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Milk Thistle Extracts Inhibit the Oxidation of Low-Density Lipoprotein (LDL) and Subsequent Scavenger Receptor-Dependent Monocyte Adhesion

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Silymarin encompasses a group of flavonolignans that are extracted from *Silybum marianum* (Asteraceae) fruits. The silymarins have previously been reported to lower low-density lipoprotein (LDL) levels associated with high-fat diets. The present study reports the efficacy of the silymarins in inhibiting oxidized low-density lipoprotein (oxLDL) generation and subsequent scavenger receptor (SR) mediated monocyte adherence to oxLDL. The flavonolignans that comprise silymarin include silichristin (SC), silidianin (SD), silibinin (SBN), and isosilibinin (IS). These flavonolignans (300 μM) lowered oxLDL generation, measured by the thiobarbituric acid-reacting substances (TBARS) assay, by 60.0, 28.1, 60.0, and 30.1%, respectively. SBN treatment of LDL in the presence of copper sulfate ($CuSO_4$) resulted in a significant dose-dependent inhibition of monocyte adhesion. Inhibition was paralleled by a decrease in binding of anti-oxLDL antibodies recognized by U937 monocyte Fc gamma receptors (FcγR). These results showed that silymarin and SBN, likely through antioxidant and free radical scavenging mechanisms of action, inhibit the generation of oxLDL and oxidation-specific neoepitopes recognized by SR and FcγR expressed on monocytes/macrophages.

KEYWORDS: Atherosclerosis; oxLDL; *Silybum marianum*; monocyte; scavenger receptor

INTRODUCTION

Milk thistle (*Silybum marianum* L. Gaertn.) (Asteraceae) fruits contain the bioactive and well-characterized silymarin (SM) complex. Milk thistle can be cultivated in northern climates such as Canada (*1*) as well as in southern and arid conditions (*2, 3*). Yields of fruits range from 350 to 1478 kg/ha, making milk thistle a widely available agricultural commodity (*3, 4*). Depending on rainfall, soil conditions, seed quality, and geographic location, silymarin yields can vary from 4 to 19 mg/g of seed (*5*). Silymarin is the collective term for a mixture of seven flavonolignans (**Figure 1**). Silibinin (SBN) is the best-documented of the flavonolignans in displaying health-beneficial effects. SBN has long been known to occur as two diastereoisomers, silybin A and silybin B (SA and SB). SA and SB occur in approximately 1:1 proportions. Silidianin occurs in just one form and is denoted by SD. Isosilibinin (IS) also occurs in two diastereoisomeric configurations, isosilybin A and B (ISA and ISB). The ratio of ISA to ISB occurring in silymarin is approximately 7:3. Silichristin (SC) is the most recent of the flavonolignans to have its multiple structures fully elucidated. SC has been shown to contain two distinct products, sometimes referred to as isosilichristin and silichristin, or silichristin A and silichristin B (collectively, SC) (*6, 7*). HPLC analyses of the SC standard tested in this research showed the presence of three distinct peaks; however, the third of these peaks coeluted with SD. SC was quantified as the first two peaks only and is referred to throughout this research as SC, which denotes a combination of the A and B forms. Currently, SBN and IS are solely commercially available as mixtures of SA/SB and ISA/ISB. The flavonolignan

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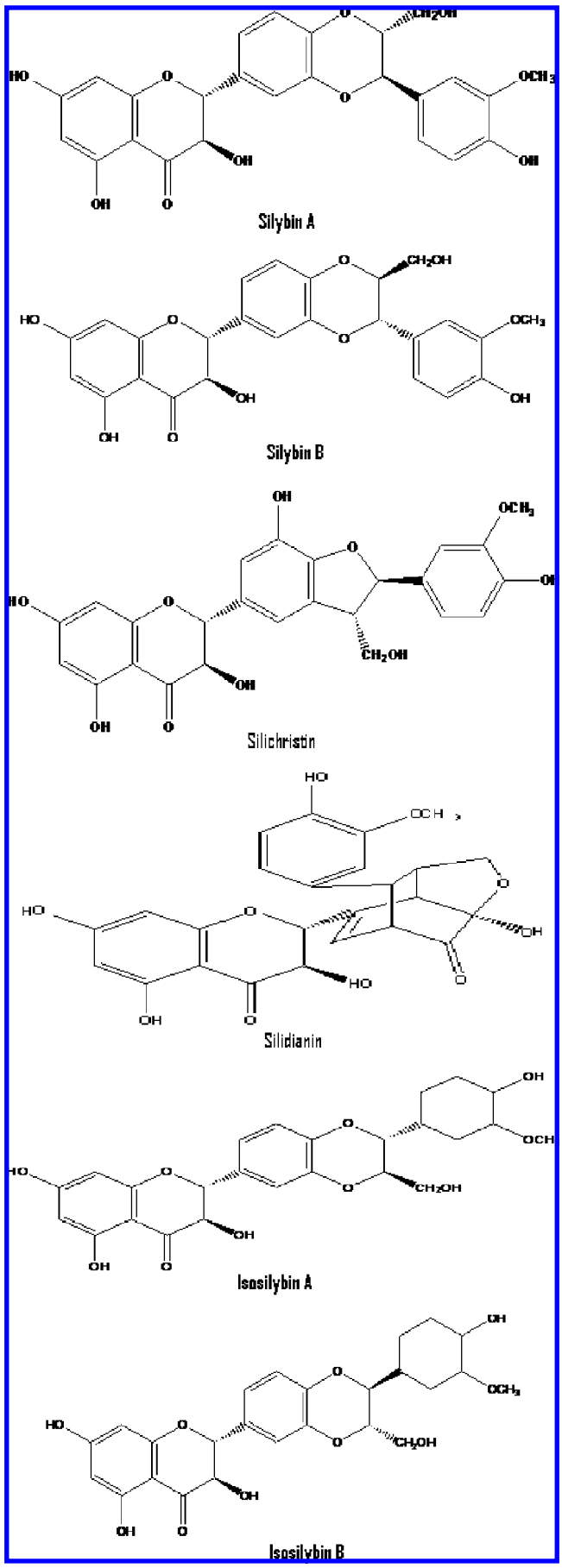

Figure 1. Structures of the flavonolignans.

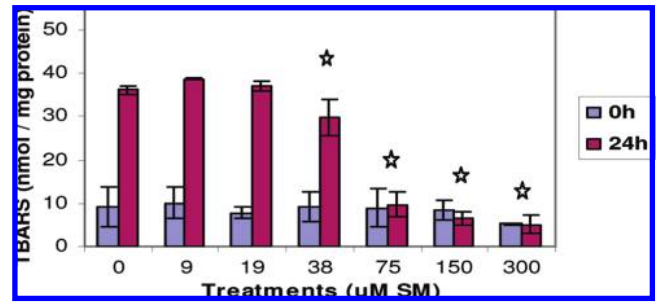

Figure 2. Inhibition of $CuSO_4$-induced LDL oxidation by 0−300 μM milk thistle fruit extracted using an ethanol Soxhlet apparatus.

proportions present in differing batches of fruits can vary with growing conditions.

In Europe, silymarin has been clinically used as a natural remedy to treat liver diseases (*8, 9*), and recent reports have demonstrated that silymarin has potent antitumor activity (*10, 11*). In addition to being well documented as a hepatoprotectant, a wealth of literature details multiple anticancer effects of silymarin and its flavonolignans (*12*).

Silymarin extracted from milk thistle fruit has been shown to have health-beneficial effects that thwart the progression of atherosclerosis by lowering low-density lipoprotein (LDL) in rats fed hypercholesterolemic diets (*13, 14*) through the removal of LDL from the livers of rats, as measured by an enzymatic assay of the recirculated perfused liver medium. The oxidative modification and inflammation hypothesis of atherogenesis is widely accepted and supported by experimental data in hypercholesterolemic animal models and human epidemiologic studies (*15*). Oxidized low-density lipoprotein (oxLDL) generated during the early phases of atherosclerosis has been shown to result in the activation and adhesion of monocytes to the vascular endothelium (*16–18*). Adhesion of monocytes to the inflamed vascular endothelium initiates a cascade of atherogenic events. Monocytes transmigrate to the intima, where they transform into macrophage (MΦ) cells that can take up oxLDL to form lipid-laden foam cells, leading to the cellular accumulation of cholesterol (*19*). OxLDL is likely a prerequisite for MΦ uptake (*15*). Strategies to inhibit the generation of oxLDL may serve to prevent the development of atherosclerotic changes or to facilitate its regression.

Studies present evidence that silymarin inhibits LDL oxidation. The protective effect of silymarin in tissues is most likely the result of free radical scavenging activities (*10, 20, 21*). Because the formation of oxLDL is a prerequisite for the development of atherogenesis, we then hypothesize that silymarin present in milk thistle may inhibit the generation of oxLDL and subsequent interactions with monocyte-expressed scavenger receptor (SR) and Fc gamma receptors (FcγR). Preliminary evidence of this hypothesis was presented by Locher et al. (*22*) using SBN, which, at doses ranging from 50 to 200 μmol/L, significantly inhibited LDL autoxidation and oxidation as measured by the delay in the lag time of conjugated diene formation. Inhibiting the cascade of atherogenic events with an easily available and relatively low cost agricultural commodity such as milk thistle would have positive benefits both for the producer and for the end user. This work reports the antiatherosclerotic activities of SM and its component SBN.

MATERIALS AND METHODS

Chemicals. Human monocytic cells (U937) from American Type Cell Culture (ATCC, Manassas, VA) were cultured in DMEM/F12 medium supplemented with 5% FBS, purchased from Hyclone (Logan,

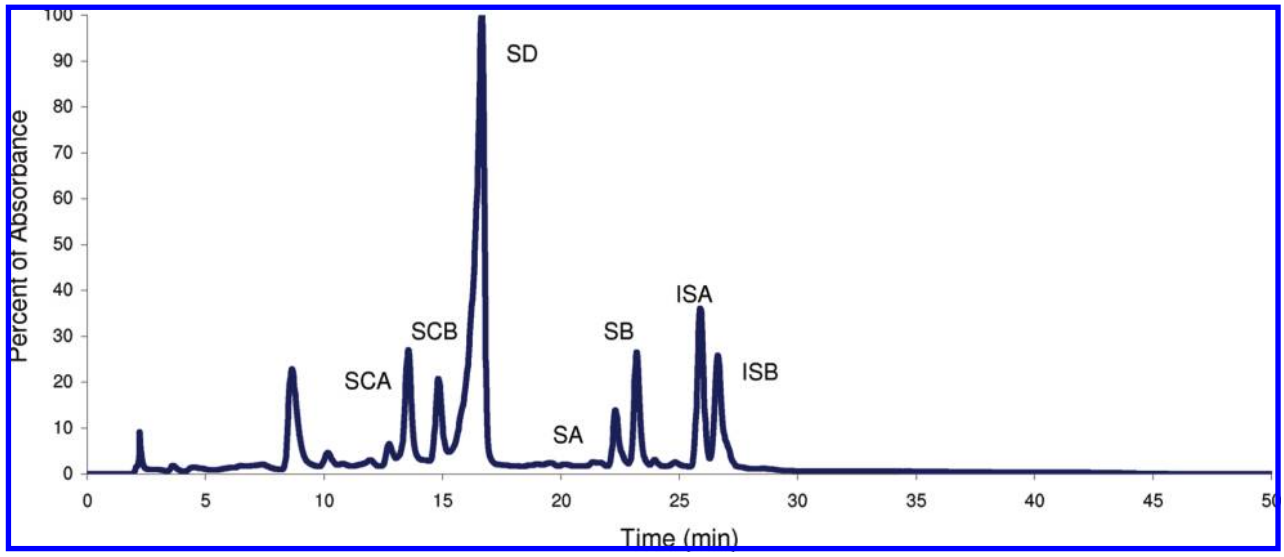

Figure 3. HPLC-UV trace of milk thistle fruit extracted with ethanol using a Soxhlet apparatus.

UT) as described by Stewart et al. (*23*). SBN, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and butylated hydroxytoluene (BHT) were acquired from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) and trichloroacetic acid (TCA) were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Dulbecco's Modification of Eagle's Medium (DMEM) was purchased from ATCC. The flavonolignans SC, SD, and IS were obtained from PhytoLab (Hamburg, Germany). LDL was purchased from Biomedical Technologies, Inc. (Stroughton, MA). Milk thistle fruits were purchased from Horizon Herbs (Williams, OR).

Preparation of the Soxhlet Extract. Ten grams of milk thistle fruits was ground in a coffee grinder to an average particle diameter of 0.4 mm according to ASAE S319.1 (*24*) and pretreated by soaking them in 200 mL of HPLC grade water for 24 h. Pretreated meal was then extracted with 200 mL of 100% ethanol. A Soxhlet apparatus was used for the ethanol extractions. A 4 h extraction cycle began when the 200 mL of ethanol began to boil. The extract was dried with a SpeedVac (Savant Instruments, Inc., Farmingdale, NY) and reconstituted in 50 mL of methanol for HPLC analysis. The total silymarin concentration was determined, in milligrams per milliliter. As the flavonolignans all share the same molecular weight, calculations could then be performed to determine the volume of extract containing the appropriate mass to deliver 300 μM silymarin to the wells. Aliquots from the extract were evaporated to dryness using nitrogen and reconstituted in DMSO for experiments. The 300 μM solution was titrated with DMSO to generate a dose response.

HPLC Analysis. Milk thistle was characterized for its silymarin content by HPLC-UV analysis as outlined by Wallace et al. (*5*). Silymarin concentrations were determined by HPLC using a Waters system (Milford, MA) composed of an Alliance 2690 separations module and a 996 photodiode array, controlled with Millennium^{32} chromatography software. Separation of the flavonolignans was obtained using a Symmetry (Waters) C_{18} precolumn placed in series with a Symmetry (Waters) C_{18} column (150 mm × 4.6 mm, 5 μm), both at 40 °C. Solvent A consisted of 20:80 methanol/water, whereas solvent B was 80:20 methanol/water and followed the gradient described by Wallace et al. (*5*). The flow rate was 0.75 mL/min, and the silymarin compounds were monitored at 290 nm. Calibration curves were prepared with individual flavonolignans in concentrations ranging from 1 to 0.06 mg/mL. The 10 μL sample volume injected resulted in a maximum of 10 μg of the individual flavonolignans being loaded onto the column. **Figure 3** presents the HPLC-UV trace of milk thistle extracted with ethanol using a Soxhlet apparatus, and **Table 1** presents the yields of the individual flavonolignans in milligrams per milliliter.

Oxidation of LDL. LDL was dialyzed against EDTA-free 50 mM Tris buffer (pH 7.4) for 24 h. LDL (100 mg/L) oxidation was initiated by adding $CuSO_4$ (5 μM). SBN, SC, SD, and IS were added such that their final well concentrations were 0−300 μM. The final well concentration of the combined flavonolignans in the milk thistle extract was 300 μM. Tested compounds were added immediately before the addition of $CuSO_4$. The reaction was stopped at 24 h by the addition of BHT at 50 μM. The extent of oxidation of LDL was determined by TBARS measurement by a microplate assay as described by Wallin et al. (*25*). Malondialdehyde (MDA) formation was calculated using a standard curve generated with TEP (or equivalents of MDA). Results were expressed as nanomoles of MDA per milligram of protein. Protein estimation was performed using the BCA protein assay kit (Pierce, Rockford, IL) with BSA as the standard.

Table 1. HPLC-UV Trace of Milk Thistle Fruit Extracted with Ethanol Using a Soxhlet Apparatus

compound	yield[a] (mg/mL)
SC	1.7 ± 0.4
SD	4.6 ± 0.4
SA	0.4 ± 0.06
SB	0.7 ± 0.07
ISA	0.9 ± 0.07
ISB	1.7 ± 0.2

[a] Values are means ± SD, $n = 3$.

Monocyte Adhesion Assay. LDL (200 mg/L) was allowed to incubate with $CuSO_4$ (5 μmol/L) in the presence of indicated concentrations of SBN for 24 h at 37 °C. LDL or oxLDL protein (at 5 mg/L in 20 mmol/L borate buffer/10 mmol/L EDTA, pH 8.5) was coated onto an ELISA plate from Nalgene (Rochester, NY) at a concentration of 50 μL/well for 16 h at 4 °C. Adhesion of fluorescent labeled human monocytic cells, U937, to the protein-coated ELISA plate was used to determine whether SBN could effectively inhibit generation of the oxidized neoepitopes in oxLDL recognized by scavenger receptors (SR) as described earlier (*23*). Using a BioTek Synergy plate reader (Winooski, VT) with a 485 nm excitation/530 nm emission filter, fluorescent intensity was measured before and after inversion of the plate. The percentage of cells adhered was calculated by the following formula: (fluorescence post inversion × 100)/fluorescence of pre-inversion. SBN-dependent inhibition was calculated by taking monocyte adhesion to oxLDL without SBN as 100%.

Anti-oxLDL IgG Binding. ELISA plates (Nalgene) were coated with LDL (50 μL of 5 mg/L in 20 mmol/L borate buffer/10 mmol/L EDTA, pH 8.5) treated with $CuSO_4$ in the presence or absence of SBN for 18 h at 4 °C. The ability of SBN to inhibit the generation of anti-oxLDL IgG specific epitopes in oxLDL was determined using rabbit anti-MDA IgG (50 μL of 10 mg/L, Academy Biomedical, Houston, TX) followed by HRP-conjugated goat F(ab′)$_2$ anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Color was developed using 3,3′,5,5′-

tetramethylbenzidine-1 component from Amresco (Solon, OH), and absorbance was read at 450 nm. Percent inhibition was determined by taking anti-MDA IgG binding to oxLDL without SBN as 100%.

Statistical Analysis. All experiments were repeated at least twice, from which standard deviations were calculated. Statistical analyses were performed with JMP software (SAS Institute, Cary, SC) or SigmaStat 9 program from the Systat Software Inc. (San Jose, CA) using one-way ANOVA, with mean separations tested by Tukey's analysis, set at $P < 0.05$.

RESULTS

Crude milk thistle extract was tested to determine its effect on LDL oxidation. LDL treated with vehicle (DMSO) and no milk thistle extract showed significant TBARS generation (**Figure 2**). However, when doses of milk thistle extract above 37.5 μM (38, 75, 150, and 300 μM) were reacted with LDL, reductions, as compared to 0 μM treatments, reached 18, 73, 82, and 86%, respectively, showing that the milk thistle extract inhibited LDL oxidation (**Figure 2**). A HPLC-UV trace of the tested milk thistle ethanol Soxhlet extract showed that it contained SCA, SCB, SD, SA, SB, ISA, and ISB, eluting at times of 13.55, 14.83, 16.65, 22.30, 23.19, 25.88, and 26.62 min, respectively (**Figure 3**). Estimation of these individual components showed SCA, SCB, SD, SA, SB, ISA, and ISB constituted 5, 3, 48, 5, 7, 10, and 22% of the total silymarin, respectively. Total flavonolignan yields, in milligrams per milliliter, are presented in **Table 1** as means and standard deviations.

Due to the high degree of protection afforded by the extract, subsequent experiments were carried out to determine which of the flavonolignans provided the most potent inhibition of oxLDL generation. A dose-dependent inhibition of oxLDL generation was observed with the flavonolignans (**Figure 4**). Of the four flavonolignans, SBN and SC were more effective in significantly inhibiting oxLDL generation at doses as low as 75 and 38 μM (**Figure 4a**,**b**). SBN, at doses of 75, 150, and 300 μM, reduced TBARS levels as compared to controls by 52, 52, and 64%, respectively. SC, at doses of 38, 75, 150, and 300 μM, lowered TBARS levels as compared to 0 μM controls by 37, 65, 61, and 74%, respectively. SD and IS showed poorer inhibitory properties even at higher concentration (**Figure 4c**,**d**). At the highest tested doses of 300 μM SBN, SC, SD, and IS, the generation of oxLDL was reduced by 64, 75, 31, and 47%, respectively, as compared to 0 μM treatments.

Because SBN is often the major component of silymarin and is often reported to be the most bioactive constituent, further studies were pursued using only SBN. To confirm the efficacy of SBN, a dose-dependent experiment was performed to evaluate reductions in monocyte adhesion. A dose-dependent inhibition of U937, a human monocytic cell line, adhesion to oxLDL generated in the presence of SBN was observed (**Table 2**). At a lower concentration of SBN (18 μM), about a 25% inhibition in monocyte adhesion was observed, and 77−95% inhibition was seen at higher concentrations of SBN (38−150 μM).

Next, it was determined whether reduced oxLDL generation would also result in inhibition of anti-oxLDL binding. Anti-oxLDL IgG bound to oxLDL in the absence of SBN. Very minimal binding was observed between anti-oxLDL IgG and LDL. The addition of SBN during oxLDL formation resulted in a dose-dependent inhibition of anti-oxLDL IgG binding as presented in **Table 3**. A 150 μM SBN preparation inhibited >70% of the anti-oxLDL IgG binding.

DISCUSSION

On the basis of the HPLC analysis, 300 μmol/L of the extract was estimated to contain 43, 56, 124, and 77 μmol/L of SA and SB, SCA and SCB, SD, and ISA and ISB, collectively. These yields are somewhat similar to other reported silymarin contents of extracted seed (*3*–*5*), although it is realized that the flavonolignan content can vary from seed batch to seed batch. The tested milk thistle extract drastically inhibited (93%) oxLDL generation. However, SBN and SC showed only 60−75% inhibition at 300 μmol/L, suggesting that a synergistic effect of SBN and SC could have contributed to the complete inhibition observed with the extract. It is also possible that the presence of other compounds in silymarin could have contributed to the observed extract activity, such as the recently discovered degradation product silyamandin (*26*). The results presented in this work show that SBN and SC have potent

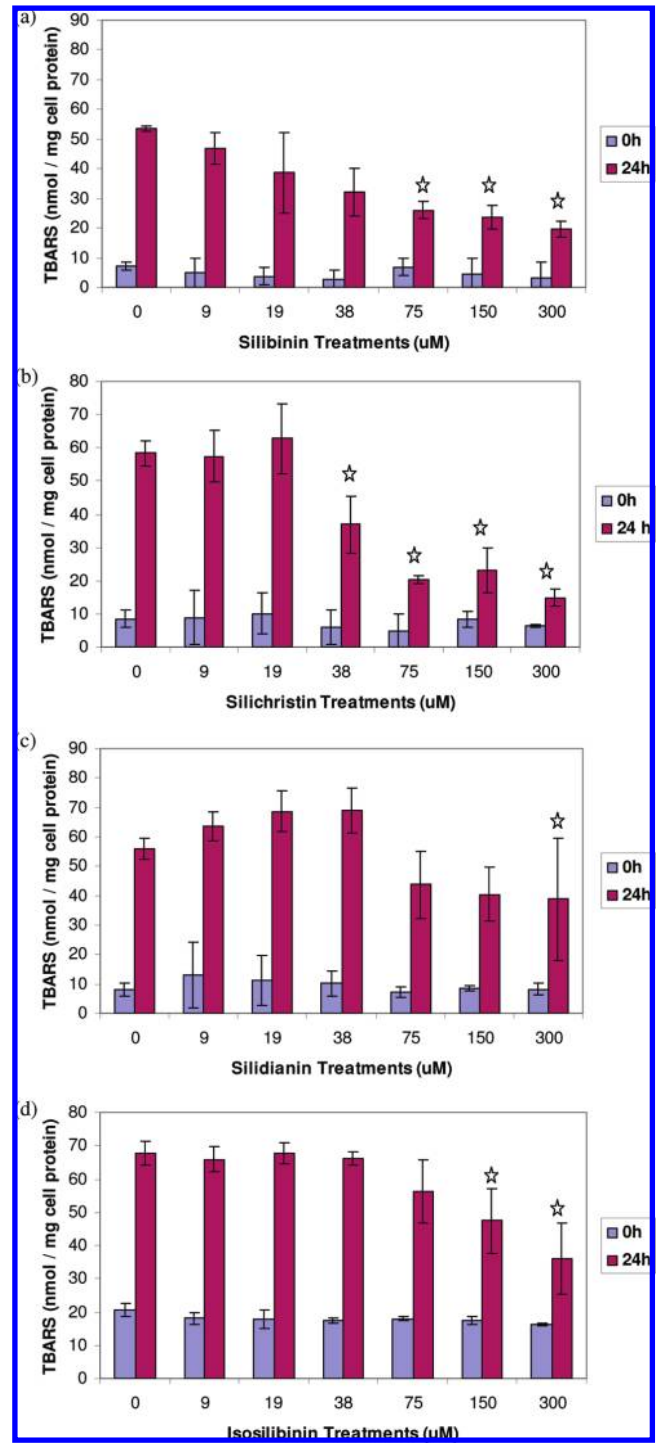

Figure 4. Inhibition of $CuSO_4$-induced LDL oxidation.

Table 2. SBN Inhibits SR-Dependent Monocyte Adhesion[a]

SBN (μmol/L)	inhibition (%)
0	1.3 ± 0.9 e
9	16.1 ± 2.7 d
19	25.3 ± 2.7 c
38	77.9 ± 6.6 b
75	92.4 ± 7.0 a
150	85.1 ± 5.7 a

[a] U937 cell adhesion to LDL incubated with $CuSO_4$ in the presence or absence of SBN was determined by the adhesion assay as described under Materials and Methods. Values are means ± SD, $n = 9$ (three independent experiments performed in triplicate). Means without a common letter differ, $P < 0.05$.

Table 3. SBN Inhibits Generation of oxLDL Epitopes Recognized by Anti-oxLDL IgG[a]

SBN (μmol/L)	inhibition (%)
0	1.0 ± 0.0 f
9	13.2 ± 2.7 e
19	20.0 ± 2.9 d
38	47.1 ± 4.6 c
75	54.3 ± 8.3 b
150	70.0 ± 5.0 a

[a] Binding of anti-MDA IgG was performed as described under Materials and Methods. Values are means ± SD, $n = 6$ (two independent experiments performed in triplicate). Means without a common letter differ, $P < 0.05$.

antioxidant function as compared to SD and IS. SBN and IS reference compounds are solely available as their diastereoisomeric mixtures. Unfortunately, the purified diastereoisomer components are not commercially available. Therefore, no definitive conclusions can be drawn in this research as to which diastereoisomer displays stronger inhibitory activity against LDL.

Earlier studies have shown that SBN increased the duration of the lag phase of LDL autoxidation as measured by diene conjugate formation (*13, 22*). Moreover, SC and SD have been shown to have pro-oxidant activity (*13*). In contrast, the results presented herein show that milk thistle extract as well as the individual components of silymarin inhibited Cu^{2+}-mediated oxLDL generation. The conflicting results could be due to the difference in method used to detect the generation of oxLDL. Whereas this research examined inhibitions in TBARS formations, prior research has focused on the delay in the lag time of conjugated diene formation in the presence of flavonolignans. The varying results could also be due to the use of copper chloride as opposed to copper sulfate (*13*) and the fact that this research was accomplished using a 5 μM copper sulfate concentration versus the 1.67 μM concentrations used by Locher et al. (*22*). SC and SD showed definite antioxidant effects in this research.

Metal chelating properties could also contribute to the ability of the flavonolignans to inhibit the $CuSO_4$-induced oxLDL generation. $CuSO_4$-induced LDL oxidation is assumed to be initiated by the reduction of Cu^{2+} to Cu^{+} (*27, 28*). Meroni et al. (*29*) have reported that SBN inhibited PHA-induced T lymphocyte proliferation, and this inhibition was prevented by the addition of $CuSO_4$, suggesting that SBN has a metal chelating property and could therefore exert its inhibitory activity on LDL oxidation via this metal chelating function. A chelating agent was not tested in this research due to the results of previous experiments. Copper sulfate (55 μmol/L) was used to chemically induce LDL oxidation in TBARS experiments, whereas 2 μmol/L copper sulfate was used to induce oxidation to LDL in cell-mediated experiments. The results showed that the flavonolignans were able to reduce TBARS formation equally well when very low doses of copper sulfate were used (*30*). This indicated to the researchers that the effectiveness of the flavonolignans in reducing oxidative stress comes down to more than just the chelation of copper ions and is at least partially due to another mechanism, such as free radical scavenging (*31*).

The results presented also show that SBN and SC have potent antioxidant function as compared to SD and IS. The differences observed between the free radical scavenging abilities of these isomers could be due to the number of phenolic groups present in flavonolignan structures, which are presented in **Figure 1**. The increased antioxidative capability of SC may be a result of the presence of the additional hydroxyl group as compared to the other isomers. The difference in antioxidant potential between SBN and IS could be due to the geometric placement of the *o*-methoxyphenol group. The proximity and geometry of the rings in SBN could lead to a highly stable conjugated system after removal of a benzyl hydrogen. However, the placement of the rings in the relatively nonactive isomers (IS and SD) probably precludes or retards the geometric requirement for efficient conjugation, which could significantly reduce the antioxidative abilities of SD and IS.

Generation of oxLDL has been shown to induce atherogenicity (*20, 32*). These effects include activation of endothelial cells (*17, 33*), induction of chemokine and pro-inflammatory cytokine expression (*17, 31*), and chemotaxis of monocytes (*34*). Moreover, oxidative modification of LDL alters its structure, allowing oxLDL to be taken up by SR expressed on MΦ, leading to the formation of lipid-laden foam cells, the hallmark of early atherosclerotic lesions (*35*). In recent years considerable attention has been dedicated to phytochemicals that can act as antioxidants to potentially prevent the development of cardiovascular diseases (*36, 37*). OxLDL molecules always express the neoepitopes, which are essential for their interaction with the SR expressed on MΦ (*35*). The data presented herein demonstrated that SBN, the major component in silymarin, decreased the concentration of oxidation-specific neoepitopes in oxLDL that are recognized by SR expressed on monocytes and MΦ, suggesting a possible mechanism by which milk thistle may prevent the progression of atherosclerosis.

Pharmacokinetics study in humans have shown that peak plasma levels of total SBN, measured as free and conjugated SBN, reached 7−9 μmol/L after oral administration of 140 mg of silymarin, equivalent to 53.2 mg of silibinin (*38, 39*). The results presented in this paper show inhibition of SR-dependent monocyte adhesion to LDL treated with SBN and $CuSO_4$ at concentrations as low as 19 μmol/L, which is twice that of the reported values in pharmacokinetics studies in humans. In any event, the in vitro observations in this paper provide a basis for the potential in vivo effects of silymarin on the prevention of atherosclerosis.

OxLDL exerts its atherogenic functions by binding to SR that are expressed on monocytes and MΦ, leading to the formation of lipid-laden foam cells (*40, 41*). In addition, oxLDL has been shown to trigger immune responses resulting in the production of autoantibodies (*16, 31, 42*). Clinical studies have suggested that the autoantibody titer correlates with the progression of atherosclerosis (*43, 44*). The presence of autoantibodies can result in the formation of oxLDL−immune complexes, which can activate monocytes and MΦ via interactions with their FcγR. Results presented in this work show that LDL oxidation in the presence of SBN resulted in the inhibition of

the generation of specific epitopes that are essential for SR-mediated monocyte adhesion. Furthermore, SBN treatment also inhibited anti-oxLDL antibody-specific epitopes, suggesting SBN may inhibit oxLDL−immune complex formation. These results show that the flavonolignans prevent the formation of oxLDL and that SBN interferes with the ability of monocyte interaction with oxLDL and/or oxLDL−immune complexes via SR and FcγR, respectively.

In summary, the in vitro results in this paper show that phytochemical(s) present in milk thistle fruit inhibited the generation of oxLDL. SBN inhibited subsequent SR-mediated monocyte interaction, a primary event in the development of atherosclerosis. These results suggest that SBN-mediated inhibition of LDL oxidation resulting in reduced SR-mediated monocyte adhesion may prevent the onset of atherosclerosis. In a recent study, SBN has been also shown to inhibit TNF-α-induced expression of the endothelial cell specific cell adhesion molecules ICAM-1 and VCAM-1 (*45*), suggesting SBN may block the interaction between endothelial cells and circulating monocytes. Thus, it is possible that the extract prepared from the fruits of an easily accessible plant could be useful to prevent the progression of atherosclerotic events. However, before important conclusions that could lead to prevention strategies are drawn, it would be of interest to determine the atheroprotective effect of silymarin in vivo using an atherosclerosis-prone apolipoprotein E or LDL receptor knockout mouse model.

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