Silymarin and hepatocellular carcinoma: a systematic, comprehensive, and critical review

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The blessed milk thistle (*Silybum marianum* L.), a flowering plant native to Mediterranean Europe, has been consumed and extensively used as a cure for various chronic liver ailments over several centuries. Milk thistle extract, known as silymarin, is a complex mixture of seven major flavonolignans and one flavonoid. The phytoconstituents of silymarin owe their therapeutic and hepatoprotective effects to their strong antioxidant and anti-inflammatory properties. Primary liver cancer, also known as hepatocellular carcinoma (HCC), occurs in a milieu of oxidative stress and inflammation. The etiology of HCC includes chronic infection with hepatitis B and C viruses, cirrhosis, and exposure to dietary and environmental hepatocarcinogens. Current therapeutic options for HCC, including surgical resection and liver transplantation, have limited benefits and are essentially ineffective. Chemoprevention, using phytochemicals with potent antioxidant and anti-inflammatory properties, represents a fascinating strategy, which has been a subject of intense investigation in the recent years. In this review, we explore the potential role of silymarin as a chemopreventive and therapeutic agent for HCC. The review systematically evaluates the preclinical in-vitro and in-vivo studies investigating the effects of silymarin and its constituents on HCC. The biochemical mechanisms involved in the anti-liver-cancer effects of silymarin have been presented. The current status of clinical studies evaluating the potential of role of silymarin in liver cancer, especially that caused by hepatitis C virus, has also been examined. Potential challenges and future directions of research involved in the 'bench-to-bedside' transition of silymarin phytoconstituents for the chemoprevention and treatment of HCC have also been discussed. *Anti-Cancer Drugs* 26:475–486

Anti-Cancer Drugs 2015, **26**:475–486

Keywords: chemoprevention, hepatocellular carcinoma, milk thistle, silibinin, silymarin, treatment

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Received 13 November 2014 **Revised form accepted** 16 December 2014

Introduction

Hepatocellular carcinoma (HCC), also known as primary liver cancer or primary hepatic carcinoma, represents malignant tumors originating from parenchymal hepatocytes. The incidence of liver cancer has been increasing in both developing and developed countries [1] and has tripled in the USA over the last 30 years [2]. Global cancer statistics have shown that HCC is the sixth most common cancer [3] and the second leading cause of cancer deaths worldwide [4]. HCC is more common in sub-Saharan Africa [5] and Southeast Asia [6] than in the USA and northern Europe [7]. Fifty percent of the estimated new cases worldwide in 2012 were in China alone [4,8,9]. According to the American Cancer Society, liver cancer is estimated as the fifth most common cause of cancer death among American men and the ninth most common cause of cancer death among American women [10].

Cirrhosis of the liver is the major cause of HCC worldwide. Hepatocarcinogenesis progresses from chronic hepatitis to liver cirrhosis in a step-by-step manner, with continuous intrahepatic inflammation and oxidative DNA damage, along with oxidative stress, resulting from the generation of reactive oxygen species (ROS) by environmental factors or cellular mitochondrial dysfunction [11,12]. Risk factors for HCC vary with respect to the location or the ethnic group of a specific population. Cirrhosis caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) is the leading risk factor for HCC in developing countries and accounts for less than 50% of all cases in the USA [6]. Aflatoxin, a naturally occurring mycotoxin produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* present in starchy vegetables and grains in high humidity during storage and at harvest, is another risk factor and cause for HCC in developing countries [13]. Other risk factors include autoimmune hepatitis [14], hemochromatosis, obesity diabetes, nonalcoholic fatty liver disease (NAFLD) [6], and nonalcoholic steatohepatitis [15,16], which can progress to HCC. In the USA, approximately three times more men than women, more Asian Americans and Pacific Islanders than

0959-4973 DOI: 10.1097/CAD.0000000000000211

other ethnic groups of people, and more people who abuse alcohol or have chronic HBV or HCV infection are likely to develop HCC [6].

Late-stage metastasized tumors are often detected during routine screening or diagnostic testing due to symptomatic complaints when size and/or location are no longer tolerable [17,18]. Early aggressive treatment with ablative therapy, surgical resection, or liver transplantation can result in an increase in the 5-year survival time from 16 to 29% [6], but without such intervention HCC will result in liver failure and death [19,20]. HCC is nonresponsive to radiotherapy, and standard chemotherapy is not effective, both having poorly accepted adverse reactions. Partial hepatectomy involves resection of the tumor, but it can only be performed in ~20–30% of patients with cirrhosis [21] and is dependent upon the cause and degree of cirrhosis [6]. Although complete removal or destruction of liver tumors provides the potential for long-term survival or cure, the number of eligible patients is limited by the number, size, and location of the tumors, as well as the degree of cirrhosis. High costs along with the lack of healthcare system are limiting factors in diagnosing and treating HCC in developing countries. Undiagnosed and untreated cases of HBV and HCV infections contribute to the continuing rise of HCC in developing countries, whereas a vaccine has nearly eradicated HBV in the USA and Europe [22]. A multikinase inhibitor, sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals Inc., Wayne, New Jersey, USA), a drug that inhibits tumor growth of HCC through antiangiogenic and antiproliferative mechanisms, has allowed some patients to live longer by an average of about 3 months [6,23–25]. Nevertheless, sorafenib has severe adverse reactions, including fatigue, hand–foot syndrome, hypertension, gastrointestinal distress and ulcers, increased bleeding, and encephalopathy [26–28]. In view of the limited treatment options, complementary and alternative approaches should be considered to reduce the burden of liver cancer.

Since the ancient Egyptians first reported their use of castor beans and garlic in their medical scrolls, the *Ebers Papyrus*, in 1500 B.C. [29], great interest in discovering natural agents to eradicate all types of diseases has prevailed. The interest in finding natural nontoxic substances to treat and prevent various types of cancers has increased over the last 50 years [30–32]. Dietary phytochemicals, such as flavonoids, betalains, chlorophylls, glucosinolates, and phenolic compounds, among other compounds found in fruits, vegetables, spices, herbs, legumes, nuts, and seeds, appear to be promising for their anticancer activities [33–36]. The underlying mechanisms of such action encompass antioxidant, anti-inflammatory, antiproliferative, and cytotoxic effects, along with apoptosis and antiangiogenesis [36–39]. Many in-vitro and in-vivo studies have offered evidence of such antineoplastic characteristics of phytochemicals [37,38,40]. Numerous efforts are being made to discover and develop various natural nontoxic chemoprotective agents that are able to diminish the risk for HCC and lower the mortality rates [41–45]. Recent research on specific phytochemicals for the chemoprevention and therapy of HCC has shown promising outcomes. Specific examples of such phytochemicals include geraniol, terpenoids in lemon, rose, and lemongrass (palmarosa) oils [46], catechins in green tea [47,48], curcumin in the Indian curry spice turmeric [49], resveratrol found in grapes, berries, and red wine [50–52], and phenols found in dried spices and dried herbs, onions, leeks, and broccoli [53–56].

Although several excellent review articles have provided a wealth of knowledge on the chemopreventive and antitumor potential of milk thistle (*Silybum marianum* L.) and its bioactive phytoconstituents against various cancers, including bladder, breast, cervical, gastrointestinal tract, lung, prostate, renal, and skin carcinomas [57–63], a systematic, comprehensive, and critical evaluation of the literature on the use of milk thistle-derived agents for the prevention and therapy of hepatic cancer has not been performed previously to the best of our knowledge and belief. Accordingly, this review explores the full potential of the bioactive constituents present in milk thistle for liver cancer prevention and intervention by presenting and analyzing the available in-vitro, in-vivo, and clinical studies. Current limitations and future directions for research on these promising natural substances against hepatocellular cancer have also been presented.

Milk thistle

Milk thistle, a natural purple flowering herbal plant indigent to Europe, which belongs to the Asteraceae (aster) or Compositae (daisy or sunflower) family, is characterized by leaves with sharp prickles and distinctive white markings or 'veins'. The name milk thistle is derived from the milky sap that exudes when the leaves are broken or torn. Milk thistle is known by many different names, including but not limited to Blessed Milk Thistle, Chardon de Marie, Holy Thistle, Lady's Thistle, Marian Thistle, Mary Thistle, Our Lady's Thistle, Shui Fei Ji, St. Mary's Thistle, and wild artichoke. Legend states that the white veins represent the Virgin Mary's milk.

Traditional and current uses of milk thistle

This herb has been used for over 2000 years as a hepatoprotectant, as a liver detoxifier, and for the treatment of disorders of the bile duct and gallbladder throughout Eurasia. The ancient Greeks and Romans also used milk thistle for snake bites. The herb has also been used for centuries for the treatment of upper gastrointestinal tract and digestive disorders, menstrual complications, and varicose veins [64]. Silymarin is an extract from the seeds (fruit) of the milk thistle plant. Silymarin, a potent

hepatoprotective agent, is used extensively to treat various hepatic disorders, including chronic alcoholic and viral hepatitis, alcoholic cirrhosis, and toxin-induced liver damage, as well as death cap mushroom (*Amanita phalloides*) poisoning. Today, folk medicine uses the powdered form of silymarin from milk thistle seeds in gel capsules, liquid extracts, and tinctures for liver disorders, including hepatitis and cirrhosis, as well as for lowering cholesterol levels and reducing insulin resistance in type 2 diabetes mellitus [65]. Although milk thistle extract is currently not approved for any medical use in the USA, it is one of the most frequently sold herbal products [66].

Phytochemicals present in milk thistle

The active ingredient extracted from the seeds of milk thistle is called silymarin. Silymarin is a mixture of seven flavolignans and one flavonoid [67]. The seven flavonolignans are as follows: silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin (Fig. 1). The only flavonoid of silymarin is taxifolin (Fig. 1). The most abundant and potent constituents of the seven flavolignans are the two diastereoisomeric compounds, silybin A and silybin B, known together as silybin and often called silibinin.

Bioavailability of silymarin, silibinin, and standardized products

Because of poor intestinal absorption (bioavailability), milk thistle constituents, such as silymarin and silibinin, are made more absorbable in the phospholipid complex form in standardized commercially available oral and intravenous products. The oral products are used in Europe and the USA as dietary supplements for hepatic support, whereas the intravenous product is used primarily for the reduction and prevention of toxin-induced liver damage caused by the death cap mushroom and is reported to block hepatocyte uptake of amatoxin [68]. Standardized preparations are usually made from the seeds of the plant and contain 70–80% of silymarin [69].

Side effects and allergy information of silymarin products

As a dietary supplement, milk thistle has been known to have a slight laxative effect, with less common side effects being nausea, diarrhea, indigestion, intestinal gas, distension, fullness or pain, and loss of appetite. People who are allergic to members of the Asteraceae/Compositae plant family such as ragweed, chrysanthemum, marigold, and daisies among many others may also be allergic to milk thistle [65,70].

Silymarin and liver cancer

The following three sections describe in-vitro, in-vivo, and clinical studies conducted worldwide by numerous researchers to explore the potential chemoprotective and chemotherapeutic effects of silymarin, silybin, that is, silibinin, and dehydrosilybin on liver cancer. Several literature databases, including PubMed, EBOSCOhost, and Google Scholar were used to find all primary literature. There were no time restraints on published articles. Only English language articles were considered for this work. Abstracts of publications were first reviewed and then useful full articles were collected. The major keywords used in various combinations included: milk thistle, silymarin, silibinin, silybin, liver, cancer, HCC, chemoprevention, prevention, treatment, *in vitro*, *in vivo*, and clinical trials. The references of review articles were also studied to retrieve relevant original research articles. For clinical studies, clinicaltrials.gov was used in addition to the aforementioned databases.

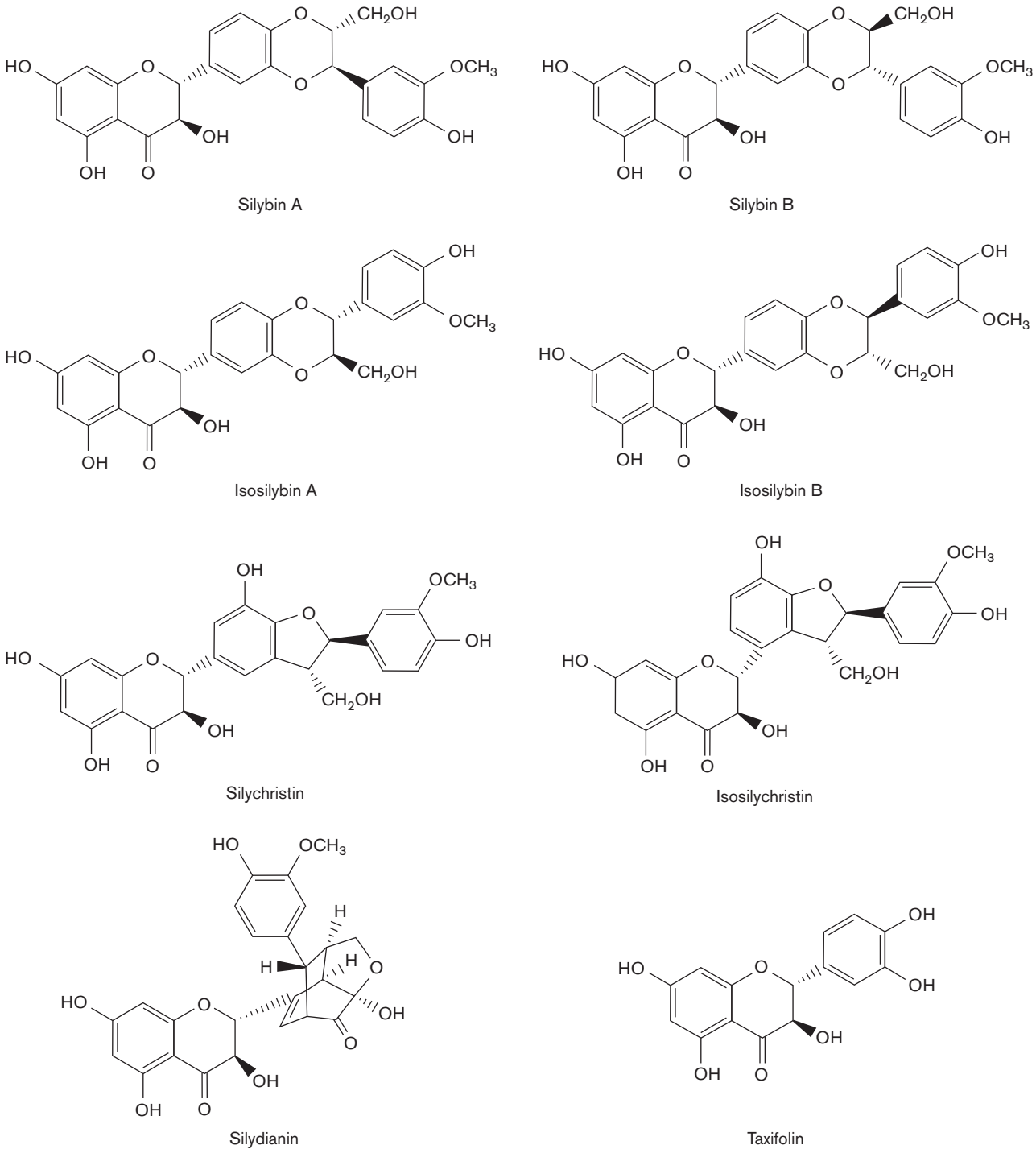
In-vitro studies

Silymarin inhibited population growth of HepG2 human hepatocellular cancer cells in a dose-dependent manner, which was associated with an increase in the percentage of apoptotic cells (Table 1). Silymarin also decreased mitochondrial transmembrane potential through an increase in the level of cytosolic cytochrome *c* (cyt. *c*) while upregulating the expressions of proapoptotic proteins, such as p53, Bax, apoptotic protease-activating factor 1, and caspase-3, and downregulating the expressions of antiapoptotic proteins, namely, Bcl-2 and survivin, and proliferation-associated proteins, for example, proliferating cell nuclear antigen, cyclin D1, c-Myc, and β-catenin [71]. A subsequent study confirmed the antiproliferative efficacy of silymarin without any effect on nontumor (Chang) liver cells. In addition silymarin increased the percentage of cells in the G_0/G_1 phase and decreased the percentage of cells in the S-phase, with concomitant upregulation of retinoblastoma protein (Rb), p53, $p21^{Cip1}$, and $p27^{Kip1}$ and downregulation of cyclin D1, cyclin E, CDK4, and phospho-Rb [72].

In one of the initial studies conducted by Varghese *et al.* [73], silibinin was found to inhibit the growth of both HepG2 and Hep3B human liver cancer cells, with more pronounced cytotoxicity in Hep3B cells, which was associated with apoptosis. Silibinin manifested both G_1 and G_2-M arrests in Hep3B cells and G_1 arrest in HepG2 cells alone. Mechanistic studies revealed that silibinin induced Kip1/p27, decreased cyclin D1, cyclin D3, cyclin E, cyclin-dependent kinase 2 (CDK2), and CDK4 levels, and inhibited CDK2, CDK4, and CDC2 kinase activities in both cell lines. In addition, silibinin also reduced the protein levels of G_2-M regulators in Hep3B cells [73]. A subsequent study showed a moderate growth-inhibitory potential of silibinin, and this effect was accompanied by the induction of apoptosis, increase in caspase 3 activity, and upregulation of Bcl-xl [74].

Silibinin reduced the growth of HuH7, HepG2, Hep3B, and PLC/PRF/5 human hepatoma cells. The silibinin-mediated growth-inhibitory effect in HuH7 was associated with apoptosis induction through downregulation of survivin and upregulation of activated caspase-3 and caspase-9.

Fig. 1



Chemical structures of the phytochemicals present in silymarin.

Silibinin also upregulated p21/CDK4 and p27/CDK4 complexes and downregulated Rb-phosphorylation and E2F1/DP1 complexes. Silibinin's antiangiogenic effect was reflected in the downregulation of metalloproteinase-2 (MMP-2) and CD34. Moreover, increased activity of phosphatase and tensin homolog deleted on chromosome ten (PTEN), decreased Akt production, and elevated acetylation of histones H3 and H4 (AC-H3 and AC-H4) were implicated in the observed antiproliferative effect of silibinin in HuH cells [75]. In another study, silibinin was

Table 1 In-vitro antitumor effects and related mechanisms of action of silymarin and silymarin-derived constituents in various liver cancer cells

Substances investigated	Cellular effects	Mechanisms	Concentration; duration	References
Silymarin	Suppressed the growth of HepG2 cells	↑Apoptosis, ↑p53; ↑Bax, ↑APAF-1, ↑capase-3, ↓Bcl-2, ↓survivin; ↓PCNA, ↓cyclin D1, ↓c-Myc, ↓β-catenin	50–200 μg/ml; 24 h	Ramakrishnan *et al.* [71]
	Inhibited the viability of HepG2 cells, not in Chang liver cells	↑Rb, ↑p53, $\uparrow p21^{Cip1}$, $\uparrow p27^{Kip1}$, ↓cyclin D1, ↓cyclin E, ↓CDK4, ↓phospho-Rb	100 μg/ml; 24 and 48 h	Chen *et al.* [72]
Silibinin	Demonstrated cytotoxic effects in HepG2 and Hep3B cells	↑Apoptosis, cell cycle arrest, ↑Kip1/p27, ↓cyclin D1, ↓cyclin D3, ↓cyclin E, ↓CDK2, ↓CDK4	100, 200, and 300 μmol/l; 12, 24, 48, and 72 h	Varghese *et al.* [73]
	Inhibited the growth of HepG2 cells	↑Apoptosis, ↑caspase 3, ↓Bcl-xl	100 and 200 μmol/l; 48 h	Pook *et al.* [74]
	Retarded the growth of HuH7, HepG2, Hep3B, and PLC/PRF/5 cells	↑Apoptosis, ↓survivin, ↑caspase-3, ↑caspase-9, ↓MMP-2, ↓CD34, ↑PTEN, ↓p-Akt, ↑AC-H3, ↑AC-H4	10–240 μmol/l; 24 h	Lah *et al.* [75]
	Curtailed the growth, proliferation, and invasive properties of HepG2 cells	↓MMP-2, ↓ERK 1/2 phosphorylation, ↑RKIP, ↑Spread-1, ↑Spread-2, ↓Hec1	25, 50 and 75 μmol/l; 72 h	Momeny *et al.* [76]
	Displayed potent antiproliferative effect in Hep3B cells	↑Apoptosis, ↓HIF-1α, ↓mTOR, ↓p70S6K, ↓4E-BP1, ↓VEGF	50–500 μmol/l; 8 h	Garcia-Maceira and Mateo [77]
	Demonstrated cytotoxic effects in HepG2 cells	↑DNA migration, ↑oxidized DNA bases, ↑oxidative stress	200 μmol/l; 24 h	Angeli *et al.* [78]
	Inhibited ethanol-stimulated cell proliferation in H4IIE cells	↓CYP2E1, ↓ROS	10 μmol/l; 2 h	Brandon-Warner *et al.* [79]
	Induced apoptotic death in Hep-55.1C cells	↑TRAIL, ↑DR5; ↑caspase-3, ↑caspase-8	150 μg/ml; 24, 48 and 72 h	Bousserouel *et al.* [80]
	Suppressed the growth of HepG2 and PLC/PRF/5 cells	↓ANGPT2, ↓ATP6L, ↓CAP2, ↓CCR6, ↓CCR7, ↓CLDN-10, ↓cortacin, ↓CXCR4, ↓GLI2, ↓HK2, ↓ID1, ↓KIAA0101, ↓mortalin, ↓PAK1, ↓RHOA, ↓SPINK1, ↓STMN1, ↑CREB3L3, ↑DDX3X, ↑PROX1, ↓MMP-2	50, 75 and 100 μmol/l; 72 h	Ghasemi *et al.* [81]
	Inhibited the viability, adhesion, and migration of HepG2 cells	↑Apoptosis, ↑caspase, ↑Bax, ↓Bcl-2, ↓survivin, ↓cyclin D1, ↑ROS, ↓GSH, ↓NICD, ↓RBP-Jκ, ↓Hes1	50, 100, and 200 μmol/l; 12, 24, and 48 h	Zhang *et al.* [82]
Silybin and DHS	Decreased the viability of HepG2 cells		1–100 μmol/l; 24 and 48 h	Dvořák *et al.* [83]
	Exhibited cytotoxicity of HepG2 cells	↑Apoptosis, ↓ROS, ↓MMP-2, ↓MMP-9	10–90 μmol/l (silybin), 5–30 μmol/l (DHS); 3 days	Huber *et al.* [84]

found to inhibit the growth, proliferation, and invasive properties of HepG2 cells through extracellular-signal-regulated kinase (ERK) 1/2 inactivation both directly by suppression of ERK 1/2 phosphorylation and indirectly through induction of Raf kinase inhibitor protein, spouty-related protein 1 with EVH-1 domain (Spread-1), and Spread-2. In addition, the inhibitory effect of silibinin on liver cancer cell proliferation could be through down-regulation of the highly expressed in cancer 1 (Hec1) gene [76]. Silibinin was found to be a potent inhibitor of Hep3B cell proliferation, with concurrent induction of apoptosis. Ancillary molecular studies have revealed that silibinin inhibited the accumulation and transcriptional activity of hypoxia-inducible factor 1α, exerted a strong dephosphorylation of mammalian target of rapamycin (mTOR) and its effectors ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1, activated Akt, and reduced hypoxia-induced vascular endothelial growth factor (VEGF) release [77].

Silybin showed genotoxic effects in HepG2 cells at a concentration of 200 μmol/l by increasing DNA migration, oxidizing DNA bases, and increasing oxidative stress, in addition to reducing cell viability. Nevertheless, it failed to demonstrate genotoxic effects in the same cell line at concentrations of up to 100 μmol/l. It is interesting that silybin was able to suppress the genotoxic effects induced by benzo[a]pyrene, bleomycin, and aflatoxin B_1 on pretreatment and simultaneous treatments, but had no significant effect on DNA damage after treatment [78]. Silibinin inhibited ethanol-stimulated cell proliferation, cytochrome P450 2E1 (CYP2E1) induction, ethanol metabolism, and ROS generation in the H4IIE rat hepatoma cell line [79]. Silibinin exerted the death of Hep-55.1C murine hepatoma cells through an extrinsic apoptotic pathway, as attested by the upregulation of TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL-death receptor 5 (DR5) transcripts, as well as the activation of caspase-3 and caspase-8 [80]. A study was conducted to examine the effects of silibinin on a battery of transcriptional markers mechanistically related to HCC recurrence and metastasis using HepG2 (HBV-negative and p53 intact) and PLC/PRF/5 (HBV-positive and p53 mutated). Silibinin was capable of suppressing the transcriptional levels of ANGPT2, ATP6L, CAP2, CCR6, CCR7, CLDN-10, cortacin, CXCR4, GLI2, HK2, ID1, KIAA0101, mortalin, PAK1, RHOA, SPINK1, and STMN1, and the enzymatic activity of MMP-2, with simultaneous transcriptional upregulation of CREB3L3, DDX3X, and PROX1 in both cell lines [81]. These interesting results suggest that silibinin could potentially function as a multitargeted antimetastatic agent and might provide a new avenue for HCC therapy, especially for HBV-related and/or p53-mutated liver cancers. Zhang *et al.* [82] assessed the antitumor activity of silybin in HepG2 cells. Silybin not only reduced the viability, but it also curtailed tumor cell adhesion and migration. These

effects were accompanied by induction of apoptosis and ROS, decrease of reduced glutathione levels, upregulation of Bax, and downregulation of Bcl-2, survivin, cyclin D1, Notch 1 intercellular domain (NICD), recombination signal binding protein for immunoglobulin κ J region (RBP-Jκ), and hairy and enhancer of split 1 (Hes1).

At least two laboratories compared the antitumor effect of silybin with that of its derivative dehydrosilybinin (DHS). Silybin and DHS decreased the viability of HepG2 cells, and the effect of the latter compound was much stronger [83]. Similar results were reported from another laboratory that showed that DHS had an IC_{50} value that was three-fold lower than that of silybin and it inhibited ROS generation in the glucose–glucose oxidase system and in HepG2 cells. DHS at a concentration of 10 μmol/l markedly inhibited MMP-2 and MMP-9 release and invasiveness, whereas silybin at 90 μmol/l had marginal effects. DHS but not silybin at 30 μmol/l induced apoptosis and loss of mitochondrial membrane potential [84].

In-vivo studies

Several laboratories have investigated the in-vivo liver cancer preventive or therapeutic effects of silymarin (Table 2). Most of these studies have used chemical carcinogen-initiated rat hepatocellular carcinogenesis models, whereas a few investigators have utilized mouse xenograft or transgenic models.

Ramakrishnan *et al.* [85] evaluated the efficacy of silymarin against diethylnitrosamine (DENA)-induced hepatocarcinogenesis in rats. Dietary exposure to silymarin (1000 ppm) before and after DENA treatment (pre- and post-treatment) or only after DENA administration reduced the total number and the multiplicity of macroscopic hepatic nodules. Electron microscopy studies supported the chemopreventive action of silymarin. Additional biochemical studies revealed that silymarin treatment suppressed lipid peroxidation, increased reduced glutathione levels, and elevated enzymatic activities of superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase in blood and liver samples of DENA-challenged animals. After treatment with silymarin at 50 mg/kg orally for 30 days, DENA-induced elevations in the serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) were found to be significantly reversed, in addition to an increase in the hepatic levels of enzymatic and nonenzymatic antioxidants [86]. All these findings suggest that silymarin impedes DENA hepatocarcinogenesis by modulating the antioxidant defense status of experimental animals.

The effects of silymarin on the levels of tumor markers and on malondialdehyde–DNA adduct formation were investigated during the course of DENA-induced HCC in rats. Dietary silymarin treatment significantly decreased

Table 2 In-vivo liver cancer chemopreventive and chemotherapeutic effects of silymarin and its constituents in preclinical animal models

Substances investigated	Effects	Mechanisms	Dose; duration	Route	References
Silymarin	Suppressed DENA-induced hepatocarcinogenesis in male Wistar rats	↑SOD, ↑CAT, ↑GSH, ↑GPX, ↑GR, ↑G6PD	1000 ppm; 10, 16 weeks	Diet	Ramakrishnan *et al.* [85]
	Exhibited hepatoprotective and antioxidative responses during DENA-induced hepatocellular damage in male Wistar rats	↑SOD, ↑CAT, ↑GSH, ↑GPX, ↑GR, ↑GST, ↑ATPase	50 mg/kg/day; 30 days	Peroral	Pradeep *et al.* [86]
	Attenuated DNA adduct formation during DENA-initiated hepatocarcinogenesis in male Wistar rats		1000 ppm; 10, 16 weeks	Diet	Ramakrishnan *et al.* [87]
	Exerted a hypolipidemic effect in DENA-evoked hepatocarcinogenesis in male Wistar rats	↓HMG-CoAR, ↓COX-2	1000 ppm; 10, 16 weeks	Diet	Ramakrishnan *et al.* [88]
	Showed antihepatocarcinogenic effects in male Wistar rats exposed to DENA	↓MCD, ↓MMP-2, ↓MMP-9	1000 ppm; 10, 16 weeks	Diet	Ramakrishnan *et al.* [89]
	Exhibited antiproliferative effects during DENA-induced hepatocarcinogenesis in male Wistar rats	↓Cyclin D1, ↑p53, ↓survivin, ↑caspase-3, ↓Bcl-2, ↑Bax, ↑cyt. *c*, ↓cyt. P450, ↓cyt. b5, ↑GST	1000 ppm; 10, 16 weeks	Diet	Gopalakrishnan *et al.* [90]
	Reversed DENA-induced hepatic histopathological alterations in male Wistar rats	↓DNA damage, ↓MDA, ↑GSH, ↑SOD, ↑GPX, ↑GR, ↑GGT, ↓VEGF	100 mg/kg; 8, 13 weeks	Peroral	El Mesallamy *et al.* [91]
	Failed to inhibit DENA-induced liver tumor formation in male Wistar rats		0.1, 0.5%; 5, 18 weeks	Diet	Imamoto *et al.* [92]
	Suppressed the progression of preneoplasia to HCC in male HBx transgenic mice	↓ROS	300 mg/kg/day; 3 months	Peroral	Wu *et al.* [93]
Silibinin	Reduced the frequency and volume of HuH7 xenografts in nude mice	↓Cell proliferation, ↑apoptosis, ↑p27/CDK4, ↓E2F1/DP1, ↓p-RB ↓p-Akt, ↑PTEN, ↓p-ERK, ↑SOD1	80,160 mg/kg/day; 5 weeks	Peroral	Cui *et al.* [94]
	Inhibited the growth of HepG2 tumor xenografts in nude mice	↑Bax, ↓Bcl-2, ↓survivin, ↓cyclin D1, ↓NICD	200 and 400 mg/kg; 5x/week for 20 days	Peroral	Zhang *et al.* [82]
	Inhibited weight and volume of Hep-55.1c orthotopic hepatocarcinoma in male C57BL/6J mice	↑TRAIL, ↑DR5, ↑caspase-3, ↓MMP-7, ↓MMP-9, ↓IL-1β	700 mg/kg; 5x/week; 4 weeks	Peroral	Bousserouel *et al.* [80]
	Failed to reduce DENA-induced tumor burden in male or female B6C3 mice		0.5% w/w; 8 weeks	Diet	Brandon-Warner *et al.* [95]

the levels of α-fetoprotein and carcinoembryonic antigen in the serum and reversed the alterations in the activities of AST, ALT, alkaline phosphatase, acid phosphatase, lactate dehydrogenase, γ-glutamyltransferase, and 5′-nucleotidase in the serum and liver. Immunohistochemical staining of liver sections indicated a silymarin-mediated reduction in malondialdehyde–DNA adduct formation [87].

Rats exposed to DENA showed severe hyperlipidemia, as indicated by elevated levels of total cholesterol, phospholipid, and triglyceride, as well as reduced levels of lipid metabolizing enzymes and HMG-CoA reductase. These effects were accompanied by an upregulation of hepatic cyclooxygenase-2 expression. Dietary silymarin supplementation diminished DENA-induced hyperlipidemia and downregulated the expression of cyclooxygenase-2 [88].

Empirical evidence suggests that mast cells play an important role in the inflammatory process that contributes to the development of neoplasia. These cells also represent a major source of MMPs involved in tumor invasion and angiogenesis [96]. Experiments were conducted to investigate whether dietary supplementation of silymarin had any role in the hepatic mast cell density and expression of MMP-2 and MMP-9 in rats with DENA-induced liver cancer. Silymarin treatment inhibited DENA-induced increases in mast cell density and downregulated both MMP-2 and MMP-9 [89].

Dietary silymarin administration, along with DENA treatment, significantly attenuated hepatic proliferation, downregulated Bcl-2 and survivin, upregulated Bax and p53, and caused the release of cyt. *c* into the cytosol. Silymarin administration also inhibited activities of microsomal phase I xenobiotic metabolizing enzymes, namely cytochrome P450, cytochrome b5, and NADPH cytochrome P450 reductase, and activated the phase II detoxifying enzyme glutathione S transferase [90].

El Mesallamy *et al.* [91] confirmed the chemopreventive effect of silymarin against DENA-induced hepatocarcinogenesis in rats. Oral administration of silymarin before or after DENA challenge reversed hepatic histopathological changes, blocked DNA damage, and reduced serum ALT, AST, and γ-glutamyl transpeptidase activities as well as VEGF levels.

In contrast to the aforementioned results, at least one study did not demonstrate the antihepatocarcinogenic effect of the silymarin chemical carcinogenesis model. Feeding of rats with 0.1% silymarin for 5 weeks or 0.1 and 0.5% silymarin for 18 weeks did not inhibit hepatic tumor formation induced by DENA. Moreover, immunohistochemical and western blot analyses revealed that the expression levels of proliferating cell nuclear antigen and glutathione S transferase-P were not significantly modified by silymarin treatment. The investigators speculated that the low bioavailability of silymarin and/or the severity of the tumor model could account for the null anti-liver-cancer effect of silymarin [92].

The potential chemopreventive effect of silymarin has also been investigated against spontaneous hepatocellular carcinogenesis using the HBV X protein transgenic mouse model. In this elegant study, oral silymarin was found to have a therapeutic effect in the early stages of hepatic damage, reversing fatty changes and recovering hepatic histopathology in a dose-responsive manner. Silymarin treatment in precancerous mice completely diminished HCC development and reduced the occurrence of small hyperplastic nodules. Although silymarin was unable to block cancer progression as well as HBV X protein expression in animals with existing HCC, it suppressed intracellular ROS, stimulated cell proliferation, and manifested the recovery of hepatocyte ultrastructure [93].

Cui *et al.* [94] used nude mice bearing HuH7 xenografts to assess the anti-HCC effect and underlying molecular mechanisms of action of silibinin. Oral silibinin treatment reduced HCC xenograft growth through inhibition of cell proliferation (Ki-67 expression), cell cycle progression, and PTEN/P-Akt and ERK signaling, and induction of apoptosis, histone acetylation, and SOD1 expression. As Notch signaling pathways play crucial roles in hepatic tumorigenesis [97], a preclinical study evaluated the possibility of silybin-mediated inhibition of tumor growth by interference with Notch signaling. Silybin treatment, indeed, reduced the growth of xenografted HepG2 tumor cells *in vivo* through suppression of various components of Notch signaling, such as NICD, RBP-Jκ, and Hes1 [82].

In an interesting preclinical study involving orthotopic syngenic grafting of Hep-55.1c liver cancer cells into the liver of C57BL/6J mice, Bousserouel *et al.* [80] evaluated the antitumor effect of silibinin using the microcomputed imaging technique. Oral administration of silibinin at 700 mg/kg for 4 weeks caused a significant reduction in tumor burden through upregulation of apoptotic mediators, such as TRAIL and DR-5, and downregulation of inflammatory components, including interleukin-1β, MMP-7, and MMP-9.

Using the DENA-initiated mouse model of liver cancer, Brandon-Warner *et al.* [95] examined the effects of dietary silibinin alone or in combination with chronic ethanol consumption on HCC progression. The results indicated that silibinin exerted only marginal hepatoprotective effects during early stages of hepatocarcinogenesis in male and female mice, but when coadministered with ethanol, it exacerbated the promotional effects of ethanol in tumor-bearing male mice.

Clinical studies

The evidence from preclinical studies set the stage for clinical studies with silymarin and its chemical constituents. As highlighted in Table 3, these clinical studies have been

performed to evaluate the safety, pharmacokinetics, and efficacy of silymarin and related compounds. Although most of the studies included patients with HCV infection, there is also at least one published report based on a clinical trial conducted in HCC patients.

A randomized, placebo-controlled clinical trial was carried out to determine whether silymarin improves the symptoms, signs, and laboratory test results in patients with clinical hepatitis, regardless of etiology. The intervention included ingestion of 140 mg silymarin or a vitamin placebo three times daily for 4 weeks, with an additional 4-week follow-up. The results of this study suggest that silymarin is safe and may be potentially effective in improving the symptoms of acute hepatitis despite the lack of a detectable effect on the biomarkers of the underlying hepatic inflammatory process [98].

The safety and dose–exposure relationships of silymarin and its effects on serum HCV RNA levels were evaluated in noncirrhotic HCV patients. Four cohorts of eight HCV patients with well-compensated, chronic noncirrhotic livers, who failed interferon therapy, were orally administered 140, 280, 560, or 700 mg silymarin every 8 h for 7 days. Steady-state exposures for silybin A and silybin B increased 11-fold and 38-fold, respectively, with a five-fold increase in dose, indicating nonlinear pharmacokinetics. Oral doses of silymarin up to 2.1 g/day were safe and well tolerated. Nevertheless, no clinically meaningful reductions from baseline in serum transaminases or the HCV RNA titer were noticed [99]. In an extended study by the same group, 420 or 700 mg silymarin administered three times per day for 24 weeks did not significantly alter biochemical or virological markers of disease activity in participants with chronic HCV infection unsuccessfully treated with interferon-based regimens [100]. Another pilot, randomized clinical trial reported a decrease in viral load and serum ALT levels following silymarin treatment for a period of 6 months in patients with chronic HCV infection. No serious adverse effects were observed apart from mild transient nausea, vomiting, giddiness, and headache in a small number of these patients [101].

The single-dose and multiple-dose pharmacokinetics of silymarin flavonolignans were investigated in patients with NAFLD or HCV infection to assess whether the disposition of silymarin and therefore its potential efficacy varies among liver disease populations. The efficacy of silymarin may be more readily observed in NAFLD patients because of higher plasma concentrations of flavonolignan and more extensive enterohepatic cycling compared with those in patients with HCV [102].

Freedman *et al.* [103] evaluated the effects of silymarin use on subsequent liver disease progression in 1049 patients of the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis (HALT-C) trial who had advanced fibrosis or cirrhosis that was nonresponsive to

Table 3 Clinical studies with silymarin and silymarin-derived compounds

Human participants	Objective(s)	Finding(s)	Dose; duration	Route	References
Patients with acute hepatitis (50)[a]	To determine the efficacy and safety of silymarin	Improved symptoms of acute hepatitis, no adverse events	140 mg; 3 times/day for 4 weeks	Oral	El-Kamary *et al.* [98]
Patients (noncirrhotic) with HCV infection (32)[a]	To evaluate safety and dose–exposure patterns of silymarin	Tolerability, nonlinear pharmacokinetics, no antiviral activity	140–700 mg; once every 8 h for 7 days	Oral	Hawke *et al.* [99]
Patients with HCV infection (154)[a]	To determine the effect of silymarin on liver disease activity	No reduction in serum ALT levels	420 and 700 mg; 3 times/day for 24 weeks	Oral	Fried et *al.* [100]
Patients with HCV infection (29)[a]	To test the safety and efficacy of silymarin	Reduction in viral load and serum ALT	140 mg; 3 times/day for 6 months	Oral	Yakoot and Salem [101]
Patients with NAFLD and HCV infection (30)[a]	To access the pharmacokinetics of silymarin	Higher plasma concentrations and enterohepatic cycling of flavonolignans	280 and 560 mg; once every 8 h for 7 days	Oral	Schrieber *et al.* [102]
Patients with HCV infection (1049)[a]	To determine the effect of silymarin on liver disease progression	Reduced disease progression from fibrosis to cirrhosis	Unspecified; 6–35 months	Unspecified	Freedman *et al.* [103]
Patients with HCV infection (25)[a]	To evaluate the mode of action of silibinin	Suppression of viral production/release	10, 15, or 20 mg/kg/day; 7 days	Intravenous infusion	Guedj *et al.* [104]
Patients with HCV infection (14)[a]	To assess the antiviral activity and safety of silibinin	Antiviral property, tolerability	20 mg/kg/day; 21 days	Intravenous infusion	Mariño *et al.* [105]
Patients with HCV cirrhosis (9)[a]	To investigate the safety and antiviral efficacy of silibinin	Lack of toxicity, antiviral effect	20 mg/kg/day; 21 days	Intravenous infusion	Bárcena *et al.* [106]
Patients with HCC (3)[a]	To determine the MTD of a silybin analog	Failure to establish MTD due to death	2 g/day; 23–69 days	Oral	Siegel *et al.* [107]

[a]The number of human participants is indicated in parentheses.

prior peginterferon plus ribavirin treatment. Silymarin use among these patients was associated with reduced progression from fibrosis to cirrhosis, but it had no impact on clinical outcomes.

Guedj *et al.* [104] analyzed and modeled HCV RNA kinetics from 25 patients infected with HCV genotype 1 or 4, who were treated with monotherapy of silibinin at 10, 15, or 20 mg/kg/day infused over 4 h for 7 days. During this study period, HCV RNA levels were measured daily. Silibinin triggered a high rate of viral decline between days 2 and 7 in all patients. The viral kinetic analyses showed that silibinin may block both viral infection and viral production/release, with its main dose-dependent effect being blocking viral production/release.

Hepatitis C recurrence following liver transplantation represents the main problem associated with most transplant procedures. A single-center, prospective, randomized, double-blind, placebo-controlled study was conducted to assess the antiviral activity and safely of silibinin administration in HCV-infected patients awaiting liver transplantation. Eleven patients received silibinin (20 mg/kg/day; infused daily over 2–4 h) and three received placebo for a maximum period of 21 days before and 7 days after liver transplantation. Among the patients who underwent more than 14 days of pretransplantation silibinin treatment, the median decrease in viral load was 2.31 $\log_{10}$ (range 0.6–4.2), compared with 0.30 $\log_{10}$ (0.1–0.6) in the placebo group. During the post-transplantation treatment, HCV RNA levels were consistently and significantly lower in the silibinin group compared with the placebo group, and they decreased below the limit of quantification in two patients and below the limit of detection in two additional patients. Pretransplant treatment with silibinin was well tolerated [105]. Another single-center, prospective, pilot study evaluated the safety and antiviral effect of prolonged intravenous silibinin treatment started immediately before liver transplantation in patients with HCV cirrhosis. Silibinin monotherapy was found to be safe during the immediate liver transplantation period, leading to potent and time-dependent antiviral efficacy and lack of HCV-RNA breakthrough during administration. Nevertheless, HCV-RNA rebounded after withdrawal, and silibinin treatment did not avoid reinfection of the graft [106].

At least one study was carried out in patients with advanced HCC and hepatic dysfunction with the objective of determining the maximum tolerated dose of a silybin derivative. In this phase I dose-escalation trial, three patients consumed 2 g/day of silybin phosphatidylcholine in divided doses. Serum concentrations of silibinin and silibinin glucuronide were found to increase within 1–3 weeks. In all patients, liver function abnormalities and tumor marker α-fetoprotein progressed; however, after 56 days, the third patient showed some improvement in these parameters. As all three patients died within 23–69 days of enrolling into this study, the maximum tolerated dose could not be established [107]. It is likely that this patient population may have been too ill to benefit from a silybin intervention to improve liver function tests.

Conclusion and future direction

On the basis of numerous studies conducted using various liver cancer cells, chemically induced xenograft, orthotopic, and transgenic animal models, and human participants, as summarized in this review, silymarin and its principal phytoconstituent silibinin were found to play an important role in the prevention and treatment of HCC. From the results of various preclinical in-vitro and in-vivo studies, it is evident that the constituents of silymarin could inhibit all stages of hepatocarcinogenesis, namely, initiation, promotion, and progression. The fundamental mechanisms of action of silymarin in hepatocellular carcinogenesis involve the mitigation of ROS-induced oxidative stress through antioxidant activity and the suppression of sustained hepatic inflammation through modulation of the prostaglandin pathway. It is also likely that the antitumor effects of silymarin are largely due to inhibition of abnormal cell proliferation and apoptosis induction through cell cycle arrest and interference of intrinsic and extrinsic mitochondrial pathways. Several signaling pathways activated in HCC, namely ERK, PTEN/Akt, Wnt/β-catenin, mTOR, and Notch, could also be targets of silymarin constituents. It is tempting to speculate that several bioactive phytochemicals present in silymarin could act through coordinated regulation of multiple discrete pathways to prevent the occurrence of liver tumors and kill established hepatic carcinoma cells. This is in line with emerging evidence that plant phytochemicals manifest chemopreventive and antitumor effects when they are used in combination rather than individually [108–110].

As with most research studies utilizing phytoceuticals and nutraceuticals, there exist substantial challenges for the development of silymarin for the prevention and therapy of human HCC. Variations in the phytochemicals present in silymarin may critically affect clinical outcome. Hence, establishment of a full phytochemical profile and standardization of the herbal extract, silymarin, are important for conducting clinical trials. Several in-vivo and clinical studies have failed to produce positive results, perhaps due to poor bioavailability of silymarin and its constituents. Chemical modification of the flavonolignan moiety to synthesize more polar derivatives may represent one approach to overcome this challenge. Novel drug formulations and delivery systems, including nanosuspensions, micelles, nanoparticles, and nanoemulsions, can be used to improve the in-vitro dissolution velocity (and thereby bioavailability) and the in-vivo efficiency of silymarin components [111]. As silymarin or silibinin can

inhibit several isoforms of the cytochrome P450 family and interfere with glucuronidation of several drugs, potential interactions may occur when milk thistle extracts are used in combination with chemotherapeutic agents. Similarly, silymarin may reduce the effectiveness of radiotherapy because of its potent free radical-scavenging activity. Moreover, promotion of tissue regeneration by silymarin, especially in the liver, and its potential estrogenic activity may promote tumor growth. All these factors should be considered before designing clinical trials of silymarin in patients with established HCC with various etiologies or patients with high risk of developing HCC. Future clinical studies should also include profiling of genes associated with molecular pathways activated in HCC with silymarin treatment, various histologic subtypes of HCC, appropriate biomarkers for monitoring drug response, and genetic polymorphism of phase I and II drug metabolism.

On the basis of an impressive body of evidence presented in this review, milk thistle-derived products, especially silymarin and silibinin, have been found to show significant promise for the prevention and treatment of liver cancer without any adverse effects. Nevertheless, well-designed clinical studies are urgently needed to evaluate the full potential of these natural agents to effectively treat or reduce the risk for liver cancer.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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