Identification of hepatoprotective flavonolignans from silymarin

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Abstract:

Silymarin, also known as milk thistle extract, inhibits hepatitis C virus (HCV) infection and also displays antioxidant, anti-inflammatory, and immunomodulatory actions that contribute to its hepatoprotective effects. In the current study, we evaluated the hepatoprotective actions of the seven major flavonolignans and one flavonoid that comprise silymarin. Activities tested included inhibition of: HCV cell culture infection, NS5B polymerase activity, TNF-α-induced NF-κB transcription, virus-induced oxidative stress, and T-cell proliferation. All compounds were well tolerated by Huh7 human hepatoma cells up to 80 μM, except for isosilybin B, which was toxic to cells above 10 μM. Select compounds had stronger hepatoprotective functions than silymarin in all assays tested except in T cell proliferation. Pure compounds inhibited JFH-1 NS5B polymerase but only at concentrations above 300 μM. Silymarin suppressed TNF-α activation of NF-κB dependent transcription, which involved partial inhibition of IκB and RelA/p65 serine phosphorylation, and p50 and p65 nuclear translocation, without affecting binding of p50 and p65 to DNA. All compounds blocked JFH-1 virus-induced oxidative stress, including compounds that lacked antiviral activity. The most potent compounds across multiple assays were taxifolin, isosilybin A, silybin A, silybin B, and silibinin, a mixture of silybin A and silybin B. The data suggest that silymarin- and silymarin-derived compounds may influence HCV disease course in some patients. Studies where standardized silymarin is dosed to identify specific clinical endpoints are urgently needed.

Keywords: hepatitis C | liver disease | milk thistle | botanical medicine | hepatoprotection

Article:

Chronic hepatitis C virus (HCV) is a major global medical problem. In the United States, millions of people are affected, the number of patients with HCV-induced end-stage liver disease

is growing (1), and this condition is already the leading indication for liver transplantation (2). The current standard of care for chronic hepatitis C, pegylated IFN-α and ribavirin, results in sustained elimination of virus in 55% of treated patients (3, 4). However, significant numbers of patients do not clear the virus and are intolerant to, have contraindications to, or opt out of therapy. Furthermore, because emerging specifically targeted antiviral therapy for HCV therapies need to be administered with pegylated IFN plus ribavirin (5), it is likely that many patients will not tolerate this therapy. Thus, there are many patients who have no other Food and Drug Administration-approved options to eliminate HCV and prevent progression of liver disease. As a result, many individuals have opted for complementary and alternative medicine-based approaches, including botanicals, to treat their chronic hepatitis C. Indeed, as many as 13 to 23% of American patients with chronic liver disease use botanical medicines, with silymarin being the most popular (6, 7).

Silymarin, an extract from the seeds of the milk thistle plant, Silybum marianum, has been used for centuries as a hepatoprotectant. Although silymarin has been described to possess antioxidant, immunomodulatory, antiproliferative, antifibrotic, and antiviral activities (8, 9), its mechanisms of action remain to be fully defined. Although its clinical efficacy is currently uncertain (8, 10), interest in this botanical medicine has been piqued by studies showing silymarin blocks HCV cell culture (HCVcc) infection (9). Intravenous administration of silibinin, composed of a 1:1 mixture of silybin A and silybin B, causes dose-dependent reduction of viral load in patients with chronic hepatitis C (11).

We previously showed that silymarin blocked HCVcc infection of human hepatoma cultures, inhibited TNF-α and TCR induced NFκB-dependent transcription, and suppressed TCR-mediated proliferation and inflammatory cytokine production from T cells (9, 12). Thus, silymarin displays antiviral, anti-inflammatory, and immunomodulatory functions in human liver and immune cells. We (13) and others (14) have recently shown that silymarin can also block in vitro HCV NS5B polymerase activity at high concentration. Therefore, in the current study, we screened the seven major flavonolignans and one flavonoid that comprise silymarin for antiviral, antipolymerase, anti-NF-κB, and immunomodulatory actions. In addition, we assessed the antioxidant potential of the pure compounds.

Results

Silymarin is a complex of eight major compounds, including seven flavonolignans: silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and one flavonoid, taxifolin (15). Compounds were purified as previously described (16) (Fig. S1). We then tested the effects of silymarin and pure compounds in assays for HCVcc infection, NS5B polymerase activity, HCVcc-induced oxidative stress, TNF-α-induced NF-κB transcription, and TCR-mediated induction of T-cell proliferation.

We first performed cytotoxicity dose-response experiments by measuring ATP levels, a sensitive marker of cell viability. All compounds were well tolerated by Huh7.5.1 human hepatoma cells up to 80 μM, except for isosilybin B, which was toxic to cells above 10 μM (Fig. S2). Silydianin, silychristin, isosilychristin, and taxifolin were not toxic at 100 μM.

Fig. 1*A* depicts the antiviral effects of the pure compounds against in vitro HCV infection, based on HCV protein expression. The concentrations that inhibited HCV protein by 50% (IC50) are shown in Table 1. Taxifolin, isosilybin A, and silybin A were the most effective in inhibiting JFH-1 infection, and these compounds were more potent than silymarin extract. Silybin B and silibinin, a 1:1 mixture of silybin A and silybin B, also inhibited HCV infection with potencies similar to silymarin. Isosilychristin, silydianin, and isosilybin B did not inhibit HCV protein expression.

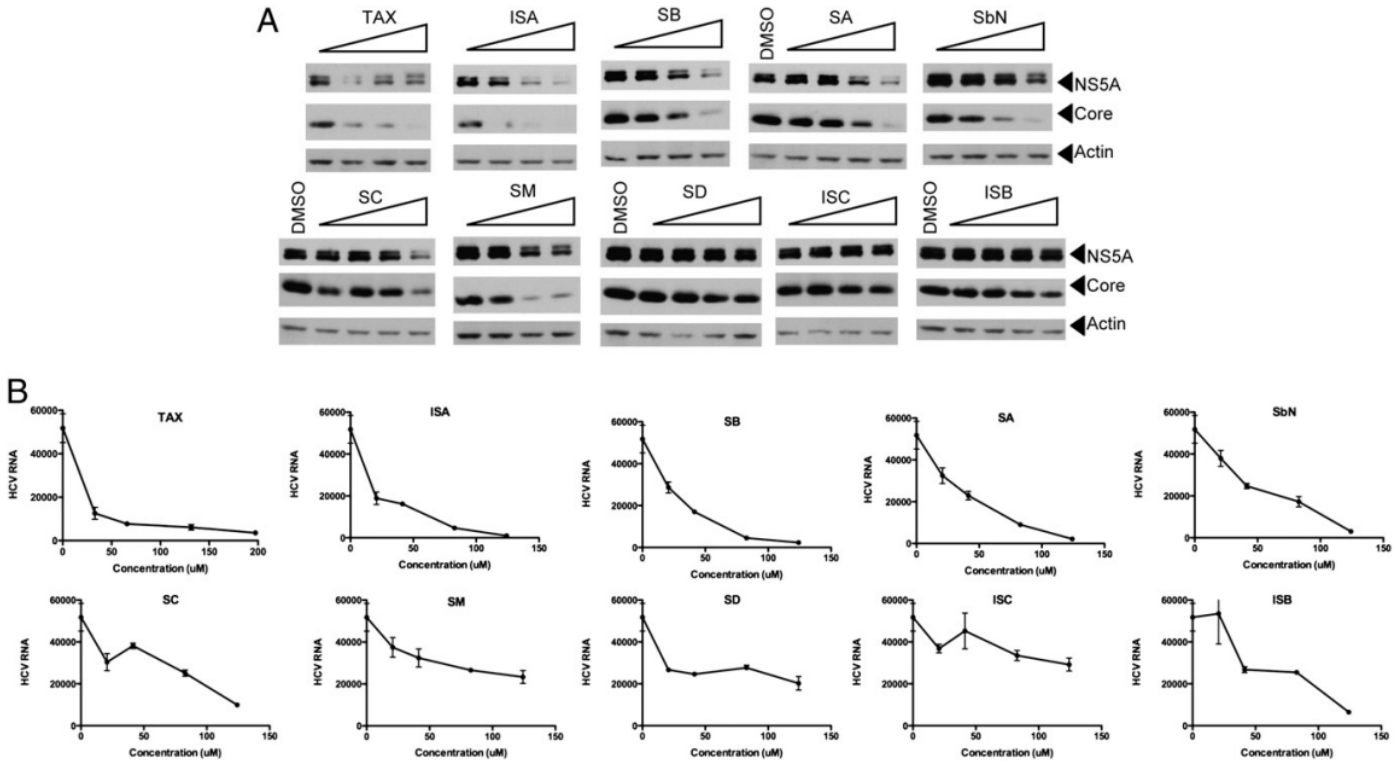

Fig. 1. Antiviral effects of silymarin-derived flavonolignans. (A) Effects on HCV protein expression. JFH-1 virus at a multiplicity of infection of 0.05 was adsorbed to Huh7.5.1 cells for 5 h. After the inoculum was removed, fresh medium was added containing DMSO or 20.7, 41.4, 82.8, or 124.2 μM of pure flavonolignan (mw 482), or 32.9, 65.8, 131.6, or 197.4 μM of taxifolin (mw 304). Protein lysates were harvested 72 h later and HCV protein expression detected by Western blot analysis. Actin served as a loading control. (B) Effects on HCV RNA. JFH-1 virus at a multiplicity of infection of 0.05 was adsorbed to Huh7.5.1 cells for 5 h. After the inoculum was removed, fresh medium was added containing DMSO or pure flavonolignan or taxifolin at the concentrations listed above. Total RNA was harvested 72 h later and HCV RNA expression quantitified by real-time RT-PCR. The y axis presents the number of copies of HCV RNA per 10 ng of total RNA. Silydianin (SD), silychristin (SC), isosilychristin (ISC), silybin A (SA), and silybin B (SB), taxifolin (TAX), isosilybin A (ISA), isosilybin B (ISB), silibinin (SbN), and silymarin (SM).

	Antiviral (IC50)	Antiviral (IC50)	Antiviral (IC50)	Anti-Inflammatory (IC50)	Antioxidant (percent inhibition)	Immunomodulatory (percent inhibition)
Compound	HCV Protein*	HCV RNA†	RdRp*	NFkB*	ROS (%)	T-cell proliferation (%)
Silymarin	60	89.6	300	80	99.2	86.2
Silibinin	80	41	800	80	99.5	60.6
Silybin A	50	31.7	>800	40	68.7	71.4
Silybin B	80	24.5	600	40	74.0	47.5
Isosilybin A	40	14.6	>1,500	80	95.3	61.6
Isosilybin B‡	>40	53.9	>1,500	40	91.0	100
Silychristin	100	60	800	>80	78.9	30.3
Isosilychristin	>100	245	>1,500	>80	80.4	11
Silydianin	>100	>100	~1,000	>80	52.1	2.8
Taxifolin	40	6.8	>1,500	40	96.3	0

IC50 data are expressed as concentrations in micromolars, both estimated* and actual†. Antioxidant data reflect the percent inhibition of JFH-1-induced oxidative stress for flavonolignan treated infected cells versus DMSO solvent controls. For immunomodulatory function, data are expressed as percent-inhibition of CD3 induced proliferation at a compound concentration of 40 μM.

‡Note that isosilybin B was toxic above 10 μM, so the concentrations for the reported activities are largely because of toxicity rather than a specific hepatoprotective function.

Table 1. Hepatoprotective effects of silymarin-derived flavonolignans

Fig. 1*B* and Table 1 summarize inhibition of HCV RNA replication among the different compounds. Taxifolin was the most potent at blocking HCV RNA replication, followed by isosilybin A, silybin B, silybin A, silibinin, and silychristin, which were all more potent than silymarin. Silydianin and isosilychristin were inactive.

Silymarin inhibits HCV NS5B polymerase activity, albeit at concentrations 5- to 10-fold higher than required for HCVcc inhibition (13). We therefore tested the pure compounds for their ability to inhibit JFH-1 NS5B polymerase activity. NS5B polymerase inhibition was only observed at very high concentrations, with silymarin and silybin B displaying the most potent activities, with IC50 values of 300 to 600 μM (Table 1).

In summary, the data indicate that certain silymarin-derived pure flavonolignans can inhibit HCV infection as measured by blockade of HCV protein and RNA expression, but that inhibition of NS5B RdRp activity can only partly explain the antiviral activity. Indeed, we have shown that silymarin blocks virus entry and fusion, and virus production (13).

To determine if silymarin contains antioxidant functions in the context of HCV infection, we treated infected cells with silymarin, then labeled cells with the oxidant reactive dye, H2DCFDA, and measured fluorescence by microscopy. JFH-1 infection of Huh7.5.1 cells caused large

increases in fluorescently labeled cells compared with mock-infected cells, indicative of oxidative stress (Fig. 2*A*). Treatment of cells with three different preparations of silymarin (MK-001, USP, and Legalon) caused significant reductions of cellular fluorescence (Fig. S3). Treatment with all eight pure compounds inhibited JFH-1-induced oxidative stress (Fig. 2*B*). IFN, a known antiviral for HCV, also inhibited JFH-1-induced oxidative stress. Silibinin (a 1:1 mixture of silybin A and silybin B), taxifolin, and isosilybin A had antioxidant activity equal to or greater than silymarin, causing greater than 90% reduction in JFH-1-induced oxidative stress. Compounds that lacked antiviral action, such as silydianin and isosilychristin, still possessed antioxidant function.

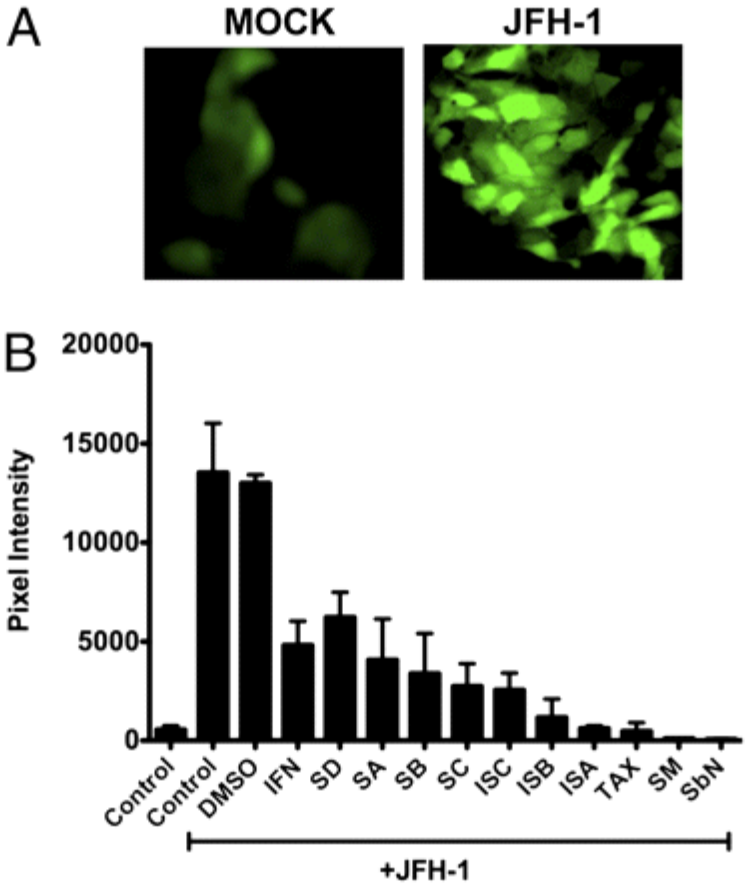

Fig. 2. Silymarin and silymarin-derived flavonolignans block HCV induced oxidative stress. (A) Huh7.5.1 cells were infected or mock-infected with JFH-1 at a multiplicity of infection of 0.01 and 72 h later, cells were labeled with the dye H2DCFDA for 30 min using the Image-IT Live Green Reactive Oxygen Species kit (Molecular Probes/Invitrogen). On oxidation, H2DCFDA becomes highly fluorescent. Images were captured on a Nikon Microscope using MetaMorph software. (B) Huh7.5.1 cells were treated as described above except in addition to silymarin (SM), cells were also treated with 41.4 μM of each flavonolignan, 65.8 μM of taxifolin, or 100 U/mL of IFN-α. ROS were quantitated by pixel intensity. Abbreviations of compounds are listed in the legend to Fig. 1.

NF-κB is the key transcription factor that is central to the induction of an inflammatory response (17). NF-κB transcription factors are homo- and heterodimeric complexes of proteins with molecular weights of 50 and 65 kDa (18). Inactive NF-κB is found in the cytoplasm via its association with inhibitory proteins of the IκB family. Stimulation of NF-κB activity involves phosphorylation of IκB-α on serine amino acid 32, phosphorylation of NF-κB on serine 536, followed by degradation of IκB-α, permitting NF-κB translocation to the nucleus and expression of inflammatory response genes (19, 20).

Silymarin blocks NF-κB-dependent transcription from a canonical NF-κB promoter (9). Silymarin also blocked TNF-α activation of NF-κB dependent transcription from the positive regulatory domain (pRDII) domain of the IFN-B promoter, a key gene in antiviral defense (21) (Fig. S4*A*). Silymarin reduced both p65 and IκB-α serine phosphorylation by about 20% and caused an approximate 20% decrease in TNF-α induced p50 and p65 nuclear translocation (Fig. S4 *C* and *D*). However, silymarin did not affect the ability of p50 or p65 to bind to the NF-κB promoter (Fig. S4*E*). The data suggest that silymarin partially inhibits phosphorylation of IκB-α and p65/RelA and nuclear translocation of p50 and p65 subunits of NF-κB, but does not impair the ability of NF-κB to bind to DNA.

Fig. 3 depicts examples of the effects on the pure compounds' abilities to block TNF-α induced NF-κB-dependent transcription. In this assay, we were able to test higher concentrations of silymarin because the flavonolignans were only incubated with cells for 4 h. Silybin A and silybin B caused dose-dependent blockade of TNF-α induced NF-κB dependent transcription, with IC50s of 40 μM (Fig. 3 *A* and *B* and Table 1). Compared with silybin A and B, taxifolin induced the most-pronounced inhibition of NF-κB transcription at the lowest doses, but its effect appeared to plateau (Fig. 3*C*).

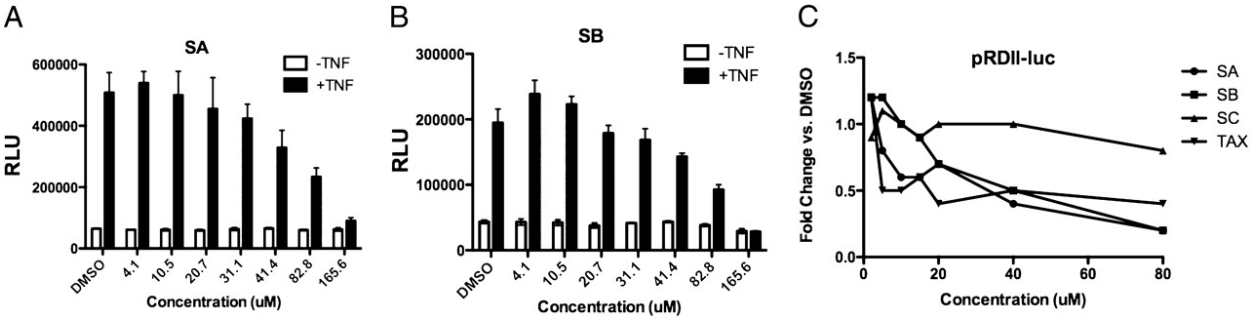

Fig. 3. Silymarin flavonolignans inhibit NF-κB transcription. (*A* and *B*) Silybin A and silybin B inhibit NF-κB transcription. Huh7 cells were transfected with a luciferase reporter gene under control of the pRDII domain from the IFN-B promoter and 24 h later, cells were treated with the indicated micromolar doses of each flavonolignan or DMSO control for 30 min before addition of 10 ng/mL TNF-α. Luciferase activity was measured 3.5 h later. Error bars represent SDs. (*C*) Comparison of NF-κB inhibitory profiles of silybin A, silybin B, taxifolin, and silychristin.

Table 1 summarizes the effects of silymarin at 40 μM on T-cell proliferation induced by CD3 ligation. Silymarin, silibinin, silybin A, and isosilybin A displayed the most potent suppression of T-cell proliferation. Isosilychristin, silydianin, and taxifolin were essentially inactive. Isosilybin B also blocked T-cell proliferation, but this was because of toxicity. In contrast to the

other measures of hepatoprotection, where some pure compounds were more active than silymarin, silymarin was the most potent in blocking T-cell proliferation. Silymarin and pure compounds also inhibited inflammatory cytokine expression from T cells (Fig. S5).

Discussion

To our knowledge, this report is unique in characterizing silymarin and silymarin-derived pure compounds in four different measures of hepatoprotection in human cells and in the context of hepatitis C virus infection. The natural compounds inhibit virus infection, virus-induced oxidative stress, NF-κB-dependent transcription, and TCR mediated proliferation. Table S1 summarizes the data of compound activity in the five hepatoprotective assays. Isosilybin A, taxifolin, and silibinin were the most effective hepatoprotectors because they displayed potent activity in four of five assays, followed by silybin A and silybin B, which were active in three of five assays. Taxifolin was particularly noteworthy because hepatoprotection was observable at lower doses than the other compounds, and tended to plateau after reaching a nadir at low doses. In contrast, the other compounds showed more linear dose-response relationships. Intriguingly, all compounds inhibited virus-induced oxidative stress regardless of whether they had antiviral activity.

With the exception of T cell proliferation, several purified compounds were more potent than the parent silymarin extract. Although some complementary and alternative medicine practioners may emphasize that it is the combination of bioactive molecules in a botanical extract that defines its unique biological properties, our data suggest that combinations of flavonolignans may provide optimal hepatoprotection. Because the structures of the flavonolignan stereoisomers are similar, there may be competitive interactions between flavonolignans for cellular targets. This possibility implies that the hepatoprotective potential of select mixtures of pure compounds could in theory be greater than the silymarin extract itself. Moreover, different combinations of flavonolignans might be selected, depending on the hepatoprotective functions one is targeting. Given that isosilybin B is very toxic in cell culture, as shown here, select combinations may be especially relevant if one is trying to minimize cytotoxicity for treatment of liver diseases of both viral and nonviral origin. Further studies that examine combinations and define the cellular targets of the pure compounds are needed to resolve these issues.

In the current study, although silymarin and silybin B appeared to be the most potent polymerase inhibitors, the IC50 concentrations were much higher than the concentrations that caused cytotoxicity. Moreover, silymarin displays weak polymerase inhibition against four clinical isolates of genotype 1b HCV RdRps, and silymarin does not inhibit HCV replication in noninfectious replicon cell lines (13). Therefore, we propose that polymerase inhibition by the natural compounds, although demonstrable in vitro, may not contribute to anti-HCVcc action in vitro, and plays a limited role in antiviral effects in patients with chronic hepatitis C when silymarin is taken orally. However, the i.v. formulation of silibinin that displays antiviral effects, Legalon-SIL, is actually the dihydrogen disuccinate disodium versions of silybin A and silybin B. This chemical modification makes silibinin water soluble, so this formulation may have a different antiviral profile and mechanism of action than the natural compounds.

NF-κB serine phosphorylation is required for maximal transcriptional activation by NF-κB (22). We showed that silymarin partially inhibits NF-κB-dependent transcription via partial blockade of phosphorylation of Ser536 on the p65 subunit of NF-κB and serine32 on IκB-α. Silymarin also partially inhibited nuclear translocation of the p50 and p65 NF-κB subunits. These results are in agreement with previous studies in prostate cancer cells, where silibinin was shown to block p50/p65 nuclear translocation and IKK-α activity (23).

HCV infection induces oxidative stress and inflammation (24–27). Unchecked oxidative stress can modify proteins and lipids, damage DNA, alter mitochondrial membrane potential, and be a predisposing event in the progression of liver disease. In the present study, we demonstrated that JFH-1 infection caused large increases in oxidative stress. These effects were abrogated by silymarin and pure compound treatment. We suggest that silymarin inhibits HCV-induced oxidative stress by at least two mechanisms. First, silymarin's demonstrated antiviral actions could have reduced HCV replication and the ensuing oxidative stress. Second, flavonolignan components of silymarin may have direct antioxidant functions without antiviral functions. Evidence in support of both modes of action was provided in the present study. These data extend silymarin's previously documented antioxidant functions (28) to the context of HCV infection. Because silymarin inhibits oxidative stress independently of suppression of virus, it is possible that the hepatoprotective actions of the botanical may be extended to liver diseases of nonviral origin.

Although silibinin, which is a mixture of silybin A and silybin B, has been largely touted to contain many of the hepatoprotective functions (28, 29), we are unique in showing that there are other flavonolignans in silymarin that are equal to, if not more potent, than silibinin. The best examples are isosilybin A and taxifolin, which have antiviral activities that were superior to silibinin. Yet in T cell proliferation silibinin, but not taxifolin, displayed significant activity. These data underscore the importance of careful evaluation of all flavonolignans and that the activity of each flavonolignan may vary depending on the biological assay.

Our data suggest that silymarin and silymarin-derived compounds could influence HCV disease course in some persons. Although standardized silymarin has an exceptional safety record (8), the effects observed in vitro occur at relatively high concentrations, so additional studies are necessary to assess if adverse effects occur in humans at these levels. Therefore, we caution against the self-administration of large amounts of these botanicals, particularly those purchased as dietary supplements, because there is inconsistency in the standardization of these products, the entire contents may not be known, they may contain contaminants, and misuse of herbals can cause hepatotoxicity (30). On the other hand, the activities observed in the present study using highly pure natural compounds suggests great promise for the treatment of hepatitis C, for which previously unexplored therapeutics are urgently needed.

Silymarin is an interesting botanical extract that has a multitude of biological activities. The four activities we measured: antiviral, antioxidant, anti-inflammatory, and immunomodulatory, are all likely to be directly related to its well-described hepatoprotective actions (31), but seemingly much harder to demonstrate in humans (32). Although the latter may be primarily because of the metabolism and bioavailability of silymarin and a dearth of properly designed clinical studies, it

is clear that further studies are necessary to define the molecular mechanisms by which silymarin exerts such pleiotropic effects.

Methods

Subjects. Subjects with and without chronic HCV infection were recruited at Harborview Medical Center after written informed consent was obtained through a University of Washington Institutional Review Board–approved protocol.

PBMC Isolation and Proliferation Assay. Peripheral blood mononuclear cells (PBMCs) were isolated within 24 h of venipuncture and immediately assayed. PBMC were stimulated for 24 h at 37 °C 5% CO_2 using plate-bound anti-CD3 (UCHT1, 10 μg/mL, BD Biosciences) in RPMI medium 1640 supplemented with 10% human serum (Gemini Bio-Products). Cellular proliferation detected by ^{3}H-thymidine incorporation into replicating DNA was measured by adding 1 μCi to each replicate well of 10^5 PBMC for an additional 24 h before quantitative analysis using a Topcount Liquid Scintillation Counter (Perkin-Elmer). All experiments used four replicates per condition. Data were obtained as mean cpm incorporated per condition tested. Percent inhibition was calculated using the following formula: 100 × (vehicle-treated mean cpm − compound-treated mean cpm)/vehicle-treated mean cpm.

Cells, Virus, and Plasmids. Huh7.5.1 cells were grown in Huh7 medium as described (9). JFH-1 viral stock preparation, cell infection, and titration was performed as described (33–35). For antiviral assays, cells were in infected at a multiplicity of infection of 0.05 and protein and RNA harvested 72 h postinfection. To measure NF-κB–dependent transcription, we used a luciferase reporter gene under control of the positive regulatory domain (pRDII), which contains the NF-κB site from the IFN-B promoter.

Silymarin Preparations, and Pure Compounds. Three preparations of silymarin were used in the current study. The first was MK-001, prepared as described (9, 36). The second preparation was commercially prepared (Legalon; Madaus). The third preparation was purchased from US Pharmacopeia. Silymarin was solubilized in DMSO at 50 mg/mL for Huh7 cell studies; methanol was the solvent for PBMC studies. Pure flavonolignans were solubilized at 50 mg/mL in DMSO for all studies except PBMC studies, where methanol was used as the solvent.

Silymarin treatment of Huh7.5.1 cultures consisted of adding silymarin immediately after removing the viral inoculum. For PBMC studies, silymarin or pure compounds were added to cells at the same time that they were exposed to plate-bound anti-CD3 (T-cell receptor antibody).

Cytotoxicity Determinations. The toxicity of silymarin extracts and pure flavonolignans on Huh7.5.1 cells was determined by measuring ATP levels using the ATPlite system (Perkin-Elmer), as described (9).

Reporter Gene Assays. Reporter gene assays were performed as described (37). Endotoxin free plasmid DNA was purified (Endofree kit, Qiagen), and was introduced into cells with Lipofectamine 2000 according to manufacturer's recommendations (Invitrogen). Next, 100 ng of the pRDII-luciferase gene was transfected into cells in quadruplicate. Eighteen hours later, cells

were preincubated with silymarin or pure compounds for 30 min before rhTNF-α (15 ng/mL; Sigma Aldrich) was added. Four hours later, luciferase activity was measured on cell lysates using the Britelite Assay System (Perkin-Elmer).

Western Blot Analysis. NS5A and core proteins in JFH-1 infected cells were detected with serum from patients infected with HCV genotype 2a. The HCV core protein was detected with a monoclonal antibody (Affinity BioReagents). GAPDH was detected with polyclonal antiserum (Santa Cruz Biotechnology). NF-κB antibodies consisted of a monoclonal antibody to RelA/p65 Serine 536, IκBα Serine 32 (Cell Signaling), and antibodies to p50 and p65 subunits (Upstate/Millipore). For Western hybridizations and washings, both the standard seal a meal bag approach and the Snap-ID system (Millipore) were used.

Oxidative Stress Measurements. Huh7.5.1 cells were incubated with 20 μg/mL of silymarin or pure flavonlignans immediately after virus adsorption. Seventy-two hours later, cells were labeled with the dye 2',7'-dichlorodihydrofluorecein diacetate (H2DCFDA) for 30 min using the Image-IT Live Green Reactive Oxygen Species kit (Molecular Probes/Invitrogen). H2DCFDA is a cell-permeable indicator for reactive oxygen species that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. On oxidation, H2DCFDA becomes highly fluorescent Pharmacopeia. Images were captured on a Nikon Microscope using MetaMorph software (Molecular Devices) with a 20× objective.

HCV NS5B Polymerase Assays. NS5BΔC21 C-terminally fused to a hexa-histidine tag was expressed and purified for HCV JFH1 and for the genotype 1b isolates as described (38). JFH-1 RNA dependent RNA polymerase (RdRp) assays contained buffer, 5 μCi of [α-^{32}P]GTP, 50 μM GTP, 1 mM each ATP, CTP, UTP, 2 μg polyC (GE Healthcare), 1 μg purified polymerase, and given amounts of purified compounds in DMSO in a total volume of 25 μL. All reaction components except nucleotides and template were preincubated for 15 min at room temperature; the reaction was started by adding the nucleotide mixture and polyC and was incubated for 1.5 h at room temperature. Reaction products were precipitated, passed through microfilters (GE Healthcare), washed five times with 1% trichloroacetic acid and 0.1% tetra-sodium pyrophosphate, and air-dried. After addition of 6 mL of Ultima Gold (Perkin-Elmer), samples were subjected to liquid scintillation counting. All measurements were performed in triplicate and the IC50 values were calculated with GraphPad Prism.

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Supporting Information

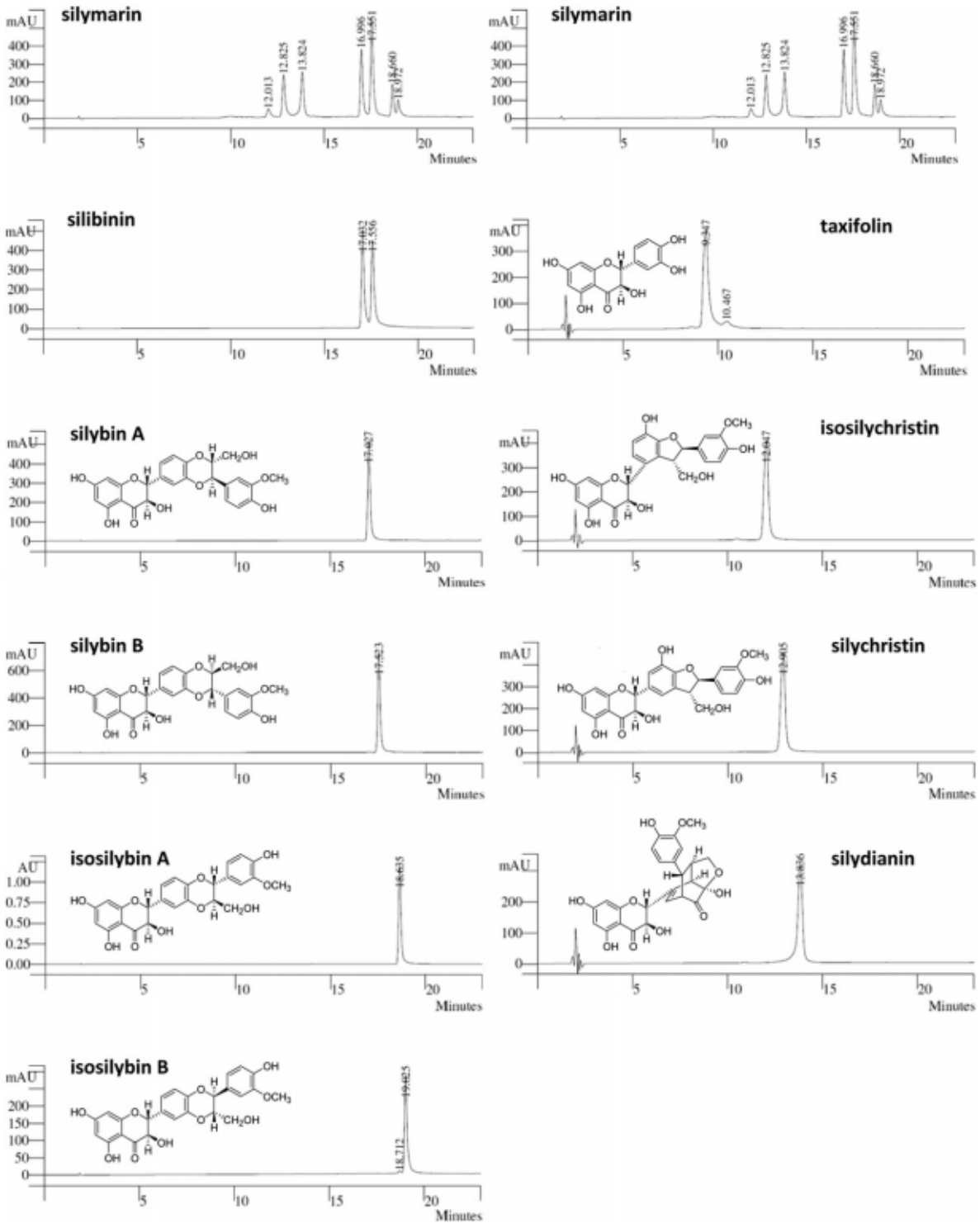

Fig. S1. HPLC chromatograms of milk thistle extract (silymarin), the 1:1 mixture of silybin A:silybin B (silibinin), the flavonoid (taxifolin), and the seven flavonolignan

diastereoisomers (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin). The structures for all of the pure compounds
are also shown.

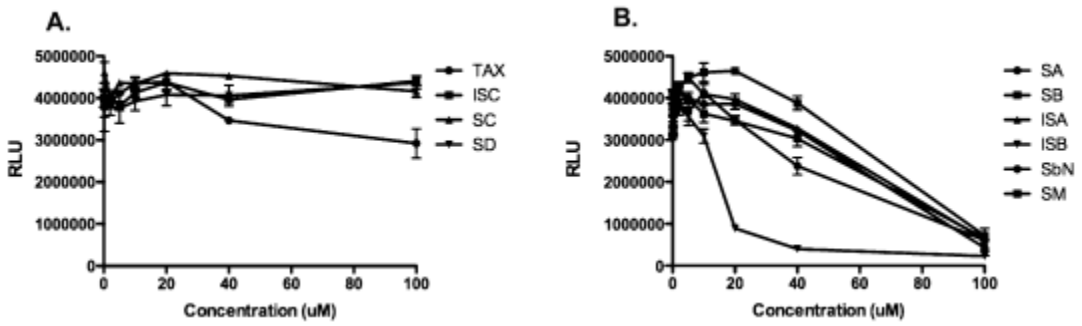

Fig. S2. Cytotoxicity profiles of silymarin flavonolignans. Huh7 cells were incubated with the indicated micromolar concentrations of pure compounds or DMSO and 96 h later, cell viability was assessed by measuring ATP levels, expressed as relative light units. Error bars represent SDs of triplicate cultures. (A) Profiles of noncytotoxic compounds including silydianin (SD), silychristin (SC), isosilychristin (ISC), and taxifolin (TAX). (B) Profiles of compounds that show cytotoxicity at high dose including silybin A (SA), and silybin B (SB), isosilybin A (ISA), isosilybin B (ISB), silibinin (SbN), and silymarin (SM). ISB is toxic above 10 μM.

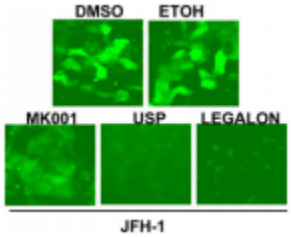

Fig. S3. Silymarin preparations block Hepatitis C virus (HCV)-induced oxidative stress. Huh7.5.1 cells were incubated with 41.4 μM of various preparations of silymarin: MK-001, US Pharmacopeia, or Legalon (Madaus) immediately after HCV infection. Cells were separately treated with DMSO or ethanol as solvent controls. Seventy-two hours later, cells were labeled with the dye H2DCFDA for 30 min using the Image-IT Live Green Reactive Oxygen Species kit (Molecular Probes/Invitrogen), and images captured by fluorescence microscopy.

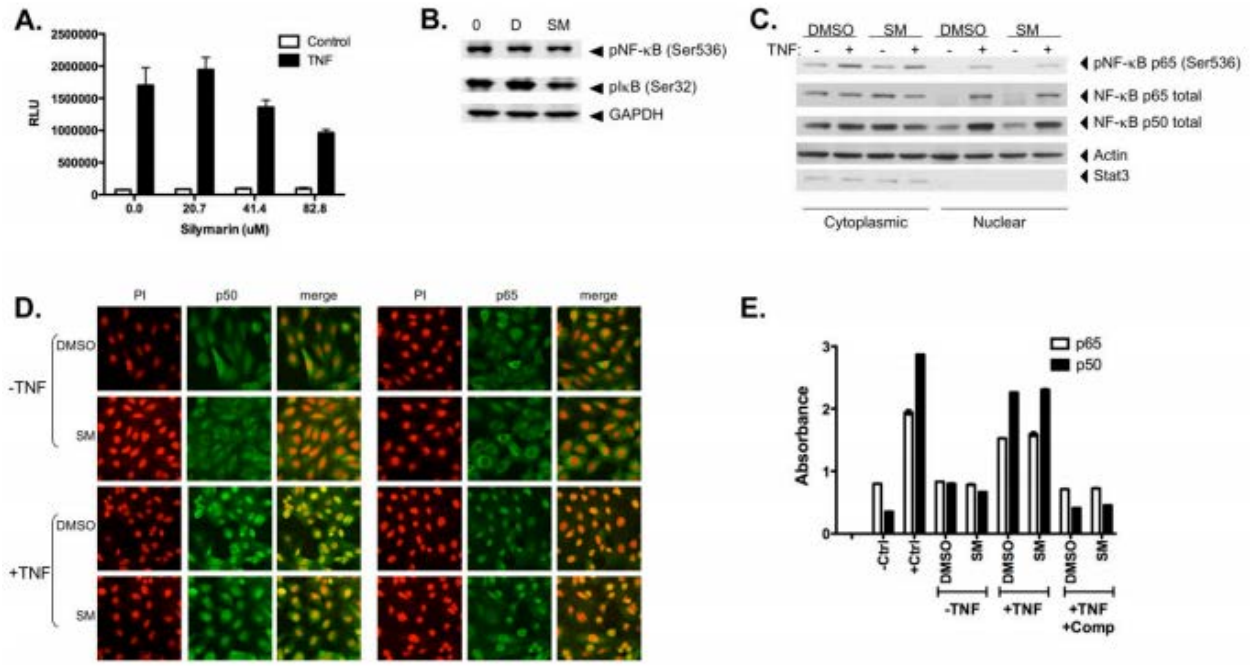

Fig. S4. Effects of silymarin (SM) on NF-κB. (A) SM blocks NF-κB transcription from pRDII. Huh7 cells were transfected with a luciferase reporter gene under control of the pRDII domain from the IFN-B promoter, and 24 h later cells were treated with the indicated doses of silymarin or DMSO control for 30 min before addition of 10 ng/mL TNF-α. Luciferase activity was measured 3.5 h later. Error bars represent SDs. (B) SM blocks TNF-induced IkB and NF-κB phopshorylation. Huh7 cells were treated for 30 min with DMSO (D) or 41.4 μM silymarin before stimulation with 10 ng/mL TNF-α for 15 min. Protein lysates were run on SDS/ PAGE gels and probed for phosphorylated forms of NF-κB (Ser536) and IκB (Ser-32). GAPDH was probed as a loading control. (C) SM blocks NF-κB Ser536 phosphorylation in human hepatoma cells, and has modest reduction in p65 and p50 nuclear translocation. Huh7 cells were treated for 30 min with DMSO or 41.4 μM SM before stimulation with 10 ng/mL TNF-α for 15 min. Cytoplasmic and nuclear extracts were prepared and total forms of p65 and p50 NF-κB subunits as well as Ser536 p65 were detected by Western blot. Actin served as a loading control; Stat3 served as a control for a cytoplasmic protein. (D) SM partially blocks p50 and p65 nuclear translocation. Huh7 cells were treated for 30 min with DMSO or 41.4 μM SM before stimulation with or without 10 ng/mL TNF-α for 20 min. Cells were fixed and immunofluorescent detection of p50 and p65 NF-κB subunits was performed. Nuclei were counterstained with propidium iodide (PI). Images were visualized by confocal microscopy. (E) SM does not block binding of p50 and p65 to the NF-κB promoter. Huh7 cells were treated for 30 min with DMSO or 41.4 μM SM before stimulation with or without 10 ng/mL TNF-α for 20 min. Nuclear extracts were added to ELISA plates containing oligonucleotides containing WT NF-κB binding sites, followed by detection of p50 and p65 subunits with secondary antibodies. Negative and positive controls (−ctrl, +ctrl; Leftmost bars) for p50 and p65 were included in the commercial kit. Specificity was demonstrated by adding excess free WT competitor to the reaction (Rightmost bars).

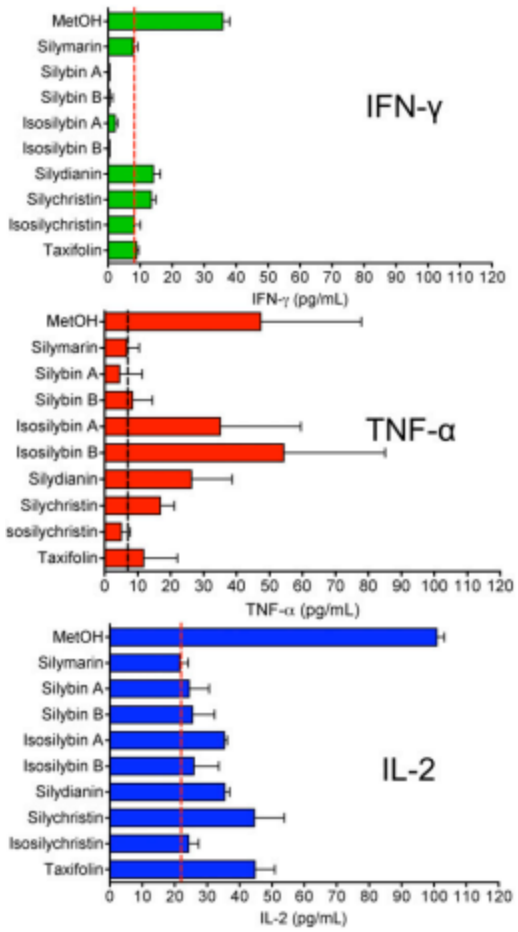

Fig. S5. PBMC proinflammatory cytokine production is inhibited by purified silymarin components. For all assays, a single dose of silymarin and pure compounds or vehicle control (methanol; MetOH) was added at culture initiation at 20 μg/mL (41.4 μM for flavonolignans and 65.8 μM for taxifolin). Freshly isolated PBMC from a healthy subject were tested in triplicate for cytokine responses to plate-bound anti-CD3 (10 μg/mL) stimulation. Supernatants collected 24 h after anti-CD3 stimulation were tested in triplicate by ELISA for IFN-γ, TNF-α, and IL-2 levels by multiplex immunobead ELISA (Biosource/Invitrogen) and the Luminex xMAP system (Luminex Corp.). Error bars indicate 1 SD among replicates for each condition. Dashed lines indicate the level of inhibition of cytokine secretion by silymarin, for comparison with the activity of other subcomponents.

Table S1. Summary of the most potent purified compounds

	Antiviral	Antiviral	Anti-Inflammatory	Antioxidant	Immunomodulatory	TOTAL
Compound	HCV protein	HCV RNA	NFkB	ROS	T Cell Proliferation	
Silybin A (SA)	√	√	√			3/5
Isosilybin A (ISA)	√	√	√	√		4/5
Taxifolin (TAX)	√	√	√	√		4/5
Silibinin (SbN)	√	√	√	√		4/5
Silybin B (SB)	√	√	√			3/5
Silychristin (SC)	√	√				2/5
Isosilychristin (ISC)						0/5
Silydianin (SD)						0/5
Isosilybin B (ISB)						0/5

The four hepatoprotective functions are listed across the top of the table, with the five assays listed below each function. Check marks indicate activity was equal to or more potent than silymarin. Please refer to Table 1 for details.