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Silybin–Phosphatidylcholine Complex Protects Human Gastric and Liver Cells from Oxidative Stress

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Abstract. *Background/Aim: Silybin is the main component of silymarin with antioxidant, anti-inflammatory and cytoprotective actions. Our aim was to compare the effect of silybin used as single substance, silybin–phosphatidylcholine complex (SilPho), and derivatives of silybin (MannpSil, GalpSil, GlcpSil, LactpSil) on MKN28 and HepG2 cell viability and cell death, in vitro, after induction of oxidative stress. Materials and Methods: Oxidative stress was induced by incubating HepG2 and MKN28 cells with xanthine oxidase in the presence of its substrate xanthine. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide assay. Determination of Malondialdehyde (MDA) in MKN28 cells was performed by high-performance liquid chromatography. Quantitative analysis of apoptotic cells was carried-out using annexin. Results: SilPho and new silybin glycoconjugates did not affect cell viability, while silybin induced about 50% cell death in both MKN28 and in HepG2 cells. Pre-treatment of cells with silybin and new silybin glycoconjugates (before oxidative stress induction) did not affect cell viability, while SilPho had a protective effect. Exposure of MKN28 cells to oxidative stress caused a two-fold increase in cellular MDA concentration compared to untreated cells. Moreover, pre-treatment with SilPho, but not with silybin, significantly prevented oxidative stress-induced increase in cellular Malondialdehyde. Moreover, silybin induced apoptosis potentiated by oxidative stress, while SilPho did not induce any effect. Oxidative stress caused cell death primarily by necrosis, antagonized by SilPho. Conclusion: The protective effect of SilPho is partially due to inhibition of radical oxidative species.*

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Silybin is the main component of silymarin (famous anti-oxidant) with an increasing number of effects (1). Silibinin is a semi-purified, commercially available fraction of silymarin: Silibinin is an approximately 1:1 mixture of two diastereoisomeric compounds, silybin A and silybin B (2). Therefore, purified silybin and silibinin are practically synonymous (2).

The main effects attributed both *in vitro* and *in vivo* to silybin are related to its anti-oxidant, anti-inflammatory and cytoprotective actions (3-5). Silybin is also considered a chemopreventive and cancer-protective agent because it modulates a series of mitogenic signaling and cell-cycle regulators (6, 7), mediating a pro-apoptotic effect (8, 9).

Both bioavailability and therapeutic efficacy of silybin *in vivo* are rather limited by low water solubility, low bioavailability, and poor intestinal absorption (10). To improve these pharmacological limitations, a silybin phytosomecomplex (silybin plus phosphatidylcholine; SilPho) has been co-formulated with vitamin E [Realsil (RA), Istituto Biochimico Italiano, Lorenzini S.p.a., Italy] (11, 12). Pharmacokinetic analyses indicated that the bioavailability of silybinphytosome is much higher than that of silymarin, and in this pharmaceutical preparation, silybin is widely distributed in plasma and tissues, which include the liver, lung, stomach, skin, and prostate (13, 14).

In vivo, silymarin and silybin have been used as therapeutic herbal products for treatment of acute and chronic liver diseases: in particular, alcoholic liver disease and cirrhosis (15-17), non-alcoholic fatty liver disease (18) and hepatic fibrosis (19, 20). In animals, silybinphytosome complex reduces oxidative stress, lipid peroxidation, collagen accumulation and consequently liver damage (19). In men, RA ameliorates certain serum and histological parameters of liver damage and fibrosis (18).

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Recently, Zarrelli *et al*. obtained new 9''–phosphodiester silybin conjugates with different mono- and di-saccharide labels through the anomeric hydroxyl group in order to enhance the biological efficacy of the derivatives by increasing their *in vivo* stability, binding affinity, and overall uptake (21). These silybin derivatives have water solubility well above that of silybin. Despite a large series of studies reported in the literature, confusion about the different actions of silybin exists. Therefore, the following merit investigation: i) if the effects of silybin are similar in different cell lines of different histogenesis; ii) the influence of the concentrations used in different experimental models; iii) the effects of the different silybin derivatives.

In the present study, we compared the effect of silybin used as a single agent or as SilPho, and different silybin derivatives on MKN28 and HepG2 cell death *in vitro* after the induction of oxidative stress. We used two cell lines (MKN28 and HepG2) to verify the results obtained by excluding the possibility of interference of the cell type.

Materials and Methods

Materials. Silybin was a gift from Indena (Milan, Italy). SilPho was provided by Istituto Biochimico Italiano (G. Lorenzini S.p.A. Milan, Italy). Silybin derivatives were synthesized according to Zarrelli *et al*. (21): in detail, an efficient synthetic procedure leads to new 9''–phosphodiestersilybin conjugates with different mono- and di-saccharide labels through the anomeric hydroxyl group. In this approach a suitable 9''-phosphoramidite was used as silybin building block and 1-OH full protected mono- and di-saccharide derivatives chosen as sugar starting materials (21). The new silybin conjugates were: silybin-900-phosphoryl-D-mannopyranoside (MannpSil), silybin-900-phosphoryl-D-galactopyranoside (GalpSil), silybin-900-phosphoryl-D-glucopyranoside (GlcpSil), silybin-900-phosphoryl-D-Lactopyranoside (LactpSil).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay was purchased by Sigma (Milan, Italy). Annexin Apoptosis Detection Kit was obtained from BD Biosciences (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM):F12, penicillin, streptomycin, fetal bovine serum, L-glutamine and trypsin/EDTA were obtained from Life Technologies Inc. (Gaithersburg, MD, USA).

Agent preparation. Pure silybin was dissolved in dimethyl sulfoxide (DMSO) and used at final concentration of 10, 25, 50, 75, 100 and 200 μM. SilPho was dissolved in DMSO to achieve final concentrations of silybin similar to those employed for testing silybin alone (10, 25, 50, 75, 100 and 200 μM). Silybin derivatives were dissolved in water and used at final concentration of 10, 25, 50, 75, 100 and 200 μM.

Cell culture. HepG2 cells were derived from human hepatocellular carcinoma (22) and MKN28 cells were derived from a human well-differentiated gastric tubular adenocarcinoma and showing gastric-type differentiation (23) (Cell Bank Interlab Cell Line Collection, IST Genova, Italy). HepG2 and MKN28 cells were grown as monolayer in DMEM supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution (Life Technologies Inc.) at 37˚C in a humidified atmosphere of 5% CO_2 in air. Cytotoxicity experiments were conducted using serum-free medium.

Induction of oxidative stress. Oxidative stress was induced by incubating HepG2 and MKN28 cells with xanthine oxidase (XO; 10-100 mU/ml) in the presence of its substrate xanthine (X; 1 mM) for periods of up to 3 h. Exposure of cells in culture to X-XO (1 mM + 50 mM) for 2 hours causes significant cell injury (50%) and this has been demonstrated to be due to generation of radical oxidative species (ROS) and in particular of OH produced from H_2O_2 by iron catalyzed Fenton reaction (22).

We examined the effect of silybin, SilPho, and the new silybin glycoconjugates (MannpSil, GalpSil, GlcpSil, LactpSil) on X-XO-induced cell damage. Cells were incubated with serum-free medium (Control) for 1-48 hours; with serum-free medium for 1-48 h and then with X-XO (1 mM + 50 mM) for 2 h (X-XO control); with silybin, SilPho, and new silybin glycoconjugates (10-200 μM) for 1-48 h and then, after washing, with X-XO (1 mM + 50 mM) for 2 h.

Cell viability. Cell viability was determined by the MTT assay. Briefly, 10 μl of MTT (5 mg/ml saline) were added to each well, and treated cells were incubated for 90 min at 37˚C and centrifuged for five minutes. After aspiration of supernatant, cells were lysed and solubilised by addition of 100 μl of 0.04N HCl containing isopropanol. The absorbance of each sample was analyzed at 540 nm. Cell viability (%) was calculated by dividing the absorbance of samples obtained from cells incubated with test drugs by the absorbance of samples obtained from cells incubated with tissue culture medium only (control) and multiplying this ratio by 100. Data are presented as the mean±standard deviation (SD) of three experiments run in duplicate.

Determination of lipid peroxidation. Malondialdehyde (MDA) is considered a presumptive biomarker for lipid peroxidation in live organisms and cultured cells (24). Determination of MDA in MKN28 cells was performed by High Performance Liquid Chromatography (HPLC) with fluorimetric detection, according to the method of Bergamo and colleagues (25). Cellular pellets were extracted with 250 ml of Milli-Q water in an ultrasonic bath for 30 minutes after the addition of 250 ml of cold 10% tricloracetic acid (TCA). Samples were vigorously mixed (three minutes) and centrifuged (5 minutes, 10,000 × *g*). The supernatant was added to 700 ml of thiobarbituric acid prepared using thiobarbituric acid in 2 M acetate buffer at pH 3, degassing by a vacuum pump (5 min), and flushing the final solution with nitrogen for 10 min. The mixtures were degassed and then incubated for 30 min at 90˚C. At the end of the incubation period, samples were cooled, centrifuged (5 min, 10,000 × *g*) to remove particulate material and, finally, sample aliquots (20 ml) were analyzed by HPLC. Quantification of MDA was obtained from a calibration curve constructed by injecting increasing amounts of standard MDA. MDA concentration was expressed as mg/10^6 cells.

Quantitative analysis of apoptotic cells by flow cytometry. Quantitative analysis of apoptotic cells with and without treatment of silybin was carried-out using the Annexin Apoptotic Detection Kit II (BD Biosciences).

Briefly, MKN-28 cells were treated with silybin alone or SilPho at the doses previously described for 24 h with or without X-XO-induced cell damage. Cells were harvested, washed twice with cold Phosphate Buffered Saline (PBS) and then resuspended in 1X

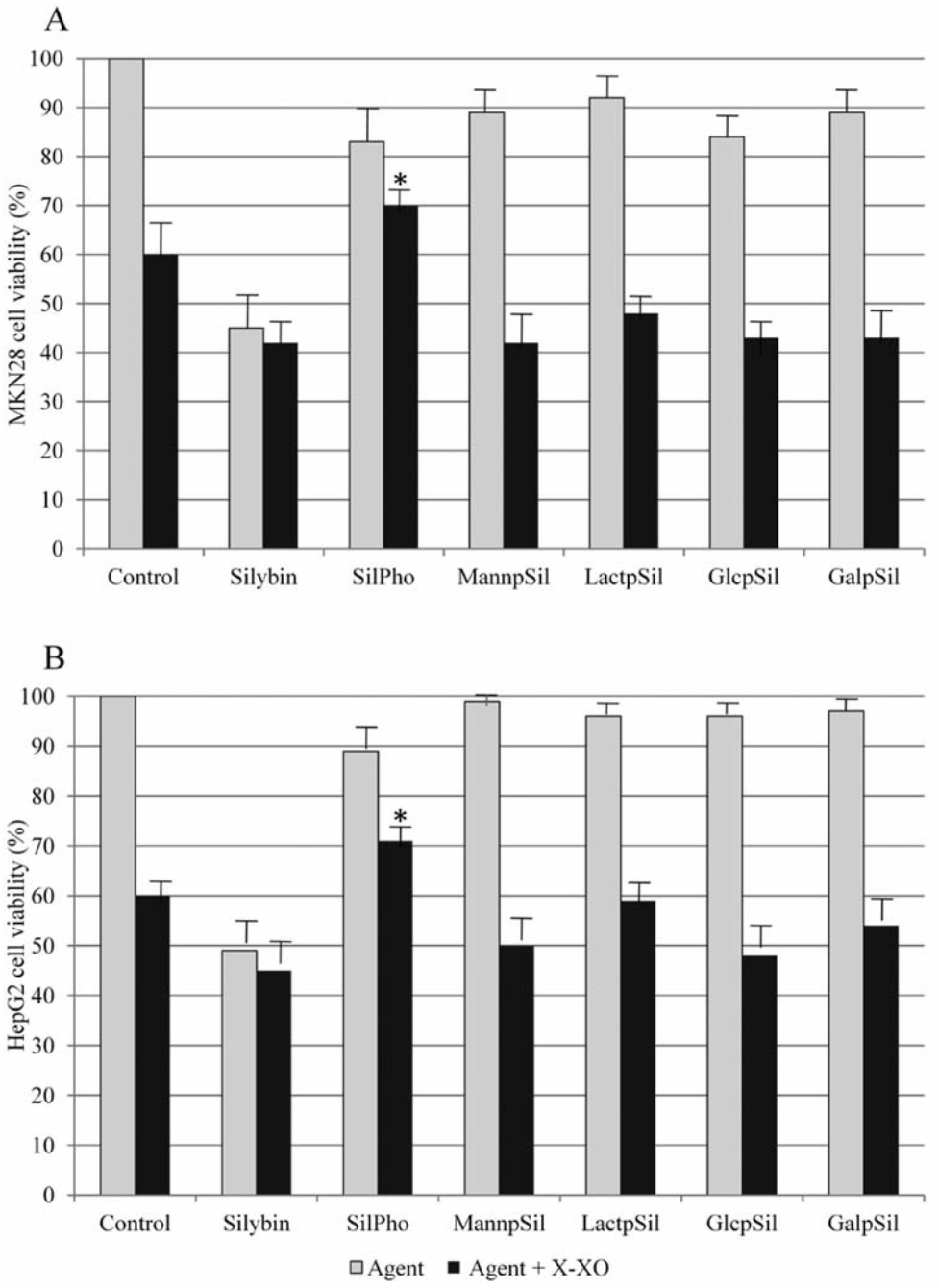

Figure 1. *Evaluation of cell viability in MKN28 (A) and HepG2 (B) cultured cells after incubation with silybin, silybin–phosphatidylcholine complex (SilPho), silybin-900-phosphoryl-D-mannopyranoside (MannpSil), silybin-900-phosphoryl-D-galactopyranoside (GalpSil), silybin-900-phosphoryl-D-glucopyranoside (GlcpSil), silybin-900-phosphoryl-D-Lactopyranoside (LactpSil), before and after induction of oxidative stress with xanthine-xanthine oxidase (X-XO). The data are reported as means±SD of three experiments. The concentration of agents utilized was 50 μg and the time of observation was 24 h. *p<0.01 vs. Control and SilPho alone.*

binding buffer at a density of 1×10^6 cells/ml. Cellular pellets were subjected to annexin and propidium iodide staining at room temperature for 15 min in the dark and analyzed by flow cytometry within 1 h after The addition of 400 μl of 1X binding buffer. Apoptotic cells, stained with annexin and propidium iodide, were analyzed by fluorescence activated cell sorting using a Cell Quest 3.4 software (FACS Calibur; BD Biosciences, San Jose, CA, USA). The apoptotic cells stained with annexin exhibited green fluorescence, whereas the cells stained with propidium iodide exhibited red and green fluorescence.

Experiments were conducted as it follows: I: Evaluation of silybin, SilPho, MannpSil, GalpSil, GlcpSil and LactpSil toxicity in MKN28 and HepG2 cultured cells under basal conditions; II: evaluation of cell viability after the induction of oxidative stress; III: evaluation of cell viability in MKN28 and HepG2 cultured cells after incubation with silybin, SilPho, MannpSil, GalpSil, GlcpSil and LactpSil and subsequent induction of oxidative stress; IV: determination of MDA as a marker showing the induction of oxidative stress in cultured cells after incubation with silybin alone and with SilPho; V: quantitative analysis of apoptotic cells with and without treatment of silybin and SilPho.

Statistical analysis. Data are expressed as the mean±SD. Significance of differences was assessed by one-way analysis of variance (ANOVA) and, when the F value was significant, by Tukey-Kramer test for multiple comparisons or by Student's *t*-test for comparison between two means. Differences were considered to be significantly different if $p<0.05$.

Results

Effect of oxidative stress on MKN 28 and HepG2 cell viability. Oxidative stress was induced by incubating MKN28 and HepG2 cells with XO (10-100 mU/ml) in the presence of its substrate (1 mM) for periods of up to 3 h. Two-h incubation with X-XO (1 mM and 10-100 mU/ml) caused a dose-dependent and significant reduction in cell viability, as assessed by the MTT assay (Figure 1). For the subsequent experiments, a concentration of X-XO of 1 mM plus 50 mU/ml was selected that led to a decrease in cell viability close to 60%.

Effect of silybin, SilPho, and new silybin glycoconjugates on X-XO induced cell damage. Underbasal conditions, the incubation of MKN28 and HepG2 cells with silybin, SilPho, and new silybin glycoconjugates led to two different results (Figure 1). SilPho and new silybin glycoconjugates did not affect cell viability, while silybin induced cell death of about 50%, even at the lower dose used, both of MKN28 and HepG2 cells. The pre-treatment of cells with silybin and new silybin glycoconjugates (before X-XO incubation) did not affect cell viability, while SilPho had a protective effect (Figure 1). In Figure 1, the concentration of molecules utilized and the time of observation reported are 50 μg and 24 h, respectively. With the exception of the SilPho, the same results have been verified at 10, 25, 50, 75, 100, 200 μM and at 1-48 h of observation.

As the oxidative damage induced by X-XO and the effect of pre-treatment were similar in MKN28 and HepG2 cells we decided to perform the following experiments only in MKN28 cell line.

Effect of silybin and of SilPho on X-XO induced lipid peroxidation in MKN28 cells. ROS-induced cell damage is associated with cell membrane disruption due to lipid peroxidation. Therefore, we hypothesized that silybin and SilPho might prevent lipid peroxidation caused by ROS generated by X-XO. In this light, we evaluated whether pre-treatment with silybin and SilPho was able to counteract X-XO-increased cellular MDA, a marker of lipid peroxidation. Exposure of MKN28 cells to X-XO caused an approximately two-fold increase in cellular MDA concentration compared untreated cells (Figure 2). Moreover, pre-treatment with

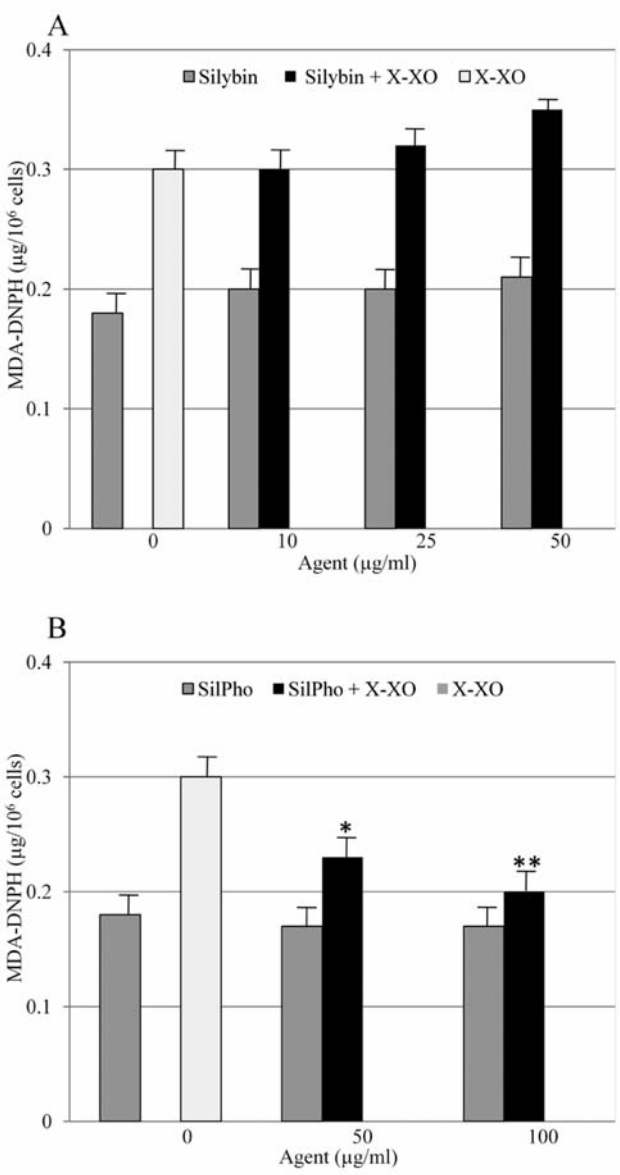

Figure 2. *Effect of silybin (A) and silybin–phosphatidylcholine complex (SilPho) (B) on Malondialdehyde (MDA) in MKN28 (A) and HepG2 (B) cells before and after induction of oxidative stress with xanthine-xanthine oxidase (X-XO). The data are reported as means±SD of three experiments. *$p<0.05$ and **$p<0.01$ vs. 0 μg/ml.*

Table I. *Cell viability on treatment with silybin–phosphatidylcholine complex (SilPho) and xanthine-xanthine oxidase (X-XO). SilPho protects against X-XO-induced damage in MKN28 and HepG2 cells.*

	Cell viability (%)						
	0 μg/ml	10 μg/ml	25 μg/ml	50 μg/ml	75 μg/ml	100 μg/ml	200 μg/ml
MKN28							
Control	100	-	-	-	-	-	-
X-XO	60	-	-	-	-	-	-
SilPho	-	85	80	83	84	83	80
SilPho+X-XO	-	61	64	70	67	68	66
HepG2							
Control	100	-	-	-	-	-	-
X-XO	60	-	-	-	-	-	-
SilPho	-	88	85	89	85	88	84
SilPho+X-XO	-	60	63	71	68	66	65

SilPho (Figure 2B) (25-100 μg) but not with silybin (10-50 μg) (Figure 2A), significantly prevented X-XO-induced increase of cell MDA. These results suggest that the protective effect of SilPho was partially due to inhibition of ROS-induced lipid peroxidation.

Data regarding new silybin glycoconjugates are not reported because experimental results were similar to those obtained with silybin.

Effect of silybin and SilPho on MKN28 cell apoptosis and necrosis before and after induction of oxidative stress with X-XO. The pre-incubation with SilPho showed a dose-dependent protective effect (Table I). These effects are, almost in part, explained by the results obtained by flow cytometry (FACS). In fact, as reported in Table II, we found that the two assessed substances differently affected cell vitality. Under basal conditions, silybin induced apoptosis and SilPho did not induce any effect. Oxidative stress caused cell death primarily by inducing cell necrosis. The concomitant presence of silybin and oxidative stress enhanced the ability of the latter to induce apoptosis. SilPho had no effects on apoptosis, but significantly counteracted cell necrosis. The increase of silybin and SilPho concentrations up to 100 μg did not modify these results.

Table II. *Cell death with pre-incubation of cells with silybin and silybin–phosphatidylcholine complex (SilPho) under basal conditions and after the induction of oxidative stress.*

	Apoptosis (%)	Necrosis (%)
Basal	2.99	1.65
Silybin, 25 μg/ml	7.52*	6.38*
SilPho, 25 μg/ml	2.89	2.22
Oxidative stress	5.50*	12.97*
Oxidative stress + silybin, 25 μg/ml	22.05*	6.40*
Oxidative stress+ SilPho, 25 μg/ml	3.69	8.41*

*$p<0.05$ *vs*. basal.

Discussion

Several reports have been published on silymarin and its flavonolignan obtained from the seeds of milk thistle (*Silybum marianum*) constituents regarding their liver-protective, anti-oxidant, and free-radical scavenging activities (1, 3-5). Silybin acts, both *in vitro* and *in vivo*, as a radical scavenger by increasing the levels of superoxide dismutase and glutathione peroxidase and by reducing MDA and 4-hydroxynonenal (26), markers of lipid peroxidation. Similarly, vitamin E and phospholipids are well-known anti-oxidants and the conjugation of these three substances without any alteration in their stability enhances anti-oxidant action (27, 28). The conjugation of silybin with phospholipids was performed in order to modify its solubility and absorption *in vivo*. In fact, while silybin has very low solubility in water, its conjugation with other substances allowed its intravenous administration and enhanced its oral bioavailability (11). *In vivo*, the complex of silybin with phospholids and vitamin E (RA) is rapidly absorbed, with a blood peak concentration at 2 h and a large inter-organ distribution (14).

The new silybin derivatives obtained by Zarrelli *et al*. have a higher water solubility than that of silybin, with enhanced biological efficacy, binding affinity, and overall uptake (21).

In the present study, we assessed the effects of all these compounds on cell viability and evaluated whether silybin or SilPho pre-treatment were able to counteract X-XO-induced increase of intracellular MDA. Exposure of MKN-28 cells to

X-XO caused an approximately two-fold increase in MDA level as compared to untreated cells. Moreover, pre-treatment with SilPho and silybin prevented X-XO-induced intracellular MDA increase. This suggests that the protective and anti-oxidant effect of SilPho and silybin is, at least in part, due to inhibition of ROS-mediated lipid peroxidation. *In vitro* studies revealed that flavonoids can have considerable anti-oxidant activity in a wide range of chemical oxidation systems (29, 30). In our study, silybin and SilPho exhibited powerful spontaneous anti-oxidant capacity in human gastric and liver cells. Moreover, we evaluated the protective effect on X-XO induced injury in MKN-28 cells line measuring cell viability, and we found that only SilPho had a dose-dependent protective effect. It is likely that phospholipids have a protective effect against X-XO-induced cell death by stabilizing plasma membranes.

In our experimental system, cell death induced by oxidative stress followed two different patterns. The first led to necrosis, a typical consequence of acute metabolic perturbation, and the second to apoptosis, the consequence of programmed death (31). Silybin enhanced X-XO-induced apoptosis and reduced X-XO-mediated necrosis, whereas SilPho significantly counteracted cell necrosis only. Previously, we demonstrated that RA induced a normalization of circulating lipids in patients with non-alcoholic steatohepatitis, probably by improving liver function (32).

In conclusion, our results showed that both silybin and SilPho act as anti-oxidants in an *in vitro* cell system, reducing MDA levels induced by oxidative stress. Moreover, SilPho protects MKN-28 cells from X-XO-induced cell death, being more active than silybinin protecting cells from oxidative stress.

Conflicts of Interest

All Authors have declared no personal or family conflicts of interest in regard to this study. This study was not funded.

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