World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i18.2288

World J Gastroenterol 2011 May 14; 17(18): 2288-2301
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Silybin and the liver: From basic research to clinical practice

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Received: November 18, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: May 14, 2011

Abstract

Herbal products are increasingly used, mainly in chronic liver disease. Extracts of milk thistle, Silymarin and silybin, are the most prescribed natural compounds, with different indications, but with no definitive results in terms of clinical efficacy. This review analyzes the available studies on the effects of the purified product silybin, both as a free and a conjugated molecule, on liver cells or on experimentally induced liver damage, and in patients with liver disease. We searched PUBMED for articles pertaining to the *in vitro* and *in vivo* effects of silybin, its antifibrotic, anti-inflammatory, and antioxidant properties, as well as its metabolic effects, combined with the authors' own knowledge of the literature. Results indicate that the bioavailability of silybin phytosome is higher than that of silymarin and is less influenced by liver damage; silybin does not show significant interactions with other drugs and at doses < 10 g/d has no significant side effects. Experimental studies have clearly demonstrated the antifibrotic, antioxidant and metabolic effects of silybin; previous human studies were insufficient for confirming the clinical efficacy in chronic liver disease, while ongoing clinical trials are promising. On the basis of literature data, silybin seems a promising drug for chronic liver disease.

Key words: Milk thistle; Silymarin; Liver disease; Hepatic fibrosis; Hepatic inflammation; Radical species

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Loguercio C, Festi D. Silybin and the liver: From basic research to clinical practice. *World J Gastroenterol* 2011; 17(18): 2288-2301 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2288.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2288

INTRODUCTION

The terms milk thistle, flavonoids, silymarin, and silybin are generally used interchangeably; however, each of these compounds has specific characteristics and actions, with an intrinsic beneficial or toxic effect. In the last 10 years, about 12 000 papers have been published on these substances, used as antioxidants or chemopreventives and anticancer agents, and especially as hepatoprotectants. This publication volume indicates that scientific interest in these molecules, or classes of molecules, is high worldwide. In the US and Europe, about 65% of patients with liver disease take herbal preparations; in Europe, the cost of the use of silymarin reaches $180 million in Germany alone. Despite the wealth of literature, no firm clinical evidence exists to recommend the use of these substances in clinical practice[1-10]. This discrepancy is attributable to various factors, such as quality of clinical trials, heterogeneity of diagnoses, lack of standardized preparations, and frequently inconsistent dosing and outcome parameters. At a time when the use of herbal products is increasing, whether driven by individual choice or industry promotion, in our opinion it is necessary to focus more intently on these compounds that may have beneficial, placebo, or toxic effects.

This review analyzes studies of the effects of the

purified product silybin, both as a free and a conjugated molecule, on liver cells or on experimentally induced liver damage, and in patients with liver disease.

DEFINITION AND CHARACTERISTICS OF SILYBIN

As reported, silybin and silymarin are not synonymous[1,3,6]. Silymarin is a complex of at least seven flavonolignans that are the most common class of compounds present in milk thistle extract, and one flavonoid, taxifolin. The relative abundance of each compound may vary depending on the source of botanical material, supplier, and extraction processes. Silybin represents about 50% to 70% of the silymarin extract. Silybin can be resolved into two 1:1 diastereoisomers, silybin A and silybin B. In addition, silybin may be present as isosilybin, a 1:1 mixture of two diastereoisomeric compounds, isosilybin A and isosilybin B[11-17]. The concentrations of silybin in the main pharmaceutical products containing silymarin present in the US and other countries range from 20% to 40%[16].

PHARMACOKINETICS AND PHARMACODYNAMIC ASPECTS

Flavonolignans are known for their poor and erratic bioavailability; for example, silymarin absorption rate levels vary between 20% and 50%. Silybin has been separated commercially as a pure substance[11-16], and the study of silybin pharmacokinetics properties using an HPLC method has shown that the concentration-response relationship is linear over a concentration range of 0.5-100 μg/mL[16]. After administration to rats, the disposition of silybin in the plasma and bile fluid is due to rapid distribution and equilibrium between the blood and hepatobiliary system, and the bile levels of unconjugated and total silybin are greater than those in plasma[18-22].

Similarly to other flavolignans, limiting factors for the use of silybin are its low solubility in water, low bioavailability, and poor intestinal absorption. To counteract this aspect, different more soluble derivatives of silybin have been synthesized, such as silybin bis-hemisuccinate, β-cyclodextrin complex, silybin-N-methyl-glucamine, silybin 11-O-phosphate, and silybin-phosphatidylcholine. Another strategy for improving silybin solubility is represented by the enzymatic synthesis of its β-glycosides, such as silybin β-galactoside, silybin β-glucoside, silybin β-maltoside, and silybin β-lactoside. A soluble silybin prodrug has been finally synthesized with a high aqueous soluble polymeric carrier (polyethylene glycol)[23-30].

Some conjugations may in part affect the activity of silybin. For example, the radical-scavenging activity of the silybin 20-O-β-D-glucuronide is considerably lower than that of free silybin. In contrast, a considerable increase in radical-scavenging activity is observed in the other silybin, 7-O-β-D-glucuronide, in which position C-20 is free. The increase in the radical-scavenging activity of this latter conjugate cannot be simply ascribed to the addition of the glucuronyl moiety. The change in antiradical activity is, therefore, the result of the overall structural change. It is also quite interesting that one diastereoisomer of silybin (B) undergoes conjugation faster than the other (A); this difference indicates that the two silybin diastereoisomers are metabolized at different rates. These findings will aid the development of improved silybin formulations designed to inhibit the C-20 conjugation or, alternatively, to contribute to a modified dosage scheme that maintains free plasma silybin at sufficient levels[31-35]. Both free and conjugated silybin have a rapid plasma and tissue distribution that reaches maximum levels within one hour after 50 mg/kg silybin administration in mice[36]. The protein binding of silybin in rat plasma is 70.3% ± 4.6%[21].

In humans, the pharmacokinetics of silybin was evaluated after the administration of variable doses of silymarin, pure silybin, and silybin conjugated with phytosome in healthy volunteers. Hoh *et al*[37] for the first time identified the silybin plasma metabolites and measured the silybin tissue levels in humans who had ingested silybin. They demonstrated that silybin undergoes multiple conjugation reactions in humans and clearly identified the conjugated species: silybin monoglucuronide, silybin diglucuronide, silybin monosulfate and silybin glucuronide sulfate. The administration in humans of 240 mg of pure silybin induces a peak concentration of 240 ± 54 ng/mL in about 2 h that persists for 4 h. After administration of a single oral dose of 560 or 600 mg silymarin (about equivalent in total to 240 mg of silybin), the fractions of the free, sulfated, and glucuronidated silybin in human plasma are about 17%, 28%, and 55% of the total dose, with a higher plasma percentage of glucuronidated silybin B (71%) than of glucuronidated silybin A. The total percentage of dose recovered in urine, as free and conjugated, is very low, ranging from 1% to 7% of the dose (mean: 2.8 ± 0.6 ng/mL)[29,38-42]. The complex silybin phytosome (silybin + phosphatidylcholine) was recently formulated with the addition of vitamin E (Indena, IBI-Lorenzini spa Italy: Realsil®). This conjugation induces a greater solubility of silybin, as reported in Table 1. In 12 healthy volunteers aged 20-53 years, silybin was administered as Realsil® in two pharmaceutical forms (capsules and granules, both corresponding to 47 mg of silybin). Data were compared to those obtained by administering, in a cross-over manner after 1 wk of wash-out, a silymarin capsule containing 58 mg of silybin and silymarin granules containing 80 mg of silybin[43]. As summarized in Tables 1 and 2, the global results of the pharmacokinetic analysis indicated that the bioavailability of silybin phytosome is much higher than that of silymarin.

EFFECT OF LIVER DAMAGE ON PHARMACOKINETICS OF SILYBIN

In patients with well-compensated liver cirrhosis, silybin was administered as silybin phytosome at a dose of 120 mg

Table 1 Pharmacokinetics of silybin phytosome and silymarin in healthy participants[38-43]

	Silybin phytosome	Silymarin
Peak concentration (ng/mL)	298 ± 96	102 ± 22[a]
Time of peak (h)	1.6 ± 0.3	1.4 ± 0.3
Mean residence time (h)	3.6 ± 0.4	3.5 ± 0.4
AUC (ng/mL/h)	881 ± 207	257 ± 66[b]

[a]$P < 0.05$, [b]$P < 0.01$. NB: The analyses were performed using HPLC.

Table 2 Pharmacokinetics of silybin after administration to 12 healthy volunteers as silymarin or silybin-phosphatidylcholine-vitamine E complex (RA)[43]

Product	Cmax (ng/mL)	Tmax (h)	AUC (ng/mL per hour)
RA granules (47 mg silybin)	213 ± 166	0.5	246 ± 114
RA capsules (47 mg silybin)	117 ± 93	1.0	161 ± 85
Silymarin gran (58 mg silybin)	18 ± 17	0.5	25 ± 18
Silymarin caps (80 mg silybin)	5 ± 7	1.0	11 ± 5

× 3 /d, or as silymarin containing 84 mg of silybin 4 fold/d. The results showed that liver cirrhosis does not modify the kinetics of silybin and that in liver patients, the bioavailability of silybin is higher when it is administered as a phytosome adduct[44]. Similar data were obtained in rats with experimentally induced cirrhosis[45]. Table 3 summarizes the data obtained in humans.

More recently, the pharmacokinetics of silybin has been evaluated in patients with chronic hepatitis [hepatitis C virus (HCV) or non-alcoholic fatty liver disease (NAFLD)] and compensated liver cirrhosis[46,47]. A single, 600 mg p.o. dose of milk thistle extract was administered to healthy volunteers and to three patient cohorts, and blood samples were obtained over 24 h. Silybin A and B accounted for 43% of the exposure to the sum of total silymarin flavonolignans in healthy volunteers and only 31% to 38% in liver disease cohorts as a result of accumulation of silychristin (20%-36%). Area under the curve (AUC, 0-24 h) for the sum of total silymarin flavonolignans was 2.4, 3.3, and 4.7-fold higher for the HCV or NAFLD ($P \leqslant 0.03$) and HCV cirrhosis cohorts ($P \leqslant 0.03$), respectively, compared with healthy volunteers. Silymarin kinetics was correlated with plasma levels of caspases as an index of liver inflammation; caspase 3/7 activity correlated with the AUC (0-24 h) for the sum of all silymarin conjugates among all participants ($R^2 = 0.52$) and was 5-fold higher in the HCV cirrhosis cohort ($P \leqslant 0.005$ versus healthy participants). These findings suggest that the presence of liver damage, particularly as chronic inflammation, may affect the bioavailability of the different components of silymarin, possibly explaining the low beneficial effects of flavonoids in patients with liver damage.

Interactions and toxicity

There are substantial differences between silymarin and silybin in their interactions with metabolizing enzymes, and the reasons for these differences remain unknown. Silybin alone or as silybin β-galactoside, β-glucoside, β-lactoside, or β-maltoside at a final concentration of silybin 100 μmol/L has been tested *in vitro*. Under these conditions, silybin showed slow inhibitory effects (IC_{50} > 200 μmol/L) on marker substrates of CYP2E1, CYP2D6, CYP2C19, and CYP2A6. Silybin and silybin β-glycosides did not induce expression of CYP1A2 and CYP3A4 and did not affect the inducible expression of either of these enzymes[48-53].

Table 3 Comparison between silybin phytosome and silymarin pharmacokinetics in patients with liver cirrhosis[44]

	Silybin phytosome (360 mg)	Silymarin (336 mg)
C_{max} (ng/mL)	860 ± 166[b]	83 ± 15
Tmax (h)	2.7 ± 0.7[b]	2.6 ± 2.1
/2 (h)	3.3 ± 0.7[b]	2.6 ± 0.4
AUC	515 ± 665[b]	262 ± 39
Total bioavailability	252 ± 39[b]	19 ± 23

[b]$P < 0.001$.

In *in vivo* work, Sridar *et al*[54] investigated metabolic interactions involving silybin at doses ranging from 25 to 250 μmol/L and substrates metabolized by CYP3A4 or CYP2C9, showing that silybin may be a modulator/inactivator of P450s 3A4 and 2C9. What remains to be elucidated is whether this effect is brought about by competition at the site or inhibition for substrate binding and for metabolism at a particular binding pocket in the active site; another possibility is that it results from a conformational change in the active site. Despite these results, silybin has shown no effect on the metabolism of indinavir[55,56], which is mediated by CYP3A4, and others[57-60] have documented that silybin, at a concentration of 100 μmol/L, has no effect on either basal or inducible expression of CYP3A4 mRNA.

In any case, the silybin-drug interaction of these enzyme substrates is not clinically relevant, and the inhibitory effects of silybin occur only at concentrations that massively exceed the physiologically used doses[60-62]. *In vitro*, silybin inhibits the UGT glucuronyl transferases UGT1A6 and UGT1A9, but it is 14- to 20-fold more selective for UGT1A1[48,49,61-64] (see Table 4 as summary). The clinical relevance of this phenomenon is presently unknown because there are no published reports indicating the existence of a clinical interaction with bilirubin. UGT1A1 is the only enzyme responsible for bilirubin glucuronidation, and it contributes to the glucuronidation of several drugs. In animals, silybin affects the hepatobiliary elimination of various drugs[52,53,57,58,64]; oral feeding of pure silybin at doses of 100 and 200 mg/kg/bw/d showed a moderate to highly significant increase in both glutathione-S-transferase and quinine reductase activities in the liver, lung, stomach, skin, and small bowel in a dose- and time-dependent manner[58]. Recently, Flaig *et al*[27] provided the best evidence that silybin can be administered to humans at doses producing anticancer-relevant concentrations, with minimal or no side effects.

Table 4 Interference of silybin with cytochromes[48-64]

Interference	Possible interference	No interference
UGT1	CYP3A4	CYP2E1
	CYP2	CYP2D6
		CYP2C19
		CYP1A2
		CYP2A6

That study employed the largest doses ever used, ranging from 2.5 to 20 g of silybin-phosphatidylcholine (Indena's Siliphos® "silybin phytosome"), given daily in three divided doses for 4 wk to 13 men with a history of prostate carcinoma. At a dose escalation from 15 to 20 g/d, silybin was discontinued because of asymptomatic hyperbilirubinemia, most likely because of inhibition of the glucuronyl transferase UGT1A1. However, in all patients, this mild hyperbilirubinemia improved with treatment cessation.

The limitations of available clinical trials with regard to establishing safety are the same as those for establishing efficacy. Clinical trials testing safety are poor predictors of the fate of extracts in real-world settings, where patients ingest multiple drugs and herbs, take different formulations of the same product, and add alcohol and other compounds, often for extremely long periods. In the randomized trials that reported adverse effects, their frequency was approximately equal in the silybin and control groups. The majority of the observed adverse events were unrelated to the product or difficult to separate from the concomitant disease, and in available reports, causality is rarely addressed. There are no safety data in children or older adults, as there are no reported studies in children and very few studies that included patients older than 65 years. Adverse effects associated with oral ingestion of silybin include mainly gastrointestinal problems, but these are rare. Headache/dizziness and pruritus were reported in one clinical trial. Asymptomatic liver toxicity has been observed in recent clinical trials performed in cancer patients, in whom hyperbilirubinemia and increases in alanine aminotransferase (ALT) levels were observed; however, these effects were present only when very high dosages of silybin phytosome (between 10 and 20 g/d) were used. At high doses, a laxative effect of silybin phytosome is possible because of increased bile secretion and bile flow. Mild allergic reactions have also been noted, but they were not serious. Therefore, the available data indicate that milk thistle has few side effects when doses lower than 5 g/d are used, with possible adverse effects at doses greater than 10 g/d[6,7,62].

The documented effects of silybin in basic research

In liver cells, as well as in other types of cells, the common effects of silybin may be summarized as follows: (1) Antioxidant; (2) Direct and/or indirect (through the antioxidant capability) modulator of inflammation and fibrogenesis; and (3) indirect and/or direct modulator of some intrahepatic metabolic pathways.

Antioxidant action

The antioxidant effects of silybin have been demonstrated in all cells studied. Silybin acts as an antioxidant because it inhibits radical formation, binds some radical species (scavenger), interferes with lipid peroxidation of membranes (and therefore modulates membrane permeability), and increases the intracellular content of scavengers[65]. In fact, in the presence of oxidative and nitrosative stress, silybin inhibits the formation of superoxide anion radicals and of nitric oxide (NO), increases ATP content through the phosphorylation of ADP, decreases the content of malondialdeyde (MDA) and totally abolishes the decrease of glutathione, of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase[66-73]. These results, which are dose-dependent, have been documented in isolated rat Kupffer cells, hepatocytes, HEPG2 cells, isolated mitochondria from rat hepatocytes, and in models of ischemia-reperfusion of rat liver. In experimentally induced liver damage, one hour after intragastric administration to rats of conjugated silybin (0.6 g/kg bw), the content of silybin in the microsomes measured using a specific HPLC assay was approximately 2.5 μg/mg of protein, corresponding to a final concentration of 10 μmol/L of silybin. Under these conditions, lipid peroxidation, mitochondrial permeability and respiration, and membrane potential as well as cell death were reversed by silybin, particularly if it was conjugated as silybin + phosphatidylcholine. This last compound, at a dose of 20 μmol/L, directly scavenges hydroxyl, hydroxyethyl, lipodienyl, methyl, and trichloromethyl radicals[74,75].

Finally, silybin acts as an antioxidant because it also serves as an iron chelator[76-78]. More recently, it has been suggested that dehydrosilybin (DHS), an oxidized form of silybin, has greater antioxidant activity than silybin (about three times better), probably because of the presence of unsaturated bonds that contribute to hydrogen-donating capacity. The better scavenger activity of DHS may also be the result of its greater ability to react with cell membranes because it has higher lipophilicity than silybin[32,65].

Table 5 summarizes the main antioxidant effects of silybin.

Anti-inflammatory action

In general terms, silymarin and silybin interfere with the NF-κB-controlled transduction cascade. NF-κB is an inducible and ubiquitously expressed DNA-binding protein, acting as a transcription factor for genes involved in inflammation, cell survival, differentiation, and growth[79,80]. In unstimulated cells, NF-κB is sequestered in the cytoplasm by interaction with inhibitory protein 1 κ B α (IκBα). Upon activation from oxidative stress, NF-κB dissociates from IκBα, and IκBα is degraded. NF-κB translocates to the nucleus and, through kinase phosphorylation, drives the activation of genes supporting inflammation. Consistent with its antioxidant activity, silybin has been demonstrated to inhibit NF-κB activation and translocation through suppression of IκBα phosphorylation and degradation[81-83]. In an acute model of liver damage

Table 5 Antioxidant effects of silybin[65-75]

Doses	Cells	Targets
From 10 to 100 μmol/L	Hepatocytes	Decreased formation of reactive oxygen species from mitochondria; iron chelator
	HepG2	Decreased formation of superoxide anion
Mean dose with documented effects: 20 μmol/L	Kupffer cells	Decrease of NO production
	Monocytes	Scavenger of lipodienyl, methyl, trichloromethyl radicals
	Endothelial cells	Decrease of hydrogen peroxide concentration
	Cancer cells	Block of membrane lipid peroxidation

Table 6 Anti-inflammatory/antifibrotic effects of silybin[79-99]

Doses	Cells	Targets
From 5 to 50 μmol/L	Hepatocytes	Inhibition of NF-κB-mediated signaling
Mean: 15 μmol/L	Endothelial cells	Suppression of IκBα phosphorylation
	Platelets	Inhibition of protein kinase kinase
	Cancer cells	Inhibition of c-jun N-terminal kinase
	Phagocytes	Inhibition of leukotriene formation
	Stellate cells	Inhibition of release of cytochrome c
	HepG2	Inhibition of ERK, MEK, and Raf phosphorylation; inhibition of release of caspase 9 and 3, IL-8, and of PDGF- and TGF-β-mediated signaling; decrease of MMP2; increase of TIMP2; inhibition of HCV replication

in which mice were treated with concanavalin A, silybin reduced plasma levels of transaminases and the plasma and liver content of pro-inflammatory cytokines, inhibited hepatic NF-κB activation, and increased plasma and tissue levels of IL-10[84]. In rats with dimethyl-nitrosamine-induced chronic liver damage, silybin conjugated with phosphatidylcholine and vitamin E (Realsil®, Ibi-Lorenzini, Italy) administered by gastric gavage could prevent loss of body and liver weight, as well as reducing the degree of liver injury, as determined by ALT values and necroinflammatory scores. This outcome was associated with reduced hepatic stellate cell activation and proliferation both after 1 and 5 wk of treatment[85]. The anti-inflammatory action of silybin is also related to its interference with multiple cytokine-induced signaling pathways to down regulate inducible nitric-oxide synthase (iNOS) expression[86-88]and to the inhibition of cyclooxygenase (COX)-2 expression and activity and leukotriene formation in human platelets, white blood cells, and endothelial cells. Finally, silybin inhibits activation of the protein kinases and of a c-jun N-terminal kinase[89-92].

In addition to its antioxidant and anti-inflammatory actions, silybin also shows an antiviral effect. In fact, at a concentration of 20 μmol/L, it inhibits the protein expression and the replication of HCV virus in infected polymorphonucleated cells derived from patients with chronic HCV infection[93-95].

Antifibrotic action

In an *in vitro* model of human hepatic fibrogenesis, silybin demonstrated both direct and indirect antifibrotic properties. In fact, in stellate cells from human liver, silybin reduced platelet-derived growth factor (PDGF)-induced DNA synthesis and cell proliferation at a dose of 25 μmol/L. Silybin also reduced PDGF-induced cell migration in a dose-dependent fashion. Finally, pre-treatment with 25-50 μmol/L of silybin significantly reduced the TGF-β-induced *de novo* synthesis of procollagen type I in cell supernatants[96].

To investigate the role of silybin in modulating the pro-inflammatory properties of hematopoietic stem cells, cells were stimulated with IL-1β (20 ng/ml), a potent pro-inflammatory cytokine; silybin inhibited, in a dose-dependent manner, IL-1-induced synthesis of human MCP-1 (monocyte chemoattractant protein 1) and human IL-8 as detected in cell supernatants. This effect was related to the effect of silybin on the inhibition of IκBα phosphorylation and to its capability to inhibit ERK, MEK, and Raf phosphorylation at any concentration used[96]. Antifibrotic effects were also documented in experimental animals and in humans[85,97-99].

Table 6 summarizes the main anti-inflammatory and antifibrotic effects of silybin.

Metabolic effects

Silybin interferes with some mechanisms of action of insulin. In fact, it modulates the uptake of glucose in adipocytes by blocking the insulin-dependent glucose transporter 4. In rat hepatocytes, silybin, in concentrations ranging from 25 to 100 μmol/L, lowers glucose formation from different gluconeogenic substrates through an inhibitory effect on pyruvate kinase activity[100]. As previously reported in cultured hepatocytes, low doses of silybin reduce reactive oxygen species formation from mitochondria; this reduction leads to a decrease in oxidation of carbons arising from glycolysis[101]. Moreover, silybin inhibits, in a dose-dependent manner, gluconeogenesis and glycogenolysis, both in basal conditions and after a glucagon-dependent stimulation, by blocking glucose-6-phosphate hydrolysis. This effect was demonstrated using different substrates, such as dihydroxyacetone, lactate/pyruvate, glycerol, and fructose[102] (Table 7).

Table 7 Effects of silybin on hepatic metabolism of glucose at doses of 25-100 μmol/L in hepatocytes[100-102]

Doses	Cell	Targets
25-100 μmol/L	Hepatocytes	Inhibition of pyruvate kinase Inhibition of glycolytic flux Inhibition of glucose-6-phosphate hydrolysis Inhibition of glucose-6-phosphatase

Table 8 Other effects of silybin on cellular signaling[106-122]

Induction of apoptosis through an inhibition of IGF-IR
Down regulation of survivin and an increase in p53 expression
Block of cycle-regulator cyclins and promoter activities
Decrease of MMP-1 production
Decrease of angiogenesis
Inhibition of VEGF expression
Inhibition of HIF-1α
Increase of IGFBP-3

In an animal model of type 1 diabetes mellitus[103], in a 6-mo, double-blind, randomized trial in patients with poorly controlled non-insulin-dependent diabetes mellitus and alcoholic liver disease[104], and in a randomized, double-blind, placebo-controlled trial in patients with type Ⅱ diabetes[105], silybin significantly affected plasma levels of glucose and triglycerides, with a trend toward lower hemoglobin A1c levels.

Other effects on cellular signaling

In cancer cells, silybin alters cell cycle regulators and induces apoptosis, both through antioxidant and anti-inflammatory properties and through the inhibition of growth factor receptor-mediated mitogenic and cell survival signaling, particularly related to the activation of tyrosine kinases[106-111]. PDGF receptor (PDGFR), epidermal growth factor receptor (EGFR), Bcr-Abl, and KIT are examples of tyrosine kinases overexpressed in most human cancers. Silymarin or silybin are particularly effective in inhibiting EGFR signaling with suppression of cyclin-dependent kinase expression (i.e. CDK4) and up regulation of CDK inhibitors (CDKIs). These effects lead to G1 and G2-M arrest in cancer cells[112-119], as recently confirmed in a model of human colon cancer[120,121]. In fact, at a dose ranging from 40-75 μmol/L, silybin significantly inhibited cell proliferation through cell cycle arrest *via* inhibition of cyclin promoter activity.

Because it inhibits constitutive NF-κB activation, silybin can induce apoptosis, consistent with a significant decrease in its nuclear level of p65 subunit. In addition, it activates caspase 3 and caspase 9 and decreases survivin levels[122].

Angiogenesis refers to the growth of capillary vessels from existing blood vessels and is considered obligatory for the growth and progression of solid tumors. Angiogenesis critically depends on several conditions; in fact, endothelial cells must: (1) proliferate to provide the necessary number of cells for the growing vessels; (2) secrete matrix metalloproteinases (MMPs), which are required to break down surrounding tissue matrix; and (3) be capable of movement and migration. In addition, the angiogenic stimuli like hypoxia and the production of angiogenic cytokines, such as vascular endothelial growth factor (VEGF), must be sustained. Silybin treatment decreases secreted VEGF levels and shows a strong, concentration-dependent inhibition of capillary tube formation on matrigel, retraction, and disintegration of preformed capillary network, inhibition of matrigel invasion and migration, and a decrease in MMP-2 secretion[92,106-122].

Table 8 summarizes the main cellular signaling affected by silybin.

The use of silybin in the clinical setting: efficacy and suggestions

The use of silymarin in the clinical setting has a long history; in its native Mediterranean region, it has been employed for liver damage since the Greco-Roman era. As mentioned in the introduction, "herbal therapy" use is consistently increasing worldwide, and some of the most common herbal supplement preparations are derived from the milk thistle plant (*Silybum marianum*). Despite a long history of its use and the large number of people who consume this substance, no conclusive data on its clinical efficacy can be identified. In fact, only a few well designed clinical trials have been performed. Most studies have been conducted using silymarin and with inclusion of patients with alcoholic or viral cirrhosis. Stickel *et al*[8] identified the 10 main controlled trials conducted in liver patients, with numbers of patients ranging from 20 to 200 and doses of silymarin ranging from 210 to 450 mg/d for 7 to 730 d. These clinical studies generally have suffered from the same shortcomings found in many other trials on herbal medicines, such as a small sample size, lack of appropriate randomization and of allocation concealment or blinding, very different periods of treatment, lack of information about type and dose of extract used as well as product characterization, ill-defined patient population, and a lack of etiology, severity of disease, and discussion of potential confounders.

We could argue that the history of studies on silybin reflects, in part, the epidemiological history of liver diseases. When liver cirrhosis was the only clearly identified manifestation of liver damage and alcohol was the only known pathogenetic factor, silymarin and other plant extracts represented the most frequently used drugs. Knowledge about the pathogenetic relationships between hepatitis viruses and liver damage induced researchers and industry to focus attention on antiviral drugs. In response to the discovery of metabolic liver diseases, as well as increased knowledge about the cellular and subcellular mechanisms of both induction and progression of liver damage, researchers in academia and industry have begun to reappraise natural products and to evaluate their therapeutic efficacy using appropriate methods.

The clinical experience with silybin is mainly related to its properties as a detoxifying agent and as a hepatoprotective compound in different acute and chronic liver diseases.

Silybin as a detoxifying and hepatoprotective substance

Several chemotherapeutic agents are metabolized by the liver and can exert hepatotoxicity, with the net result of drug reductions or withdrawal. Chemotherapeutic agents that are likely to produce hepatotoxicity include dactinomycin, daunorubicin, docetaxel, gemcitabine, imatinib, 6-mercaptopurine, methotrexate, and oxaliplatin. Cancer patients taking these therapies often self-medicate with milk thistle because of its reputation as a liver protectant. Clinicians also prescribe it to cancer patients for the same purpose. The rationale of milk thistle use is to provide support to the liver while it performs multiple functions, including responding to the increased metabolic demands caused by tumor growth, assisting in metabolizing products generated when a tumor is killed or reduced by chemotherapy and radiation, and aiding in the processing of drugs prescribed to cancer patients.

Silybin is also considered a potent inhibitor of human intestinal β-glucuronidase, blocking the release and reabsorption of free xenobiotics and their metabolites from their glucuronide conjugates. Because the liver is the primary organ that cleanses and detoxifies the blood, many detoxification programs include a component of liver support or what is often called liver cleansing. For these reasons, silybin is commonly included in detoxification regimens[6,7,62,108-111]. However, only one randomized, double-blind study has reported the effects of milk thistle in patients receiving cancer therapy; 50 children with acute lymphoblastic leukemia and grade 2 or higher hepatic toxicity were randomized to receive a milk thistle supplement (Siliphos, Thorne Research, Dover, Idaho) (5.1 mg/kg/day) or placebo for 28 days. The authors reported significant reductions in AST levels ($P < 0.05$) and a trend toward a significant reduction in ALT levels ($P < 0.07$). A significantly larger number of children in the milk thistle group developed a > 50% reduction in total bilirubin at day 28 compared to placebo ($P < 0.0069$)[123].

Silybin in acute liver damage

The administration of silybin within approximately 48 h after poisoning produced by the mushroom *Amanita phalloides* (death cap) seems to be an effective measure to prevent severe liver damage[124]. A retrospective analysis of 205 cases of clinical poisoning from 1971 to 1980 documented the efficacy of milk thistle silybin extract in increasing survival rates in adults and children exposed to this potentially lethal mushroom[125]. In January 2007, six family members in California suffering from aflatoxin poisoning caused by *A. phalloides* mushrooms were treated with intravenous milk thistle, provided by Madaus Pharma (Brussels, Belgium; division of Madaus AG, Cologne, Germany). The US Food and Drug Administration granted permission for the use of milk thistle after considering that all patients were at high risk of dying of liver failure. Ultimately, one of the six patients died, while the others had a full recovery after treatment. A case report mentions that silybin may offer protection from liver toxicity caused by the pharmaceutical drug phenytoin[126].

Silybin in chronic liver disease

The various flavonoid compounds that are included in the general term "milk thistle" have been recognized as a "safe and well-tolerated herb" with a limited adverse event profile[1-10]. As previously reported, the majority of studies have been conducted using silymarin and including patients with alcoholic or viral cirrhosis. The Cochrane Collaboration group[4,127] evaluated the studies related to the use of silymarin/silybin in patients with different types of acute and chronic liver diseases. Various items were analyzed to define the quality of trials, such as the number of participants, the criteria of randomization, the blinding, the modalities of follow-up, and the statistical methods. From 1,831 references, 67 publications addressed patients with alcoholic and/or hepatitis B or C liver diseases treated with milk thistle; of these, only 26 were selected according to the agreed criteria, and these were associated with 13 trials. The active treatment consisted of silymarin per os in 10 randomized clinical trials, silybin phytosome per os in two randomized clinical trials, and silybin + phosphatidylcholine in one randomized clinical trial. The Cochrane Collaboration studies concluded that milk thistle does not seem to significantly influence the course of the disease in treated patients. However, all causes of mortality were reduced by 50% in patients with alcoholic liver disease without HCV antibodies who took milk thistle extracts compared to placebo ($P < 0.05$); in trials that studied liver-related mortality, there was a significant effect of silybin (relative risk, 0.50; 95% CI 0.29 to 0.88; $P = 0.02$)[4,8,9,127, 128].

Seeff *et al*[5,129] examined the spontaneous use of herbal products in the US in patients affected by HCV chronic infection, including non-responders to a previous treatment with interferon and ribavirin. In 1145 patients, about 50% used herbal products; of these, silybin was used by 70%. Even if no changes in liver tests and/or in HCV-RNA serum levels were observed, the univariate analysis documented a lower incidence of symptoms and a better quality of life in patients who consumed silybin relative to those who did not. On multivariate analysis, adjusted for age, gender, educational status, alcohol use (n. drinks/d), physical activity, body mass index, and smoking, silybin positively affected more than one aspect of quality of life. An examination of the total number of patients treated with silymarin/silybin in both well-conducted trials and in pilot studies with a high quality identified about 2000 patients with liver cirrhosis or with chronic hepatitis of different etiology, with a mean duration of treatment of six months and a dose of silybin ranging from 160 to 360 mg/d (except in two cases, see below).

The main results regarding the efficacy of silybin in

Table 9 Main studies performed by using purified silybin as drug

Authors	Type of study, number of patients	Drug used, dose and duration of treatments	Outcomes	Results	Clinical relevance
Vailati *et al*[146]	A phase II randomised, open trial on 60 patients with chronic alcoholic or viral hepatitis	Three doses (160, 240, 360 mg) of silybin and phosphatidylcholine (IdB 1016, Indena, Italy) for two weeks. No placebo or no intervention group was used	Liver tests	Improvement of liver enzymes with all used doses	Scarce
Buzzelli *et al*[147]	Double blind with identical placebo. Twenty patients with HBV and/or HCV chronic active hepatitis	IdB1016 (complex with phosphatidylcholine and silybin) two capsules, twice a day (equivalent to 120 mg of silybin in each capsule) (480 mg/d). Duration of treatment and of follow-up: two months in total	Mortality. Liver biochemistry	Improvement of liver enzymes and bilirubin	Scarce
Buzzelli *et al*[148]	Unclear, described as double blind but the method to achieve this was not described. Trial characteristics: cross over design. Patients were assigned to the Silipide group for two months treatment, and one month washout. Ten patients with chronic hepatitis C. (non-responders) to a previous treatment with recombinant interferon α	Silipide (IdB1016) capsules 360 mg/d. Control group: placebo capsules. Duration of treatment and follow-up: two months of treatment and one month of washout	Mortality. Liver biochemistry	Results were not reported separately, only overall results. Improvement of liver tests	Data published only in abstract form
Lirussi *et al*[104]	Blinding: adequate, double blind with placebo of identical appearance. Sixty out-patients with chronic alcoholic liver disease and non-insulin dependent type 2 diabetes	Silybin-β-cyclodextrin (135 mg silybin) sachets t.i.d Duration of treatment: 6 mo	Mortality. Liver biochemistry	Decrease of fasting glucose and lipid peroxidation markers	Good
Bares *et al*[138]	Randomized study to 1 of 3 oral doses. Thirthy seven patients with chronic hepatitis C non responders to a previous IFN treatment	IdB1016 at 314, 628, 942 mg t.i.d.(120,240 and 360 mg t.i.d. silybin equivalents, respectively) for 12 wk	Effects on serum markers of iron status	There was a significant decrease in serum ferritin, that was independently associated with the stage Ⅲ-Ⅳ of liver fibrosi	Good
Falasca *et al*[134]	Observational study on forthy naïve HCV positive patients (30 treated and 10 observed without treatments)	Silybin-Vitamine E-Phospholipid Complex (Realsil®- Ibi-Lorenzini-Italy) in a dose of 4 pills per day (each pill: 47 mg of silybin) for 3 mo	Hepatoprotection and anti-inflammatory effect by determining cytokine pattern and markers of liver disease	Improvement of liver enzymes and of Il2 plasma levels. Improvement of insulin resistance markers in patients with contemporaneous liver steatosis	Medium
Federico *et al*[141]	Observational study on 85 outpatients: 59 with NAFLD and 26 with HCV related chronic hepatitis in combination with NAFLD, non-responders to previous antiviral treatment. 53 (39 NAFLD and 14 HCV) were treated, while the other 32 patients (20 NAFLD and 12 HCV) served as a control group (no treatment)	The complex silybin-vitamin E-phospholipids (Realsil®), 4 pieces/d for six months followed by another six months of follow up	Effects on insulin resistance and liver damage	US steatosis, liver enzymes, hyperinsulinaemia, and indices of liver fibrosis were improved in both treated groups	Suggestive
Ferenci *et al*[139]	Observational study on 36 patients with HCV chronic hepatitis non responders to IFN + ribavirin. Duration of the study: 7 d	Silybin i.v. (Madaus, Germany) at 5, 10, 15 and 20 mg/kg per day for 14 d	Effect on viral load. Safety	Good compliance, no side effects and potent antiviral effect against HCV	High

liver disease patients are summarized as follows.

In the past, most studies were focused on liver cirrhosis, particularly alcoholic cirrhosis, and the efficacy of silybin was evaluated in terms of improvement in liver test abnormalities and/or in mortality rate (see above for results)[126-128]. Silybin β-cyclodextrin has been studied as an antidiabetic drug in patients with alcoholic liver disease and concomitant non-insulin-dependent diabetes mellitus; in these patients, the drug (at a dose of 135 mg/d) did not influence liver function test results or insulin secretion but significantly reduced fasting glucose ($P < 0.03$) and serum triglyceride levels ($P < 0.01$) compared to placebo. The effects seem to be related to a reduction in insulin resistance[104].

More recently, studies have focused on chronic hepatitis, particularly that induced by HCV. Silybin phytosome, at a dose ranging from 240 mg/d to 942 mg t.i.d. (the highest dose used in patients with liver damage) was well tolerated without adverse effects and significantly improved liver damage as expressed by transaminase levels, oxidative stress (serum malondialdehyde levels) and both ferritin serum levels and iron global body storage[130-138]. Today, the attention of researchers is focused on a possible antiviral effect of silybin against HCV infection.

Polyak *et al*[95] tested the anti-inflammatory and antiviral action of different extracts from silymarin on polymorphonuclear cells from patients with chronic HCV infection, documenting an anti-inflammatory and antiviral effect of milk thistle extract, mainly for silybin, which presented the strongest anti NF-κB and anti-HCV replication effect. Ferenci *et al*[139] and Biermet *et al*[140] have demonstrated that silybin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. These studies also showed that very high doses of silybin i.v. (from 5 to 20 mg/kg/d for 14 d) were free of toxic effects.

Finally, non-alcoholic fatty liver disease (NAFLD) may occur as an expression of a metabolic syndrome or in association with HCV chronic infection. The simultaneous presence of NAFLD in this latter group of patients may negatively affect the progression of fibrosis and the response to antiviral treatment. Patients affected by primitive NAFLD and/or chronic HCV-related hepatitis in combination with NAFLD, all non-responders to previous antiviral treatment, were treated with four pieces/d of the complex silybin-vitamin E-phospholipids corresponding to about 200 mg of pure silybin/d for three or six months. The treatment induced a significant reduction of plasma markers of chronic inflammation (C-reactive protein and cytokines), metabolic parameters (triglycerides, cholesterol, insulin resistance), liver tests (transaminases and gamma-glutamyl transpeptidase), degree of ultrasonographic liver steatosis and, finally, of main indices of liver fibrosis TGF-β, hyaluronic acid and metalloproteinase 2)[99,141,142].

In a rodent model of non-alcoholic steatohepatitis, the same silybin phospholipid complex prevented mitochondrial dysfunction[143], and preliminary results in a large, multicenter Italian trial *vs* placebo indicate that complex silybin-vitamin E-phospholipids significantly improved liver damage in patients with NAFLD and markers of liver fibrosis in patients with HCV chronic hepatitis[144].

In Table 9 the main studies performed with silybin are reported and discussed.

CONCLUSION

The data reported in this review clearly indicate the increasing interest in silybin and its compounds as well as the continuous improvement in knowledge about the molecular actions of this substance. However, in the clinical setting, there is currently a lack of definitive data about its efficacy in patients with chronic liver disease. The only well-defined finding is the absence of adverse events at high doses. Generally, all clinical studies on herbal products suffer from similar limitations, in part related to the fact that well-designed trials require resources and natural products industries do not sponsor them with significant budgets. In addition, in the majority of cases, herbal products differ from pharmaceutical compounds because multiple ingredients could act through multiple pathways to therapeutically affect the host. In the case of silybin, clinical studies on the pure extract and/or its derivatives are few and with a limited number of patients. Silybin is the most active flavonolignan in silymarin, and most capsules are standardized to this compound, but many variations of complex mixtures and single extracts are available and may critically affect clinical outcome. A full phytochemical and biological profile is preferable before commencing any clinical study. Today, analytical techniques that examine a suite of compounds, including their respective ratios, provide a more rational approach to authentication and quality assessment of the products. There must be assurance that the administered dose increases plasma and/or target tissue concentrations consistent with those required to produce effects *in vitro*. One primary fault of many clinical studies of botanicals is that adequate pharmacokinetic analyses are not completed before initiating efficacy trials. Some botanicals may fail in efficacy trials, not because the botanical is itself without activity but because the dosing was not sufficient to achieve pharmacologically meaningful concentrations.

Several silybin clinical trials are ongoing at this time (see also online at www.nccam.nih.gov). In addition, manufacturers of milk thistle extracts are conducting clinical trials with their own products that will elucidate the effects of specific preparations.

There is a need to enhance the funding opportunities to evaluate these long-used products with evidence-based knowledge, but there is also a need to reiterate Kroll *et al*[60], "As biological studies progress, it remains important to make the distinction between silymarin and silybin and their respective and distinct compositions". This latter point should also be considered when clinical investigators turn to pooling existing studies for meta-analyses. The epidemiology of chronic liver disease is changing

worldwide: viral infections are declining, and patients with HCV/HBV chronic hepatitis are older; NAFLD and alcoholic liver diseases are increasing, and generally patients with these pathologies are younger. Finally, alcohol-related problems, metabolic disruptions, and viral infections frequently coexist in the same patient[145]. Therefore, in clinical practice, there is the need for drugs that can be used in the long term without serious adverse events. Researchers should definitively demonstrate if silybin has potential in this regard. Lastly, the absence of significant adverse effects of silybin even at high doses, the good compliance by the patient, the availability of a purified form of the compound, represent characteristics that allow to obtain commercially available products containing almost 600 mg of pure silybin to ensure a good concentration in tissues.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH