EGCG affects hepatoma cell growth by inducing apoptosis although different molecular mechanisms have been proposed (Nishikawa et al., 2006; Huang et al., 2009; Kuo and Lin, 2003). EGCG at concentrations of 50–100 μg/ml increased tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and cell cycle arrest at G1 phase in HepG2, HuH-7 and PLC/PRF/5 hepatoma cells (Nishikawa et al., 2006). However, in HLE cells, it induced growth arrest but not apoptosis through down-regulation of Bcl-2alpha and Bcl-xl by inactivation of nuclear factor κB (NF-κB) (Nishikawa et al., 2006). Huang et al. (2009) have shown that EGCG blocked the progression of cell cycle at G1 phase by inducing p53 expression and then up-regulating p21 expression in p53 positive Hep3B cells. Likewise, EGCG induced cell cycle arrest and apoptosis in HepG2 cells through induction of p53 protein and the activity of Fas/FasL apoptotic system, respectively (Kuo and Lin, 2003). Interestingly, EGCG instead of growth arrest induced apoptosis in p53 negative Hep3B cells (Huang et al., 2009). Moreover, EGCG induced apoptosis in hepatoma SMMC7721 cells by attenuation of mitochondrial transmembrane potential, modification of Bcl-2 family proteins and activation of caspase-3 and caspase-9 maybe through a pro-oxidant activity (Li et al., 2009). Furthermore, reactive oxygen species (ROS) seemed to be involved in EGCG-induced activation of JNK1 protein, a tumor suppressor protein and a major AMP-activated protein kinase (AMPK) in hepatoma Hepa 1–6 cells (Shirakami et al., 2009). EGCG has also been demonstrated to inhibit thrombin-induced hepatoma cell migration by activation of the protease-activated receptor (PAR) in Hep3B as well as in human primary hepatoma cell cultures (Kauffman et al., 2009). Similarly, two other studies showed that EGCG suppressed the invasion of HepG2 cells (Lu et al., 2007) as well as SK-Hep-1 hepatocellular carcinoma cells (Lee et al., 2007) into basement membranes by reducing the expression of mucin1 (MUC1), a glycoprotein with increased expression profile in various malignancies, and then the secretion of matrix metalloproteinases (MMPs), MMP-2 and MMP-9. It also seems that EGCG possesses anti-angiogenic activity, since it suppressed the expression of vascular endothelial growth factor receptors VEGFR-2 and p-VEGFR-2 in HuH7 cells (Shirakami et al., 2009). A similar effect was observed in HepG2 cells, in which EGCG inhibited hypoxia-induced expression of hypoxia inducible factor 1alpha (HIF-1alpha) protein and its downstream target vascular endothelial growth factor (VEGF) both playing an essential role in tumor angiogenesis (Zhang et al., 2006). It seems that EGCG-induced reduction of HIF-1alpha protein levels is associated with the blocking of both phosphatidylinositol 3-kinase P13K/Akt and extracellular signal-regulated kinase ERK1/2 signaling pathways and the enhancing of protein degradation through the proteasome system (Zhang et al., 2006).

2.1.1.2. In vivo studies. The protective activity of EGCG against HCC has also been supported in animal models. Thus, Matsumoto et al. (1996) showed that administration of EGCG (0.05 or 0.1%) to F344 rats decreased the number and total volume of glutathione-S-transferase placent form (GST-P) positive foci in diethyl nitrosamine (DEN)-induced hepatocarcinogenesis. Moreover, C3H/HeNcrj mice with spontaneous hepatoma when receiving EGCG (0.05% or 0.1% in drinking water) exhibited reduced incidence of hepatoma, from 83% (control) to 56% (0.05% EGCG), and also reduced average number of hepatomas per mouse, from 1.83 (control) to 0.72 (0.05% EGCG) (Nishida et al., 1994). Also, EGCG has been reported to enhance the activity of the anticancer drug doxorubicin in a murine model for chemoresistant HCC by inhibiting the P-glycoprotein (P-gp) efflux pump activity and reducing the expression of multidrug resistance (MDR)1 protein (Liang et al., 2010).

From the above mentioned studies, it is inferred that EGCG induces apoptosis and cell cycle arrest, and exhibits anti-angiogenic and anti-metastatic potential in hepatoma cells by modulating signal transduction pathways. Paradoxically, EGCG may exert its cytostatic effects against cancer cells through a pro-oxidant activity, although it has strong antioxidant properties (Brückner et al., 2012). In addition, a number of animal studies have shown that EGCG prevents chemical-induced HCC. Moreover, a number of studies with tea extracts rich in EGCG have given very promising results for its chemopreventive activity (Tharappel et al., 2008; Hirose et al., 1995; Umemura et al., 2003; Tamura et al., 1997), although there was not always an apparent relationship between EGCG concentration and liver tumor response (Cao et al., 1996). So far, there are no clinical or epidemiological studies available on EGCG chemopreventive activity against HCC. However, use of EGCG in clinical trials for other cancer types, such as cervical cancer, has given optimistic results (Ahn et al., 2003).

2.1.2. Quercetin

Quercetin is one of the most well-studied plant polyphenols found in onions, apples, berries, tea, and red wine.

2.1.2.1. In vitro studies. Among 68 plant polyphenols tested for their ability to inhibit liver cancer cell growth, quercetin along with 4-hydroxyflavone and luteolin were the most potent flavones (Loa et al., 2009). The molecular mechanisms proposed for the inhibitory effect of quercetin on HepG2 cell growth were the inactivation of survival signal proteins as AKT, ERK, protein kinase C (PKC-alpha) as well as the activation of death signals as c-Jun N-terminal kinases (JNK) and PKC-delta (Granado-Serrano et al., 2008). Moreover, quercetin has been shown to inhibit the growth of HepG2 by inducing inactivation of transcription factors NF-kB and subsequently activation of the activator protein-1 (AP-1)/JNK pathway regulating survival/proliferation and death signals (Granado-Serrano et al., 2010). Also, stabilization of p53 protein at the transcriptional and translational level has been suggested to be involved in quercetin-induced apoptosis and cell cycle arrest in HepG2 cells (Tanigawa et al., 2008). Subsequently, quercetin-induced p53 activation resulted in increase of p21 and suppression of cyclin D1 expression in favor of cell cycle arrest as well as in increase of Bax/Bcl-2 ratio in favor of apoptosis (Tanigawa et al., 2008). Also, Chang et al. (2006) showed that quercetin-induced cell cycle arrest of HA22T/VGH hepatocarcinogenic cells was due to production of reactive oxygen species. On the other hand, quercetin has been shown to inhibit reproduction of reactive oxygen species and cytotoxicity, and block the decrease of reduced glutathione (GSH) levels in aflatoxin B1 (AFB1)-treated HepG2 cells (Choi et al., 2010).

In addition, quercetin has been reported to enhance pro-apoptotic action of the anticancer drugs 5-fluorouracil and carboplatin in Hep3B and HepG2 cells by inhibiting expression of heat shock proteins (Hsps) helping cells to adapt drug exposure and survive (Sharma et al., 2009). Moreover, quercetin enhanced the apoptotic effect of the chemotherapeutic agent, paclitaxel, in HA22T/VGH cells (Chang et al., 2006). Finally, combined treatment with quercetin and TRAIL induced apoptosis in TRAIL-resistant HCC cells (Kim et al., 2008). This effect was attributed to increased expression of DR5, a death receptor of TRAIL, by inducing Sp-1 transcription factor as well as to decreased levels of the c-FLIPS protein, an inhibitor of caspase-8 (Kim et al., 2008).
2.1.2.2. In vivo studies. Administration of quercetin at a dose of 10 mg/kg to mice 2 h before DEN decreased by 70.3% and 66.2% the total area and number of preneoplastic lesions respectively (Vásquez-Garzón et al., 2009). The observed protective effect was attributed to enhancement of the antioxidant defense system by quercetin, since there was a reduction in lipid peroxidation levels, increase in GSH and total glutathione as well as the GSH/GSSG ratio, and increase in the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Vásquez-Garzón et al., 2009). Another study proposed that DEN treatment induced beta-glucuronidase activity in mice that converts the glucuronic conjugates of quercetin to the aglycone form that prevents carcinogenesis (Oi et al., 2008). Moreover, quercetin at a dose of 200 mg/kg prevented DEN-induced carcinogenesis in rats as shown by histopathological examination of animal livers, mediated possibly through reduction of DEN-induced liver damage. This was attributed to the antioxidant activity of quercetin, since its administration reduced malondialdehyde (MDA) and GSH levels as well as the activity of GPx and glutathione reductase (GR) (Seuff et al., 2009; Sánchez-Pérez et al., 2005). Contradictory results have been reported as regards the protective effects of quercetin against AFB(1) treatment in in vivo experiments. Siess et al. (2000) showed that quercetin did not reduce AFB(1)-induced c-glutamyl transeptidase-positive (GGT⁺) and GST-P positive pre-neoplastic foci in rat liver as well as it did not increase the AFB(1)-GSH conjugates. However, quercetin was demonstrated to protect from AFB(1)-induced pro-oxidant liver damage in mice through increased GSH levels and SOD activity, and reduced MDA levels (Choi et al., 2010). The different results between the two previous studies may be explained by the different experimental animals used and the different way of quercetin and AFB(1) administration, while none of these studies evaluated directly the effect of quercetin on AFB(1)-induced tumour development. Also, quercetin dietary administration (1%) reduced the number of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ)–induced GST-P positive foci in rat liver (Tsuda et al., 1994). In addition, in a rat model of phenobarbital (PB)-induced liver carcinogenesis, quercetin dietary administration (0.75–1.5%) reduced the evolution of preneoplastic foci into persistent and hyperplastic nodules as well as the number of hepatocellular carcinomas (Tang et al., 1993). On the other hand, quercetin has been found to increase heterocyclic-amine-induced GST-P positive foci formation in rats (Hirose et al., 1999).

Thus, quercetin seems to exert its chemoprevention potential through inhibition and induction of survival and death signaling pathways respectively in liver cancer cells. Moreover, in animal studies, quercetin protects from DEN- or AFB(1)-induced liver carcinogenesis due mainly to its strong antioxidant activity and consequent prevention of ROS-induced DNA mutations in critical genes for cell cycle control, such as p53. Although there are concerns about the toxicity and safety of quercetin, human studies have not shown adverse effects associated with the oral administration of quercetin in a single dose of up to 4 g or after one month of 500 mg twice daily (reviewed by Russo et al., 2012).

2.1.3. Luteolin

Luteolin is most often found in leaves, but it is also seen in celery, rinds, barks, thyme, dandelion, clover blossom and ragweed pollen, thyme, perilla, chamomile tea, carrots, green pepper, olive oil, peppermint, rosemary, navel oranges and oregano.

Among 68 plant polyphenols examined for their inhibitory ability against HCC cells, luteolin was one of the most potent (Loa et al., 2009). Indeed, luteolin has been demonstrated to induce cell cycle arrest at G0/G1 phase and apoptosis through increase in Bax/Bcl-XL ratio and caspase-3 activation in hepatoma cell lines as HepG2, SK-Hep-1, PLC/PRF/5, Hep3B and HA22T/VGH (Chang et al., 2005). Also, HepG2 cells apoptosis induced by luteolin involves increased expression of p53 and p21 proteins resulting subsequently in down-regulation of cyclin dependent kinase 4 (CDK4) expression (Yee et al., 2003). Moreover, luteolin-induced apoptosis in hepatoma HuH-7 cells was associated with increase in ROS levels, while a proteomic analysis exhibited changes in expression levels of peroxiredoxin 6 (PRDX6) and prohibitin (PHB) proteins involved in ROS metabolism (Yoo et al., 2009). Selvendiran et al. (2006) proposed that luteolin induces apoptosis in hepatoma HLF cells by inactivating STAT3 protein, a negative regulator of Fas/CD95 signaling, through ubiquitin-dependent degradation and blocking its CDK5–dependent phosphorylation. Thus, inactivation of STAT3 leads to increased function of Fas/CD95 signaling and then caspase-8 activation (Selvendiran et al., 2006). In the previous study, luteolin administration to mice has also been shown to inhibit xenograft tumor growth (Selvendiran et al., 2006). Also, in H4IIE rat hepatoma cells, luteolin induced apoptosis mediated mainly through the mitochondrial pathway of caspase activation (caspase-2, -3/7 and -9), and pro-oxidant activity as shown by the increased MDA levels (Michels et al., 2005). Apart from apoptosis induction, luteolin has been shown to inhibit metastasis of HepG2 cells by blocking hepatocyte growth factor (HGF) activity (Lee et al., 2006). In particular, luteolin suppresses phosphorylation of c-Met, the membrane receptor of HGF, and then inhibits activation of MAPK/ERKs and PI3K-Akt pathways involved in HepG2 cell metastasis (Lee et al., 2006). Finally, the luteolin anticancer activity, in general, is attributed to its strong inhibitory activity against topoisomerase 1 enzyme catalytic activity (IC₅₀ = 0.66 µM) (Chowdhury et al., 2002).

In summary, the mechanisms for the potential anticarcinogenic effects of luteolin against HCC include mainly induction of apoptosis and cell cycle arrest by action on critical molecular targets for cell survival such as p53, p21, cyclin dependent kinases and caspases in liver cancer cells. Indeed, the induction of caspase-8 and -9 suggests that luteolin may activate both molecular pathways for caspases, the extrinsic and mitochondrial respectively. Moreover, like other polyphenols, luteolin’s apoptosis induction in cell culture studies seems to be mediated through pro-oxidant effects. There are limited data regarding the in vivo chemopreventive activity of luteolin against HCC, and thus to fully elucidate the molecular mechanisms of its action and potential use in clinical trials, more in-depth animal studies are needed.

2.1.4. Silymarin and silibinin

Silymarin, a flavolignan, is the chemically active compound of milk thistle (Silybum marianum) extract. Silymarin is actually a mixture of four isomeric flavonoids with silybinin the most biologically active.

2.1.4.1. In vitro studies. Silibinin has been shown in HepG2 cells to induce cell cycle arrest at G0/G1 phase and apoptosis (Ramakrishnan et al., 2009a; Chen et al., 2009; Varghese et al., 2005). The proposed molecular mechanisms accounting for these effects involve increased levels of p53, Bax, APAF-1 and caspase-3 pro-apoptotic proteins as well as of cytoplasmic cytochrome c indicating mitochondrial membrane disruption (Ramakrishnan et al., 2009a). On the other side, the levels of beta-catenin, cyclin D1, c-Myc and PCNA anti-apoptotic proteins were decreased (Ramakrishnan et al., 2009a). Similarly, Chen et al. (2009) demonstrated that silymarin induced apoptosis in HepG2 cells through up-regulation of Rb, p53, p21 and p27 proteins and downregulation of cyclin D1, cyclin E, CDK4 and phospho-Rb, while it had no effect on normal Chang liver cells. In addition, silybin inhibited HepG2 cell proliferation and metastatic potential through suppression of MMP-2 activity and ERK1/2 cascade pathway, the latter mediated by increased expression of Raf kinase inhibitor protein (RKIP) and sprout-related proteins 1 and 2 (Sprd-1 and -2).
Also, silibinin suppressed accumulation of HIF-1alpha protein with a concomitant decrease in VEGF levels in Hep3B cells indicating a potential anti-angiogenic activity (García-Maceira and Mateo, 2009). Silibinin-induced downregulation of HIF-1alpha at a translational level was due to dephosphorylation of mammalian target of rapamycin (mTOR) and its downstream targets ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) (García-Maceira and Mateo, 2009).

Silymarin has also been reported to inhibit expression of hepatitis C virus mRNA and protein in Huh-7 and CN53 cells (Bonifaz et al., 2009). As mentioned above, infection from hepatitis C virus is one of the major causes of HCC.

2.1.4.2. In vivo studies. Cui et al. (2009) have shown silybinib to reduce development of hepatoma Huh-7 xenografts in nude mice. In concordance with results from in vitro studies, the observed effect was attributed to cell cycle arrest at G1 phase and apoptosis induction mediated through decrease in nuclear NFκB content, phosphorylation of Rb, survivin, ERK and Akt, suggesting involvement of PTEN/PI(3)K/Akt and ERK signaling pathways (Cui et al., 2009). Moreover, silymarin administration to rats reduced the number of DEN-induced liver nodules by enhancing the antioxidant defense system, since it was observed that there was a decrease in lipid peroxidation and increase in GSH levels and antioxidant enzyme activity such as SOD, catalase, GPx and GR (Ramakrishnan et al., 2006). The same research group showed silymarin to reduce liver density of mast cells (their recruitment in tumor regions playing an important role in invasion and angiogenesis) and MMP-2 and -9 expressions in a DEN-induced rat liver cancer model (Ramakrishnan et al., 2009b).

Thus, the potential use of silymarin and its most active constituent, silibinin, as chemopreventive agents against HCC is based on their capability to induce pro-apoptotic and reduce anti-apoptotic proteins in hepatoma cells. In addition, they have been shown to possess anti-metastatic and anti-angiogenic potential in cell culture studies. Moreover, their pro-apoptotic effects on liver cancer cells have been confirmed in in vivo experiments. Interestingly, a number of clinical studies have been performed with silymarin investigating its hepatoprotective activity (Kaur and Agarwal, 2007). For example, a study conducted with RealSIL-IBI, which basically is a complex of silibinin with vitamin E and phospholipids, in patients with non-alcoholic fatty liver disease found the complex to improve insulin resistance and fatigue (Kaur and Agarwal, 2007). Su et al. (2003) showed that silymarin reduced G2/M cell cycle arrest and apoptosis in Hep3B, Huh7, PLC and HA22T hepatoma cell lines through increased caspase-3 activity resulting in poly(ADP-ribose)polymerase (PARP) activation, reduced expression of the anti-apoptotic proteins Bcl-2 and Bcl-XL and inhibition of Cdc2 kinase activity, a key player in cell cycle regulation (Su et al., 2003). Yeh et al. (2007) demonstrated genistein to induce endoplasmic reticulum stress as indicated by increased growth arrest and DNA damage-inducible 153 (GADD153), GRP78 and caspase-12 levels, ROS production and mitochondrial damage in Hep3B cells. It was also shown that genistein-induced Hep3B cell growth inhibition was mediated through caspase-3, -8 and -9 activation, poly(ADP-ribose)polymerase (PARP) cleavage, p38-beta mitogen-activated protein kinase (MAPK) inhibition and pro-apoptotic Bid protein cleavage (Jin et al., 2009a,b). In addition, genistein-induced cell growth as well as metastasis of Bel 7402 and MHCC97-H hepatoma cells was associated with increased expression of focal adhesion kinase (FAK), a protein regulating motility, apoptosis and proliferation (Gu et al., 2009, 2005). Also, genistein ability to inhibit (Ki = 50 μM) cytochrome P450 (CYP450) phase 1 (e.g. CYP1A1) and induce phase II (e.g. sulfotransferases) xenobiotic metabolism enzymes may also account for its chemopreventive properties (Shertzer et al., 1999; Chen et al., 2008).

2.1.5.2. In vivo studies. Genistein administration (200 mg/kg) subcutaneously to rats protected from DEN-induced hepatocellular proliferation through apoptosis induction and caspase-3 activation (Chodon et al., 2007). Moreover, genistein administration inhibited growth and microvessel formation in Bel 7402 cells implanted in mice (Gu et al., 2005). In addition, in a mouse HCC model induced by MHCC97-H hepatoma cells transplantation, genistein inhibited tumour growth and cancer cell metastasis (Gu et al., 2009).

Thus, genistein induces in hepatoma cells growth inhibition, apoptosis and metastasis by modulating the expression of anti-apoptotic, pro-apoptotic and regulating motility proteins, as well as the activity of cyclin dependent kinases regulating cell cycle. These in vitro effects have also been confirmed in animal studies, since genistein induced apoptosis and inhibited metastasis of HCC induced by either chemicals or implanted hepatoma cells. Genistein has not been tested in clinical trials in HCC patients. However, it has been used in clinical trials evaluating efficacy and safety of genistein in patients with prostate cancer (Perabo et al., 2008). For example, a study by deVerre White et al. (2004) showed that genistein-rich extract reduced prostate-specific antigen levels (in one case by more than 50%) in patients with prostate cancer. However, it should be mentioned that a recent epidemiological study showed that isoflavone (i.e. genistein and daidzein) consumption was associated with increased risk for HCC in women (Kurahashi et al., 2009).

2.1.6. Daidzein

Like genistein, daidzein is an isoflavone found in a number of plants with soybeans being the main food source. Su et al. (2003) showed daidzein to inhibit hepatoma (i.e. HepG2, Hep3B, Huh7, PLC and HA22T) cell growth, induce apoptosis through caspase-3 activation and PARP cleavage. Daidzein was also demonstrated to affect the redox status in hepatoma cells although the data are conflicting since it induced mRNA catalase expression but at the same time caused a mild oxidative stress (Kampkötter et al., 2008; Röhrdanz et al., 2002). However, an in vivo study showed that daidzein administration (50 mg/kg) to rats increased antioxidant enzymes activity such as SOD, catalase, GPx, GST, DT-diaphorase (DTD) and GSH levels in liver (Mishra et al., 2009). Like genistein, daidzein was shown to inhibit phase I xenobiotic metabolism...
enzyme activity such as CYP1A1 that may activate pro-carcinogens (Shertzer et al. 1999).

2.2. Non-flavonoids

2.2.1. Stilbenes (trans-resveratrol)

The most well-known member of the stilbenes is trans-resveratrol (3′,4′,5′-trihydroxy-trans-stilbene) present in several plants which is produced when pathogens such as bacteria or fungi attack them (Jean et al., 2002). High amounts of trans-resveratrol can be found in grapes and grape skins used for wine production, raspberries, mulberries, blueberries cranberries, peanuts, and in certain types of pine (Jean et al., 2002). Trans-resveratrol is one of the most well-studied plant polyphenols due to its important biological properties including cardioprotective (Shukla et al., 2010), neuroprotective (Robb and Stuart, 2010), anti-aging (Queen and Tollefsbol, 2010), antiviral (Huang et al., 2010) and antioxidant activities (Das and Das, 2010). Also, several studies have shown trans-resveratrol to exert anticancer activity against different cancers (reviewed in Brissetti et al., 2009) as HCC.

2.2.1.1. In vitro studies. Trans-resveratrol has been shown to inhibit the growth of different HCC cells such as human hepatoma HepG2 (Kuo et al., 2002; Stervbo et al., 2006) Hep3B (Kuo et al., 2002) and H22 (Sun et al., 2002), rat hepatoma FAO (Delmas et al., 2000) and H4IIE (Michels et al., 2006), and rat ascites hepatoma AH109A (Kozuki et al., 2001). The basic mechanism accounting for the growth inhibitory effect of trans-resveratrol against cancer hepatoma cells seems to be the induction of apoptosis and cell cycle arrest. For example, Sun et al. (2002) showed that the growth inhibitory effect of trans-resveratrol against hepatoma H22 cells was attributable to apoptosis. Also, trans-resveratrol induced apoptosis through caspase activation in H4IIE cells (Michels et al., 2006). Moreover, Kuo et al. (2002) showed that trans-resveratrol at concentration of about 40–80 μM inhibited the growth of p53-positive HepG2 cells but not the p53-negative Hep3B liver cancer cells, suggesting that apoptosis induction was due to a p53-dependent pathway. Moreover, the cells were arrested in G1 phase and there was increase in the expression of p21 protein, a marker of cellular senescence, as well as of pro-apoptotic Bax protein (Kuo et al., 2002). In another study, trans-resveratrol at a concentration of 0.1 M was shown to induce apoptosis through cell cycle arrest in G1 and G2/M phases in HepG2 cells (Notas et al., 2006). However, in the same study, the antiproliferative effect on liver cancer cells was also attributed to the antioxidant properties of trans-resveratrol. In particular, trans-resveratrol at nanomolar and picomolar levels modulated the NO/NOS system by increasing iNOS and eNOS expression, NOS activity and NO production, while inhibition of NOS enzymes attenuated its antiproliferative effect (Notas et al., 2006). Another molecule playing role in trans-resveratrol-induced apoptosis of hepatoma cells is caveolin-1 (CAV1). CAV1 protein is a member of the caveolin family and its expression was shown to be reduced or absent in most human cancer cells. Over expression of CAV1 has been shown to block anchorage-independant growth of transformed cells (Chidlow and Sessa, 2010). Yang et al. (2009) have shown recently that CAV1 through its cholesterol shuttle domain enhances trans-resveratrol transport in HepG2 cells, and so over-expression of CAV1 by stable transfection in HepG2 cells increases its anti-proliferative and pro-apoptotic effects. Moreover, pre-treatment of HepG2 cells with 100 μM of trans-resveratrol induces CAV1 expression, which in turn seems to facilitate trans-resveratrol transfer in HepG2 cells and subsequently induction of cell apoptosis through the p38 mitogen activated protein kinase (MAPK) pathway and caspase-3 expression (Yang et al., 2009). In the same study, apoptosis was induced by trans-resveratrol at 100 μM concentration but after 72 h treatment (Yang et al., 2009). Furthermore, trans-resveratrol has been reported to decrease S-phase HepG2 cells at high concentration (100–200 μM), although it increased them at low concentration (10–50 μM) (Kocsis et al., 2005). In addition, Parekh et al. (2011) have found that trans-resveratrol inhibited growth of HepG2 cells due to reduction of cyclin D1, p38MAPK, Akt and PaK1 expression and activity. In the same study, there was increase in extracellular signal-regulated kinase (ERK) activity in trans-resveratrol-treated HepG2 cells suggesting sensitization to apoptosis (Parekh et al., 2011). However, in contrast to previous studies, De Ledinghen et al. (2001) have shown that trans-resveratrol decreased HepG2 cell proliferation due to not to cytotoxicity or apoptosis but to a post-receptor mechanism.

Furthermore, the chemopreventive activity of trans-resveratrol may be exerted by the inhibition of cytochrome P450 (CYP450) enzymes known to play an important role in the chemical activation of xenobiotics to carcinogens. For example, trans-resveratrol at 100 μM inhibited tert-butylhydroquinone-induced activity of CYP1A1 in murine hepatoma Hepa 1c1c7 cells, probably through binding to aryl hydrocarbon receptor (Gharavi and El-Kadi, 2005).

Also, studies in cell cultures have shown the anti-invasive properties of trans-resveratrol against hepatoma cells. For example, trans-resveratrol inhibited invasion of AH109A rat ascites hepatoma cells at lower concentrations although it suppressed proliferation only at higher concentrations (the concentration range was 25–200 μM) (Kozuki et al., 2001). Moreover, in the same study, trans-resveratrol-loaded rat serum restrained the invasion of AH109A cells suggesting that trans-resveratrol may be effective after oral administration (Kozuki et al., 2001). This anti-invasive activity of trans-resveratrol was probably due to antioxidant properties of the molecule and was independent from its anti-proliferative activity (Kozuki et al., 2001). The latter conclusion was supported by another study demonstrating the anti-invasive effect of resveratrol-loaded rat serum on ROS-induced invasion of AH109A cells (Miura et al., 2004).

Another mechanism accounting for the chemopreventive activity of resveratrol is its anti-angiogenic effect. For example, Zhang et al. (2005) have shown trans-resveratrol to reduce vascular growth endothelial factor (VEGF) expression through hypoxia-inducible factor-1α (HIF1α) inhibition in HepG2 cells growing under hypoxia conditions.

Apart from trans-resveratrol, other stilbenes have also shown chemopreventive activity against HCC. For example, pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene), a natural dimethylated analog of trans-resveratrol, has been reported to suppress 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced invasion, migration and metastasis of HepG2 cells (Pan et al., 2009). These effects were attributed to the inhibitory effects of pterostilbene on TPA-induced increase in MMP-9, VEGF, epidermal growth factor (EGF), and epidermal growth factor receptor (EGFR) expression (Pan et al., 2009). The pterostilbene-induced inhibition of MMP-9 expression was probably due to inhibition of the transcriptional factors activity nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1). The latter conclusion was also supported by the fact that pterostilbene inhibited the TPA-induced activation of molecules being upstream of AP-1 and NF-kB, namely ERK1/2, p38 MAPK, c-Jun N-terminal kinases1/2 (c-JNK1/2) and phosphatidylinositol 3-kinase (P13K)/Akt and protein kinase C (PKC) (Pan et al., 2009).

2.2.1.2. In vivo studies. A number of studies have also shown the chemopreventive potential of trans-resveratrol in animal models. Khanduja et al. (2004) found that administration of trans-resveratrol for two weeks at a dose of 2.5 mg/kg body weight in mice inhibited DEN-induced levels of ornithine decarboxylase (ODC) in liver as well as cyclooxygenases activities. ODC is a rate limiting
enzyme in polyamine biosynthesis pathway and is closely related to cellular proliferation and carcinogenesis (Wolter et al., 2004). Cyclooxygenases are enzymes responsible for the formation of prostanooids which are involved in inflammation process, and specifically cyclooxygenase-2 (COX-2) is implicated in progression and angiogenesis of cancers (Wu et al., 2010). Bishayee and Dhir (2009) showed that orally administration of trans-resveratrol at doses from 50 to 300 mg/kg body weight for 20 weeks reduced the incidence, total number and multiplicity of visible hepatocyte nodules in rats in a two-stage carcinogenesis model involving initiation with DEN and progression with phenobarbital. The anti-carcinogenic effect was due to apoptosis induction through increase of pro-apoptotic protein Bax and decrease of the anti-apoptotic protein Bcl-2 (Bishayee and Dhir, 2009). The previous chemopreventive activity of trans-resveratrol was also attributed to attenuation of oxidative stress and suppression of inflammatory response mediated by nuclear factor E2-related factor (Nrf2) (Bishayee et al., 2010a) as well as by heat shock protein (Hsp70), COX-2 and NF-KB (Bishayee et al., 2010b). The same research group has also examined the possible cardio toxic effects of trans-resveratrol since it was found to be a COX-2 inhibitor but the results showed that it did not have any toxicity on rat heart (Luther et al., 2011). However, in another study, administration of trans-resveratrol (0.005% w/w) in the diet did not prevent polychlorinated biphenyls (PCBs)-induced tumorigenesis in rat liver (Tharappel et al., 2008).ever, in another study, administration of trans-resveratrol did not have any toxicity on rat heart (Luther et al., 2011). How was found to be a COX-2 inhibitor but the results showed that it ined the possible cardiotoxic effects of trans-resveratrol since it ined the possible cardiotoxic effects of trans-resveratrol since it 2.2.3. Phenolic acids
2.2.3.1. Caffeic acid. Caffeic acid is a hydroxycinnamic acid found in most plants including coffee beans, nuts, berries and grains.
2.2.3.1.1. In vitro studies. The hydroxycinnamic acids, caffeic acid (3,4-dihydroxycinnamic acid), one of the most important members.
2.2.2. In vitro studies. Also, curcumin inhibited DEN- and N-bis(2-hydroxypropyl) nitrosamine (DHPN)-induced multiplicity and incidence of HCC in mice (Chuang et al., 2000; Huang et al., 2008). A similar effect was observed in rats, as curcumin inhibited the development of DEN-induced altered hepatic foci (Shukla and Arora, 2003; Sreepriya and Bali, 2005). In addition, in vitro studies, curcumin administration was shown to possess anti-metastatic activity of the highly invasive hepatoma cell line SK-Hep-1 as well as in CBO140C12 cells due to, at least in part, inhibition of MMP-9 (Lin et al., 1998; Ohashi et al., 2003).

Curcumin
Curcumin is the principal curcuminoid of the spice turmeric, which is a member of the ginger family. Curcumin is also one of the most well-studied plant polyphenols regarding its effects on HCC.

2.2.2.1. In vitro studies. It has been shown that curcumin inhibited HepG2 cell growth by inducing apoptosis through increased accumulation of p53 protein (Jiang et al., 1996). Cao et al. (2006, 2007) also showed that pro-oxidant activity and mitochondrial DNA damage account mainly for curcumin-induced HepG2 cell growth inhibition. Curcumin-induced apoptosis due to mitochondrial dysfunction mediated by translationally controlled tumour protein (TCTP), Mcl-1 and Bcl-2 has also been reported in hepatoma J5 cells (Cheng et al., 2010). The previous study also showed that curcumin induced endoplasmic reticulum stress, thus increasing GADD153 protein expression, an activator of pro-apoptotic proteins (Cheng et al., 2010). In addition, curcumin inhibited HA22T/VGH hepatoma cell line growth and induced apoptosis dependent on increased caspase-3 and -9 activities (Notarbartolo et al., 2005). Curcumin also induced cell cycle arrest at G2/M phase by up-regulating Chk-1 protein, a kinase that phosphorylates cdc25, and thus preventing cells from entering to mitosis (Wang et al., 2008). In addition, the Notch1 receptor and signaling pathway, that prevents cells from differentiation and apoptosis, was shown to be inhibited by curcumin in HCC cells such as Hep3B, SK-Hep-1 and SNU449 (Ning et al., 2009). Moreover, curcumin was demonstrated to possess anti-angiogenic potential, since it inhibited levels of both VEGF and Hif-1alpha proteins in HepG2 and endothelial cells (Bae et al., 2006). Also, curcumin was shown to inhibit metastasis of the highly invasive hepatoma cell line SK-Hep-1 as well as in CBO140C12 cells due to, at least in part, inhibition of MMP-9 (Lin et al., 1998; Ohashi et al., 2003).

2.2.2.2. In vivo studies. Also, curcumin inhibited DEN- and N-bis(2-hydroxypropyl) nitrosamine (DHPN)-induced multiplicity and incidence of HCC in mice (Chuang et al., 2000; Huang et al., 2008). A similar effect was observed in rats, as curcumin inhibited the development of DEN-induced altered hepatic foci (Shukla and Arora, 2003; Sreepriya and Bali, 2005). In accordance with in vitro studies, curcumin administration was shown to possess anti-metastatic activity of the highly invasive hepatoma cell line CBO140C12 hepatoma cells implanted in mice (Ohashi et al., 2003). Furthermore, oral administration of curcumin was shown to exert anti-angiogenic effects mediated through reduction of VEGF and COX-2 levels on HepG2 cells implanted in mice (Yousungnoen et al., 2005, 2006). One of the mechanisms accounting for the chemopreventive activity of curcumin may be its ability to induce phase II xenobiotic enzymes such as GST in rats and mice, and antioxidant ones such as glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and catalase (Piper et al., 1998; Iqbal et al., 2003).

In summary, curcumin’s suppression against HCC cells is largely due to inhibition of abnormal cell proliferation and apoptosis through modulation of relevant signaling pathways. Curcumin has also been shown in vivo to inhibit HCC induced by chemicals or implanted hepatoma cells. Moreover, both in vitro and in vivo studies exhibited anti-angiogenic and anti-metastatic properties of curcumin against hepatocarcinogenesis. Although, there are not any clinical trials with curcumin in HCC patients, the results from phase I/II clinical trials have shown that curcumin’s oral administration is well-tolerated and has no or little toxic effect (Sharma et al., 2001). Moreover, recent studies have shown that curcumin exerts chemopreventive and chemotherapeutic effects on patients with different type of cancers, such as oral, breast, prostate, pancreatic and colorectal cancers (Mimeault and Batra, 2011; Cheng et al., 2001; Dhillon et al., 2008). In addition, recent studies have suggested fabrication of an injectable microparticle formulation that can sustain curcumin release over a 1-month period to overcome the extreme lipophilicity and instability of curcumin and thus enhancing its chemopreventive activity (Shahani and Panyam, 2011).
of phenolic acid group, and ferulic acid (4-hydroxy-3-methoxycinnamic acid), have been shown to inhibit HepG2 cell growth and induce apoptosis in a dose-dependent manner (Lee, 2005). Moreover, these effects were attributed to a pro-oxidant activity of the tested polyphenolic acids, and specifically to the induction of NADPH oxidase enzyme (Lee, 2005). A similar pro-oxidant activity of caffeic acid has also been reported in human cells subjected to genotoxic damage (Stagos et al., 2007). Several studies have investigated the potential hepatoprotective activity of the caffeic acid derivative, caffeic acid phenethyl ester (CAPE), found especially in honey propolis (Na et al., 2000). Different mechanisms have been reported for the anticarcinogenic activity of CAPE in hepatoma cells, such as the restoration of gap junctional intercellular communication (GJIC) in rat liver tumorigenic WB-ras2 cells (Lee et al., 2004a; Joo et al., 2003; Na et al., 2000). At a concentration of 12.5 μM, CAPE induced inhibition of COX-2 expression and increase of connexin-43 phosphorylation, a major protein modulating GJIC through inhibition of NF-kB transcriptional activity (Lee et al., 2004a). Furthermore, CAPE has been shown to inhibit the binding of NF-kB transcription factor to the promoter of S100A6 gene encoding an acidic calcium binding protein with high expression in several tumors, and consequently suppressing its expression (Joo et al., 2003). Both caffeic acid and CAPE at low concentrations (1–2 mM) have been found to be inhibitors of MMPs as MMP-9 and -2 involved in tumor cell invasion and metastasis, by inhibiting the function of NF-kB in hepatocarcinoma Hep3B liver cells (Jin et al., 2005; Chung et al., 2004). Interestingly, CAPE has shown selective growth inhibitory activity against Hep3B cells but not in normal mouse hepatocytes (Jin et al., 2005).

### 2.2.3.1.2. In vivo studies

In vivo studies have also shown the possible protective activity of caffeic acid or its analogues against HCC. For example, caffeic acid administration at a dietary level of 2% for two years to F344 rats inhibited the development of naturally occurring GST-P positive foci (preneoplastic lesions in the liver) by 58% (Hagiwara et al., 1996). Also, CAPE administration (20 mg/kg) to Wistar rats 12 h before cancer initiation with DEN prevented liver hepatocarcinogenesis (Carrasco-Legleu et al., 2006, 2004). In particular, at the 25th day after DEN treatment, CAPE reduced the increase of GST+ foci (preneoplastic lesions in the liver) by 84%, the liver expression of both GST and GST mRNA by 100% and 90%, respectively, the nuclear localization of the p65 subunit of the nuclear transcription factor NF-kB (NF-kB activation leads to tumor cell proliferation) by 85%, and the induction of thioribarbituric acid reactive species (TBARS) by 100% (Carrasco-Legleu et al., 2006, 2004). Thus, these results showed that CAPE exerts protective effects against promotion of hepatocarcinogenesis possibly through an anti-oxidative mechanism (Carrasco-Legleu et al., 2006). Also, another study demonstrated that the protective activity of CAPE against DEN-induced rat liver damage is due to modification of enzyme activity related to CYP450 isoforms proposed to function as DEN bioactivators (Beltrán-Ramírez et al., 2008).

Concluding, both in vitro and in vivo studies have shown that caffeic acid and its derivative, CAPE, inhibit growth and metastasis of HCC through modulation of expression of proteins involved mainly in NF-kB molecular pathway. On the other hand, there is a discrepancy between in vitro and in vivo studies since the former showed caffeic acid to induce apoptosis due to increase in ROS production, while the latter suggest that antioxidant activity accounts for its hepatoprotection. The same contradiction exists for other herein mentioned polyphenols, and further research is needed for its elucidation. In addition, clinical trials are needed to examine the chemoprotective properties of these phenolic acids in HCC patients.

### 2.2.3.2. Ellagic acid

Ellagic acid is a natural phenolic acid found in numerous fruits and vegetables including blackberries, raspberries, strawberries, cranberries, walnuts, pecans, pomegranates, wolfberry and other plant foods. Dietary administration (0.4%) of ellagic acid has been reported to decrease the size of GST-P positive foci, although it increased their number in a rat model of DEN-induced hepatocarcinogenesis (Tharappel et al., 2008). Moreover, the number of AFB1-induced GST+ foci were reduced after dietary administration (0.005%) of ellagic acid to rats (Sonii et al., 1997). In addition, in the previous study, ellagic acid also prevented AFB1-induced mutations in Salmonella strains TA98 and TA100 (Sonii et al., 1997). Also, ellagic acid has been demonstrated to reduce N-2-fluorenylacetamide (FAA)-induced GGT+ foci and hepatocellular neoplasms in rats (Tanaka et al., 1998). In another study, the protective effects of ellagic acid against DEN-induced liver tumors in C3H mice were ambiguous, since it decreased the number of adenomas without affecting the number of foci of altered hepatocytes and of total lesions (Pereira, 1995).

### 2.2.3.3. Protocatechuic acid

Protocatechuic acid is widely distributed in plants as a constituent of many aromatic compounds, catechol tannins, resins, wood gums, lignified wood, and various flavone and anthocyan pigment. Protocatechuic acid has been shown to inhibit HepG2 cell growth through induction of JNK and p38 proteins (Yip et al., 2006). Yin et al. (2009) showed protocatechuic acid to inhibit cell growth, induce apoptosis through mitochondrial membrane disruption and caspase-3 and -8 activation, exhibit anti-metastatic potential by reducing intercellular adhesion molecule (ICAM)-1 level, and possible anti-angiogenic and anti-inflammatory activity by reducing VEGF, interleukin (IL)-6 and (IL)-8 levels.

### 2.2.4. Capsaicin

Capsaicin is the active component of chili peppers, which are plants belonging to the genus Capsicum. In vitro studies have shown capsaicin to induce apoptosis in HepG2 cells (Huang et al., 2009; Baek et al., 2008; Joung et al., 2007; Kim et al., 2005; Lee et al., 2004b). In particular, Huang et al. (2009) demonstrated that capsaicin-induced apoptosis in HepG2 cells is associated with increased levels of ROS, intracellular Ca²⁺, p53 protein, cytochrome c protein, an indicator of mitochondrial membrane disruption, growth arrest and DNA damage-inducible 153 (GADD153) protein, and caspase-3 activity, and decreased levels of the anti-apoptotic proteins Bcl-2 and Bax. Another study suggested that the capsaicin-induced inhibition of NAD(P)H:quinone oxidoreductase (NQO1) enzyme activity leads to increased ROS levels in HepG2 cells (Joung et al., 2007). The increased ROS levels, in turn, result in activation of Akt and increased nuclear translocation of NF-E2-related factor (Nrf2) that binds to the antioxidant response element (ARE), thus causing the expression of heme oxygenase-1 (HO-1), an enzyme conferring cytoprotection against oxidative stress (Joung et al., 2007). Likewise, Kim et al. (2005) have reported association of capsaicin-induced apoptosis in HepG2 cells with increased Ca²⁺ levels. The previous research group has also suggested that inhibition of NADPH oxidase is involved in capsaicin-induced ROS levels in HepG2 cells (Lee et al., 2004b). The results from a proteomic study support the capsaicin-induced increased ROS levels in HepG2 cells since the protein expression pattern showed mainly changes in the levels of oxidative stress and antioxidant enzymes (Baek et al., 2008). Also, capsaicin has been shown to induce apoptosis in SK-Hep-1 HCC cells mediated through down-regulation of anti-apoptotic Bcl-2 protein and upregulation of pro-apoptotic protein Bax and caspase-3 (Jung et al., 2001).

However, apart from the above in vitro and in vivo studies, a number of epidemiological studies suggest that consumption of hot peppers, which are rich in capsaicin, may increase cancer risk,
including liver cancer (Bode and Dong, 2011; Lopez-Carrillo et al., 1994). On the other hand, hot pepper consumption should not be considered equivalent to the use of pure capsaicin. Moreover, clinical studies have shown natural capsaicin to inhibit the growth of leukemic cells (Luo et al., 2011; Ito et al., 2004).

### 3. Future directions for studying the chemopreventive effects of plant polyphenols against HCC

As described above, there is a considerable body of evidence from in vitro and in vivo studies (Tables 1 and 2) suggesting that plant polyphenols are promising candidate agents for HCC chemoprevention. Of course, more research is needed to understand better the mechanisms through which they can exert their protective effects. A very promising approach for elucidating the molecular mechanisms accounting for the chemopreventive effects of plant polyphenols is the use of omics methods.

This omics approach involves measurement of gene expression profiles, with microarray technology or next-generation deep sequencing, focusing on mRNA. The application of proteomic or metabolomic technologies is also being investigated intensely, but gene expression technologies appear to be more mature at this time (see MAQC initiative) (Shi et al., 2006). The multistep development of tumors requires the acquisition of certain biological capabilities (e.g. sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction), described as the hallmarks of cancer (Hanahan and
### Table 2

In vivo effects of plant polyphenols on the development and growth of liver cancer.

<table>
<thead>
<tr>
<th>Plant polyphenol</th>
<th>Species, strain and sex</th>
<th>Agent and dose to induce the tumour</th>
<th>Biomarkers affected</th>
<th>Outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin 10 mg/kg in water</td>
<td>Male Fischer-344 rats</td>
<td>DEN 200 mg/kg i.p.</td>
<td>antioxidant defense system; lipid peroxidation levels; GSH; GSH/GSSG ratio; SOD; [catalase]; GPx</td>
<td>Decreased the total area and number of preneoplastic lesions in liver cancer</td>
<td>Vásquez-Garzón et al. (2009)</td>
</tr>
<tr>
<td>50 mg/kg i.p.</td>
<td>Male Fischer-344 rats</td>
<td>Initiation DEN 200 mg/kg; promotion 2-AAF 20 mg/kg</td>
<td></td>
<td>Prevented development of g-glutamyl transpeptidase-positive lesions in liver</td>
<td>Sanchez-Perez et al. (2005)</td>
</tr>
<tr>
<td>200 mg/kg oral</td>
<td>Male Wistar rats</td>
<td>DEN 20 mg/kg oral</td>
<td>MDA; GSH; GPx; GR; mutations in p53 gene</td>
<td>Prevented from liver cancer as shown the histopathological examination</td>
<td>Seufi et al. (2009)</td>
</tr>
<tr>
<td>15, 30, 45 mg/kg oral</td>
<td>Male ICR mice</td>
<td>AFB(1) 0.75 mg/kg oral</td>
<td>GSH; SOD; MDA</td>
<td>Protected from pro-oxidant liver damage by promoting antioxidative defense systems and inhibiting lipid peroxidation</td>
<td>Choi et al. (2010)</td>
</tr>
<tr>
<td>0.2% in diet (initiation period), 0.1% in diet (promotion period)</td>
<td>Male Wistar rats</td>
<td>Initiation DEN 100 mg/kg i.p.; promotion phenobarbital 500 ppm in diet</td>
<td></td>
<td>–</td>
<td>Siess et al. (2000)</td>
</tr>
<tr>
<td>1% in diet</td>
<td>Rats</td>
<td>Initiation IQ 100 mg/kg i.p.; promotion phenobarbital 0.05% in diet and a single dose of α-galactosamine (100 mg/kg i.p.)</td>
<td></td>
<td>Reduced the number of GST-P positive foci in liver</td>
<td>Tsuda et al. (1994)</td>
</tr>
<tr>
<td>0.75, 1, 1.5% in diet</td>
<td>Male Fischer-344 rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion 0.05% phenobarbital</td>
<td></td>
<td>Reduced the evolution of preneoplastic foci into persistent and hyperplastic nodules as well as the number of HCC carcinomas</td>
<td>Tang et al. (1993)</td>
</tr>
<tr>
<td>EGCG</td>
<td>Male Fischer-344 rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion phenobarbital 0.05% in water</td>
<td></td>
<td>Decreased the number and total volume of GST-P positive foci in liver cancer</td>
<td>Matsumoto et al. (1996)</td>
</tr>
<tr>
<td>0.05–0.1% in water</td>
<td>Male Fischer-344 rats</td>
<td>C3H/HeNCRj mice</td>
<td></td>
<td>Reduced the incidence of hepatoma</td>
<td>Nishida et al. (1994)</td>
</tr>
<tr>
<td>Luteolin 50, 200 ppm in diet</td>
<td>Male BALB/c athymic nude mice</td>
<td>–</td>
<td></td>
<td>Inhibited xenografted tumor growth in liver</td>
<td>Selvendiran et al. (2006)</td>
</tr>
<tr>
<td>Silibinin 80, 160 mg/kg gavage</td>
<td>Nude mice</td>
<td>–</td>
<td>Apoptosis; G1 arrest; NFκB; p53; Bcl-2; Bax; JNK; JNK; survivin; JNK; JNK; MEK</td>
<td>Reduced development of hepatoma</td>
<td>Cui et al. (2009)</td>
</tr>
<tr>
<td>Sillymarin 1000 ppm in diet</td>
<td>Male Wistar albino rats</td>
<td>DEN 0.01% in water</td>
<td>antioxidant defense system; lipid peroxidation levels; GSH; SOD; GPx; GR</td>
<td>Reduced the number of liver nodules</td>
<td>Ramakrishnan et al. (2006)</td>
</tr>
<tr>
<td>Genistein 15 mg/kg subcutaneous</td>
<td>Male Wistar rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion phenobarbital 0.05% in water</td>
<td>Apoptosis; caspase-3</td>
<td>Protected from HCC proliferation</td>
<td>Chodon et al. (2007)</td>
</tr>
<tr>
<td>50 mg/kg l.p.</td>
<td>Male BALB/c nu/nu mice</td>
<td>–</td>
<td></td>
<td>Inhibited growth and microvessel formation in implanted Bel 7402 hepatoma cells</td>
<td>Gu et al. (2005)</td>
</tr>
<tr>
<td>50 mg/kg l.p.</td>
<td>Male athymic BALB/c nu/nu mice</td>
<td>–</td>
<td></td>
<td>Inhibited liver cancer growth induced by MHCC97-L hepatoma cells transplantation</td>
<td>Gu et al. (2009)</td>
</tr>
<tr>
<td>Trans-resveratrol 50–300 mg/kg in diet</td>
<td>Female Sprague-Dawley rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion phenobarbital 0.05% in water</td>
<td>Bax; Bcl-2</td>
<td>Reduced the incidence, total number and multiplicity of visible hepatocyte nodules. Decreased cell proliferation and increased apoptosis in liver</td>
<td>Bishayee and Dhir (2009)</td>
</tr>
<tr>
<td>50–300 mg/kg in diet</td>
<td>Female Sprague-Dawley rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion phenobarbital 0.05% in water</td>
<td>lipid peroxidation; protein oxidation; nitric oxide synthase; 3-nitrotyrosine; Nrf2</td>
<td>Attenuation of oxidative stress; suppression of inflammatory response</td>
<td>Bishayee et al. (2010a)</td>
</tr>
<tr>
<td>Trans-resveratrol 50, 100, 300 mg/kg in diet</td>
<td>Female Sprague-Dawley rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion Phenobarbital 0.05% in water</td>
<td>Hsp70; COX-2; NRF-2</td>
<td>Anti-inflammation in liver</td>
<td>Bishayee et al. (2010b)</td>
</tr>
<tr>
<td>0.4% in diet</td>
<td>Female Sprague-Dawley rats</td>
<td>Initiation DEN 90 mg/kg i.p.; Promotion PCB 300 μM/kg l.p.</td>
<td></td>
<td>Did not prevent the tumorigenesis in liver</td>
<td>Tharappel et al. (2008)</td>
</tr>
</tbody>
</table>
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BCP 300 or diet – Reduced GGT + foci and HCC neoplasms Tanaka et al. (2003)

<table>
<thead>
<tr>
<th>Plant polyphenol</th>
<th>Species, strain and sex</th>
<th>Agent and dose to induce the tumour</th>
<th>Biomarkers affected</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>Curcumin 10, 100, 200 µmol/0.2 ml corn oil 0.2% in diet</td>
<td>Male BALB/c mice</td>
<td>DHPN 0.1% in water</td>
<td>–</td>
<td>Inhibited multiplicity and incidence of HCC in mice</td>
<td>Huang et al. (2008)</td>
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<tr>
<td></td>
<td>Male C57Bl/6N mice</td>
<td>DEN i.p.</td>
<td>p21, pPCNA, pCDC2</td>
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<td>Chuang et al. (2000)</td>
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<tr>
<td></td>
<td>Male Wistar rats</td>
<td>Initiation DEN 200 mg/kg i.p. promotion phenobarbital 0.05% in water</td>
<td>–</td>
<td>Inhibited the development of altered hepatic foci</td>
<td>Sreeepriya and Bali (2005)</td>
</tr>
<tr>
<td></td>
<td>Male Wistar rats mice</td>
<td>Initiation DEN 200 ppm in water; 2-AAF 0.05% in diet</td>
<td>–</td>
<td>Inhibited the development of altered hepatic foci</td>
<td>Shukla and Arora (2003)</td>
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<tr>
<td></td>
<td>Male Balb/c nude mice</td>
<td>–</td>
<td>–</td>
<td>Exhibited anti-metastatic activity against implanted CBO140C12 hepatoma cells</td>
<td>Ohashi et al. (2003)</td>
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<tr>
<td></td>
<td>Male Fischer-344 rats</td>
<td>–</td>
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<td>Exerted anti-angiogenic effects on implanted HepG2 cells</td>
<td>Yosyungnoen et al. (2006)</td>
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<td>Yosyungnoen et al. (2005)</td>
</tr>
<tr>
<td>Caffeic acid 2% in diet</td>
<td>Male Fischer-344 rats</td>
<td>DEN 200 mg/kg per day 2-AAF for 3 days</td>
<td>mRNA-κB</td>
<td>Reduced the increase of GST-P foci and the expression of both GST and GST mRNA in liver</td>
<td>Carrasco-Legleu et al. (2004)</td>
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<tr>
<td></td>
<td>Male Fischer-344 rats</td>
<td>Initiation DEN 200 mg/kg i.p. promotion 2-AAF 20 mg/kg</td>
<td>TBARS</td>
<td>Antioxidant defense in liver</td>
<td>Carrasco-Legleu et al. (2006)</td>
</tr>
<tr>
<td>CAPE 20 mg/kg diet</td>
<td>Male Wistar rats</td>
<td>200 mg/kg DEN i.p.; after 1 week 20 mg/kg per day 2-AAF for 3 days</td>
<td>–</td>
<td>Reduced the number of GGT + foci in liver</td>
<td>Beltran-Ramirez et al. (2008)</td>
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<tr>
<td></td>
<td>Male Wistar rats</td>
<td>Initiation DEN 200 mg/kg i.p.</td>
<td>CYP450 bioactivators of DEN</td>
<td>Protected liver damage</td>
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</tr>
<tr>
<td></td>
<td>Male Fisher-344 rats Female Sprague-Dawley rats</td>
<td>Initiation DEN 90 mg/kg i.p.; Promotion PCB 300 µM/kg i.p.</td>
<td>–</td>
<td>Decreased the size of GST-P positive foci, although it increased their number in livers</td>
<td>Soni et al. (1997)</td>
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<tr>
<td></td>
<td>Male Wistar rats</td>
<td>AFR(1) 25 µg/kg oral intubation</td>
<td>–</td>
<td>Reduced the number of GGT + foci in liver</td>
<td>Sreepriya and Bali (2005)</td>
</tr>
<tr>
<td>Ellagic acid 0.4% in diet</td>
<td>Male Fischer-344 rats Female Sprague-Dawley rats</td>
<td>Initiation DEN 90 mg/kg i.p.; Promotion PCB 300 µM/kg i.p.</td>
<td>–</td>
<td>Decreased the number of GGT + foci in liver</td>
<td>Tanaka et al. (1998)</td>
</tr>
<tr>
<td>0.005% in diet</td>
<td>Male Wistar rats</td>
<td>–</td>
<td>–</td>
<td>Reduced GGT + foci and HCC neoplasms</td>
<td>Pereira (1995)</td>
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<tr>
<td>400 ppm in diet</td>
<td>Male A/J N rats</td>
<td>FAA 200 ppm in diet</td>
<td>–</td>
<td>Decreased the number of adenomas without affecting the number of foci of altered hepatocytes and of total lesions in liver tumors</td>
<td>D. Stagos et al. / Food and Chemical Toxicology 50 (2012) 2155–2170</td>
</tr>
</tbody>
</table>

Weinberg, 2011). Genome instability and inflammation are re-
quired for cancer cells to acquire the above capabilities. In addition, the tumor needs to be understood as a population of genetically di-
verse cells, with many of them evolving rapidly, due to the inher-
ent genomic instability. That is believed to be the main reason that
tumors evolve resistance to chemotherapy of molecules that target specific genes. Each core hallmark is considered to be regulated by partially redundant signaling pathways. In addition, suppression of a certain hallmark may pressure the cancer cells to rely more on other hallmarks, as an evasive course of action. Hanahan and Weinberg (2011) suggest that future therapies should take all of the above into consideration and thus try to target simultaneously several redundant pathways from more than one hallmark. Molecular evidence shows that natural agents can target multiple cellular signaling pathways and recently, more clinical trials are being conducted to investigate the value of these agents in human cancer (see www.ClinicalTrials.gov) (Li et al., 2010). That is why plant extracts (with a complex mixture of polyphenols) that have more than one gene-target appear as an attractive choice for future cancer therapy. But, in order to select the therapeutic phytochemicals or cocktails of them, a good knowledge is required concerning the
pathways that each phytochemical modulates. Thus, gene-expression studies at the mRNA level are emerging as a prerequisite for polyphenol-cancer research. Polyphenol or plant extract activity needs to be elucidated at the molecular level and should not be treated as a black box. Food compounds, in contrast to drugs or modified analogs of a certain bioactive molecule, normally induce low levels of gene expression changes (Arola-Arnal and Blade, 2011). Therefore, a good knowledge of which pathways are modulated by certain foods or their bioactive molecules for specific types of cancer, in combination with gene expression analysis of resected tumors could (in the future) guide the diet of a post-treatment cancer patient, in order to achieve long-term chemoprotection without adverse toxic effects.

In addition, the prediction of possible plant polyphenol-induced toxic effects could be advanced by using toxicogenomic analyses. Toxicogenomics is based on the notion that microarrays can sense very quickly (faster than conventional techniques) subtle changes in the cell, that in the future may lead to toxicity. Therefore, gene expression analysis of in vitro hepatic cell cultures or in vivo studies (e.g. on rat liver) using a polyphenolic compound or plant extract should create an expression profile. In turn, this profile can be
compared against gene expression profiles of the same in vitro or in vivo systems that have been challenged with a battery of known toxic and non-toxic compounds and thus, help predict potential toxic effects.

Apart from gene-expression studies at mRNA level, studies at micro-RNA (miRNA) level may be very useful for a better understanding of the potential mechanisms of action by plant polyphenols against carcinogenesis. miRNAs are short non-coding RNAs of 20–25nt length that regulate the expression of target mRNAs by binding to their 3’ untranslated region and inhibiting their gene expression through translational repression, cleavage and decay (Zhang et al., 2007; Li et al., 2010). Some micro-RNAs may repress oncogenes or tumor suppressors, therefore, their level of expression may promote or suppress cancer. Their important role in cancer is highlighted by the fact that over 50% of the miRNA genes are located in cancer-associated regions or fragile sites (Calin et al., 2004). MiRNA studies of hepatocellular carcinoma (compared to adjacent normal tissue) revealed overexpression of miR-15 and miR-224, whereas miR-199a, miR-195, miR-199a, miR-200a, and miR-125a were underexpressed (Murakami et al., 2006). Other miRNAs involved in liver cancer are miR-21, miR-221, miR-222 (Li et al., 2010). Arola-Arnal and Blake (2011) tested anthocyanidine-rich natural extracts from grape-seed (GSPE; which is mainly composed of trimeric proanthocyanidins), from cocoa (CPE; which is mainly composed of dimeric proanthocyanidins) and also pure epigallocatechin gallate (EGCC) on HepG2 cells to determine which miRNAs each of them modulates and whether there is a common target. Dietary proanthocyanidins reach the liver in high concentrations. GSPE treatment modulated the expression of 15 miRNAs (nine up-regulated and six down-regulated). CPE treatment modulated the expression of 6 miRNAs (three up-regulated and three down-regulated) whereas EGCC treatment repressed the expression of five miRNAs. Three miRNAs (miR-1224-3p, miR-197 and miR-532-3p) were differentially regulated after treatment with the two extracts, whereas one miRNA, miR-30b was repressed in all three treatments. The mirwalk database (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) showed that 480 genes are experimentally validated targets of the miR-30b* many of which have a central role in lipid and glucose metabolism, insulin signaling, oxidative stress and inflammation. The findings for miR-1224-3p and miR-30b* were further tested and validated with qRT-PCR. Also, treatment period and treatment dose seem to play an important role in these omics-based studies. For example, as mentioned above, a study by Tsang and Kwok (2010) that was performed with EGCC treatment on HepG2 cells identified a list of differentially expressed five micro-RNAs (13 micro-RNAs were upregulated and 48 were down-regulated) that did not overlap at all with the list of differentially expressed micro-RNAs (five micro-RNAs downregulated) from the work of Arola-Arnal and Blake (2011) with EGGC treatment on HepG2 cells again. Arola-Arnal & Blake attribute this discrepancy on different doses and periods of treatment in the two experiments. Micro-RNA microarray measurements of HepG2 cells treated with ellagitannin, a polyphenol isolated from Balanophora japonica MAKINO, (a parasitic plant) has revealed the regulation of 25 micro-RNAs (17 upregulated and 8 downregulated). Subsequent bioinformatics analysis revealed that the 25 micro-RNAs target genes that are involved in the regulation of cell differentiation and proliferation. Furthermore, several of the 25 regulated micro-RNAs are situated within 2 gene clusters. In particular 7 of the 17 upregulated miRNAs are located within a 10 kb region (19q13.42), while 3 of the 8 downregulated miRNAs are located within another 10 kb region (9q22.32). In addition, 8 of the 25 regulated micro-RNAs have been reported to be regulated by other anticancer drugs (Garzon et al., 2007; Rosa et al., 2007; Sempere et al., 2007; Meng et al., 2008). Thus, it seems that a toxicogenomic approach can help in the upcoming years to elucidate the exact molecular basis of phytochemical action and prepare plupotent anticancer plant formulas for chemoprevention in liver and other cancers as well.

Conflict of Interest

The authors have declared that there is no conflict of interest.

References


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via activation of PI3K/Akt signaling: NAD(P)H:quinoine oxidoreductase as a potential target. Antioxid. Redox Signal. 9, 2087–2098.


