Biochemical Pharmacology, Vol. 26, pp. 2405–2409. Pergamon Press, 1977. Printed in Great Britain.

INHIBITORY ACTION OF SILYMARIN OF LIPID PEROXIDE FORMATION IN RAT LIVER MITOCHONDRIA AND MICROSOMES

A. BINDOLI, L. CAVALLINI and N. SILIPRANDI

"Centro per lo Studio della Fisiologia Mitocondriale" CNR, Padova and Institute of Biological Chemistry of the University of Padova 35100 Padova, Italy

(*Received* 3 *March* 1977; *accepted* 3 *May* 1977)

Abstract—Silymarin, a 3-oxyflavone present in Silybum Marianum, protected both liver mitochondria and microsomes from lipid peroxide formation induced by various agents. The antiperoxidative action exhibited by Silymarin was 10-fold higher than that of α-tocopherol, and was present when the drug was added as well as after the peroxidant agents. Data obtained rule out the possibility that the antiperoxidative action of Silymarin was due to an interaction of this drug with Fe^{2+}. The reported results are compatible with an interaction of Silymarin with free radical species responsible for lipid peroxidation.

It has been reported that Silymarin, a 3-oxyflavone occurring in the thistle Silybum Marianum [1], protects the liver of experimental animals against several hepatotoxic substances, such as amanitin, phalloidin, galactosamine, thioacetamide, and CCl_4 [2–6]. This protective action, which has been challenged, at least in part, by Leber and Knauff [7], has been described as a "membrane stabilizing action" [8, 9]. The underlying mechanism of this protection are, however, still undetermined.

On the other hand, it appeared that hepatotoxic substances, such as CCl_4, become effective following their "activation" into a free radical form [10]. Moreover, CCl_4 toxicity is lowered by substances which scavenge free radicals [10, 11].

On the basis of these considerations, it appears possible that Silymarin might act by preventing, or inhibiting, lipid peroxide formation which is induced by various agents through a radical mechanism. Indeed the results reported in the present paper show that Silymarin strongly inhibits peroxide formation induced by peroxidative agents both in liver mitochondria and microsomes.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose buffered with 3 mM Tris–HCl at pH 7.4, as indicated by Myers and Slater [12]. Rat liver microsomes were prepared as described by Ernster [13]. Protein content was estimated by the biuret method [14]. Oxygen uptake was followed at 25° with a platinum electrode assembly of the Clark type [15]. Mitochondria swelling was determined by following the change in optical density at 520 nm [16]. Malondialdehyde (MDA), was determined by the thiobarbituric acid method of Wilbur [17] as described by Ottolenghi [18], and malondialdehyde content was calculated by using $\epsilon = 1.56 \cdot 10^5$ litre $mole^{-1}$ cm^{-1} at 532 nm [19]. Silymarin was a gift of the Istituto Biochimico Italiano, Milan, Italy.

RESULTS

Figure 1 shows the traces regarding oxygen uptake by rat liver mitochondria, induced by the peroxidative system Fe^{2+}-ascorbate [20]. When Silymarin is added to the mitochondrial suspensions prior to Fe^{2+}-ascorbate, a dual, dose dependent, effect is seen: the time lag is lengthened, and oxygen uptake is decreased (Fig. 1a). At 20 μM concentration, Silymarin fully prevented oxygen uptake. Fig. 1b shows that Silymarin not only prevented, but also inhibited peroxide formation sparked by Fe^{2+}-ascorbate, but in this case, however, higher concentrations of Silymarin were required. MDA production was also prevented, or inhibited, by the same concentrations of Silymarin (Fig. 2). It is known that MDA formation reflects, as does oxygen uptake, the extent of peroxide formation, and the parallelism between these two parameters clearly emerges from data reported in Fig. 2. The same Fig. also shows the effect of α-tocopherol on MDA formation. It can be observed that the antiperoxidative action of α-tocopherol was 10-fold lower than that of equimolar concentrations of Silymarin. After a time lag of 4 min, rat liver mitochondria suspended in isoosmotic medium showed rapid swelling following the addition of Fe^{2+}-ascorbate (Fig. 3), and this can be related to peroxidative membrane alterations [20]. Preincubation of mitochondria in the presence of Silymarin completely inhibited swelling at 10 μM concentration (Fig. 3). In this experiment also, the protective action of Silymarin was 10-fold higher than that exhibited by α-tocopherol.

All the above reported results (inhibition of oxygen uptake, MDA formation and mitochondrial swelling induced by Fe^{2+}-ascorbate) concordantly demonstrate that Silymarin explicates a strong antiperoxidative action. The reasonable possibility that the antiperoxidative action of Silymarin was due simply to an interaction of the drug with added, or exogenous, Fe^{2+} seems untenable in the light of the results reported in Fig. 4, which shows that Fe^{2+} exhibited

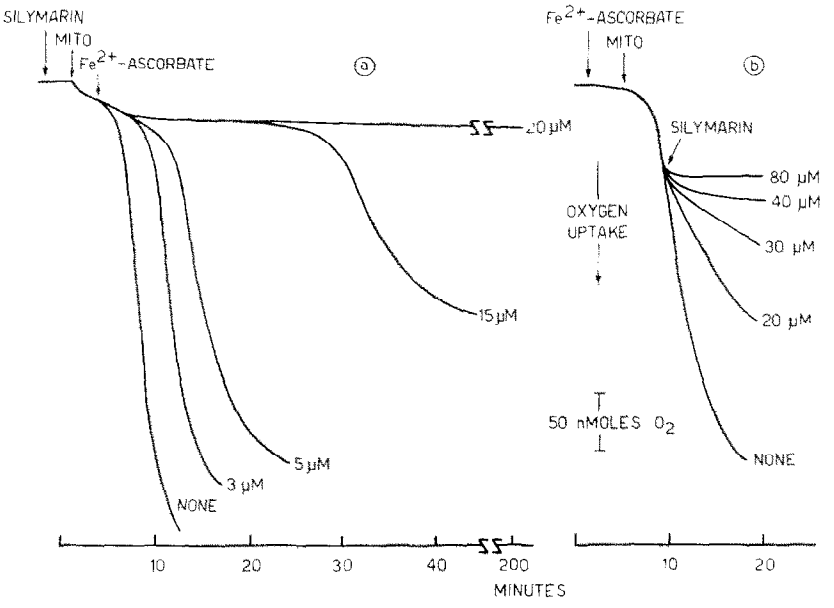

Fig. 1. Effect of Silymarin on Fe^{2+}-ascorbate induced oxygen uptake by rat liver mitochondria. The assay system, consisting of 125 mM KCl and 25 mM Tris-buffer, pH 7.4, contained 0.65 mg/ml of mitochondrial protein in a final volume of 2 ml. When indicated, 10 μM $FeSO_4$ and 200 μM ascorbate were added. Silymarin was added at the concentrations indicated for each trace.

its maximum peroxidative effect when added to rat liver mitochondria at 20 μM concentration. At higher concentrations, induced peroxide formation rapidly slowed down, probably due to the antiperoxidative effect of excess Fe^{2+} [21]. Preincubation of rat liver mitochondria in the presence of 100 μM Silymarin, followed by repeated washings in order to eliminate excess drug, showed an almost complete inhibition of MDA formation at all Fe^{2+} concentrations.

Silymarin also prevented peroxide formation in microsomes (Fig. 5), where peroxide formation was induced by the NADPH-Fe^{3+}-ADP system [13]. It can be observed that the concentration of Silymarin required for preventing peroxide formation in microsomes was approximately the same as in mitochondria, despite the different system used for inducing peroxidation.

The results reported in Table 1, which compares the two peroxidative systems, show that Fe^{2+}-ascorbate was very active with mitochondria and poorly active with microsomes, while the NADPH-Fe^{3+}-ADP system was very active in microsomes and poorly active in mitochondria. In spite of this difference, Silymarin was effective in inhibiting lipoperox-

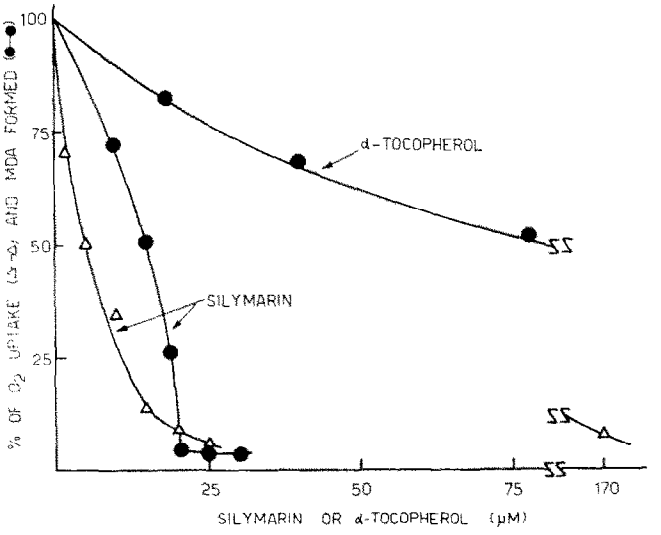

Fig. 2. Effect of Silymarin and α-tocopherol on Fe^{2+}-ascorbate induced peroxidation of rat liver mitochondria. Experimental conditions as in Fig. 1. In all experiments Silymarin or α-tocopherol were added at the indicated concentrations 2 min before addition of 10 μM $FeSO_4$ and 200 μM ascorbate. MDA formation and O_2 uptake (nmoles/min/mg protein) are reported as per cent of the control.

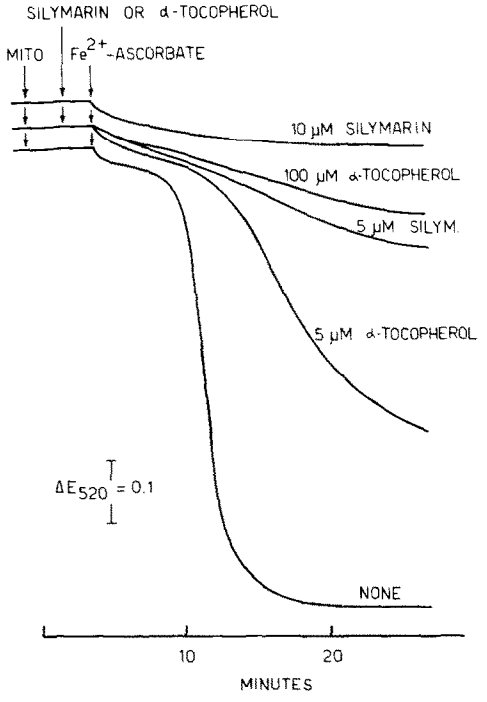

Fig. 3. Inhibitory effect of Silymarin and α-tocopherol on Fe^{2+}-ascorbate-induced swelling of rat liver mitochondria. Assay system as in Fig. 1 except mitochondrial protein was 0.4 mg/ml. Silymarin or α-tocopherol were added 2 min before addition of 10 μM $FeSO_4$ and 200 μM ascorbate at the concentrations indicated for each trace.

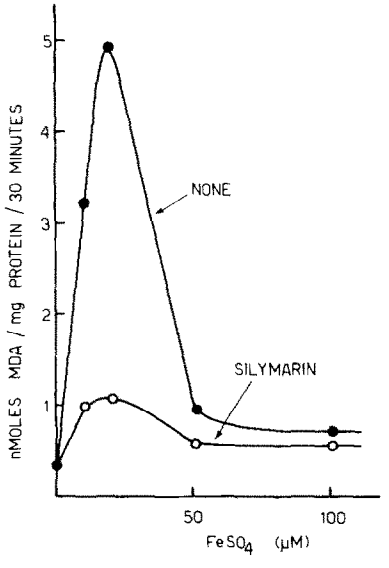

Fig. 4. Lipoperoxidation induced by increasing amounts of ferrous ions on rat liver mitochondria pretreated with Silymarin. Preincubation of rat liver mitochondria with 0.1 mM Silymarin (absent in the control) was carried out in 125 mM KCl buffered with 25 mM Tris pH 7.4 at 25° with 5 mg protein per ml; after 20 min mitochondria were rapidly centrifuged, washed twice, resuspended and diluted to a concentration of 1 mg protein per ml in the same medium. The indicated concentrations of ferrous ions sparked the lipoperoxidative reaction in aliquots of this suspension; MDA was determined after 30 min.

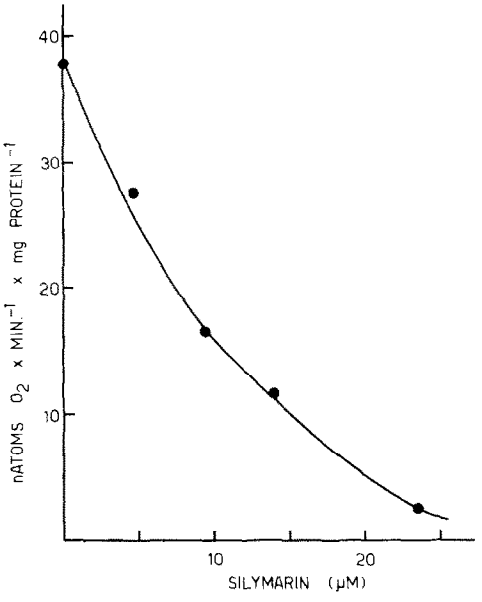

Fig. 5. Effect of Silymarin on NADPH-Fe^{3+}-ADP induced oxygen uptake by rat liver microsomes. The assay system, consisting of 125 mM KCl and 25 mM Tris-buffer pH 7.4, contained 1.7 mg/ml of microsomal protein, 0.18 mM NADPH, 12 μM $FeCl_3$, and 1 mM ADP in a final volume of 2 ml. Silymarin was added at the indicated concentrations 2 min before the addition of the lipoperoxidizing system NADPH-Fe^{3+}-ADP.

ide formation in all cases. This observation provides further evidence against a possible interaction of Silymarin with the lipoperoxide generating system.

As shown in Fig. 6, Silymarin also inhibited lipid peroxidation induced in microsomes by 0.1 mM

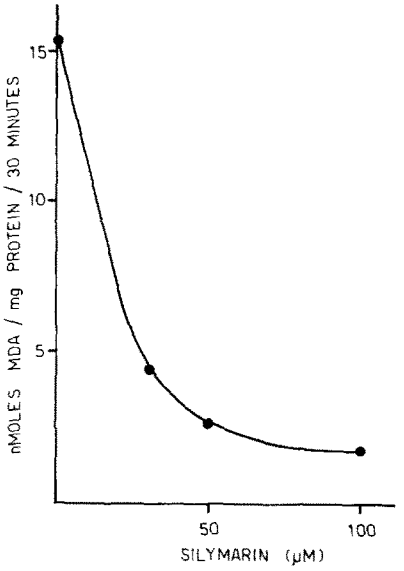

Fig. 6. Effect of Silymarin on cumene hydroperoxide induced lipid peroxidation in rat liver microsomes. The assay system, consisting of 125 mM KCl and 25 mM Tris-buffer pH 7.4, contained 1.2 mg/ml of microsomal protein and 0.1 mM cumene hydroperoxide in a final volume of 2 ml. Silymarin was added at the indicated concentrations 2 min before cumene peroxide.

Table 1. Silymarin inhibition of mitochondrial and microsomal lipo-peroxidation induced by different systems*

	$FeSO_4$-ascorbate		NADPH-$FeCl_3$-ADP	
	Mitochondria	Microsomes	Mitochondria	Microsomes
None	39.63	3.11	7.93	42.79
Silymarin	0.32	0.30	1.29	0.95

* The reaction mixture contained a basic medium composed of 125 mM KCl, 25 mM Tris pH 7.4, and 1 mg protein per ml of mitochondria or microsomes. Other reagents were 10 μM $FeSO_4$, 200 μM ascorbate, 200 μM NADPH, 10 μM $FeCl_3$ and 1 mM ADP. Silymarin was added at a final concentration of 50 μM, 2 min before the addition of the peroxidizing system. Reaction was carried out for 30 min at 25° with constant shaking and the values are expressed as nmoles of MDA per mg protein.

cumene hydroperoxide. It should be underlined that at all the concentrations employed this reagent failed to promote any lipid peroxidation in mitochondria (results not reported).

DISCUSSION

Based mostly on *in vitro* experiments carried out on red blood cells [8] and on isolated hepatocytes [9], the protective pharmacological action of Silymarin on liver has been attributed to its "stabilizing" action of the structure of biological membranes. However, the mechanism of this stabilizing action has not been determined.

The results reported in the present paper provide unequivocal evidence that Silymarin exerts a remarkable antioxidant action both on liver mitochondria and microsomes when these are exposed to a peroxidative system.

In liver mitochondria, the antioxidant action of Silymarin is demonstrated by the concomitant inhibition of Fe^{2+}-ascorbate-induced swelling, oxygen uptake, and MDA formation, and all these parameters are an expression of lipid peroxide formation. Silymarin was able to prevent peroxide formation (Fig. 1a) as well as inhibit it when added to the system after the process had started (Fig. 1b). As known, mitochondrial swelling induced by peroxidants [21] reflects irreversible alterations in membrane structure caused by lipid peroxidation. Since Silymarin inhibits lipid peroxidation (oxygen uptake and MDA formation) to exactly the same degree as it inhibits mitochondria swelling (compare Figs 2 and 3) it seems reasonable to assume that the "stabilizing" action of Silymarin is a consequence of its antioxidant effect. Moreover, the antioxidant action of Silymarin is about 10-fold higher than that of equimolar concentrations of α-tocopherol (see Fig. 2).

An antioxidant action for other flavonoids has already been described [22], as a possible consequence of their capacity to chelate metal ions, and in particular, Fe or Cu. Although chelation of added or endogenous Fe cannot be excluded in absolute also for Silymarin, the results reported in Fig. 4 indicate that this possibility is improbable. In fact, the antiperoxidative action of Silymarin persisted even after the pre-treated mitochondria were repeatedly washed, and then placed in contact with amounts of Fe^{2+} far in excess of the original Silymarin concentration. It appears very improbable that the residual Silymarin could chelate all the Fe^{2+} ions added to the system successively.

A further indication that there is no interaction between Silymarin and added Fe^{2+} is provided by the results obtained with cumene hydroperoxide (Fig. 6). The peroxidation induced by this reagent, which has been observed only with microsomes and seems to be dependent on cytochrome P-450 [23], does not require any added Fe^{2+}. The possible involvement of cytochrome P-450 in Silymarin action which could be reasonably inferred from this experiment, is in line with the previous assumption that Silybin, a component of Silymarin, could inhibit the formation of drug-cytochrome P-450 complex [24].

The results reported in Fig. 4 suggest, as previously reported by Ramellini and Meldolesi for isolated liver hepatocytes [9], that Silymarin binds to the mitochondrial membranes so that these become less susceptible to the deleterious action of oxygen radicals. In other words, our results are compatible with the assumption that the protective action of Silymarin involves an interaction of the drug with radical species critical for lipid peroxidation. If this is the case, Silymarin may be included in the class of free radical scavengers.

Whether Silymarin acts as radical scavenger and, or by protecting membrane lipids from the deleterious effect of free radicals is currently under investigation.

It appears improbable that the described antiperoxidative action of Silymarin could be the only explanation for the *in vivo* protective effect of this drug against the hepatotoxicity of different drugs [2–6]. For instance as far as galactosamine is concerned, this monosaccharide induces on rat liver microsomes a slight and scarcely significant lipid peroxidation only at concentrations (5 mM: results not reported) much higher than those presumably active *in vivo*.

Therefore the unravelling of the mechanism involved in the *in vivo* antagonism of Silymarin on galactosamine effects, will probably depend on the clarification of the very complex and not yet fully understood nature of galactosamine hepatotoxicity [25].

Acknowledgement—The secretarial work of Mrs. Maurizia Cuccia is gratefully acknowledged.

REFERENCES

1. B. Janiak and R. Hänsel, *Planta Medica* **8**, 71 (1960).
2. G. Vogel, *Arzneimittel-Forsch.* **18**, 1063 (1968).

3. G. Vogel and J. Temme, *Arzneimittel-Forsch.* **19**, 613 (1969).
4. H. Schriewer and H. M. Rauen, *Arzneimittel-Forsch.* **23**, 159 (1973).
5. H. Schriewer, R. Badde, G. Roth and H. M. Rauen, *Arzneimittel-Forsch.* **23**, 160 (1973).
6. H. M. Rauen and H. Schriewer, *Arzneimittel-Forsch.* **23**, 148 (1973).
7. H. W. Leber and S. Knauff, *Arzneimittel-Forsch.* **26**, 1603 (1976).
8. R. Seeger, *Arzneimittel-Forsch.* **21**, 1599 (1971).
9. G. Ramellini and J. Meldolesi, *Arzneimittel-Forsch.* **26**, 69 (1976).
10. T. F. Slater, *Nature, Lond.* **209**, 36 (1966).
11. H. M. Rauen, H. Schriewer and H. Fels, *Arzneimittel-Forsch.* **23**, 141 (1973).
12. D. K. Myers and E. C. Slater, *Biochem. J.* **67**, 558 (1957).
13. L. Ernster and K. Nordenbrand, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman) Vol. 10, p. 574. Academic Press, New York and London (1967).
14. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
15. R. W. Estabrook, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman) Vol. 10, p. 41. Academic Press, New York and London (1967).
16. L. Wojtczak and A. L. Lehninger, *Biochim. biophys. Acta* **51**, 442 (1961).
17. K. M. Wilbur, F. Bernheim and O. W. Shapiro, *Archs Biochem. Biophys.* **24**, 305 (1949).
18. A. Ottolenghi, *Archs Biochem. Biophys.* **79**, 355 (1959).
19. R. O. Sinnhuber and T. C. Yu, *Food Technol.* **12**, 9 (1958).
20. S. R. Fortney and W. S. Lynn, Jr., *Archs Biochem. Biophys.* **104**, 241 (1964).
21. F. E. Hunter, Jr., J. M. Gebicki, P. E. Hoffsten, J. Weinstein and A. Scott, *J. biol. Chem.* **238**, 828 (1963).
22. D. E. Pratt and B. M. Watts, *J. Food Sci.* **29**, 27 (1964).
23. E. G. Hrycay, J. A. Gustafsson, M. Ingelman-Sundberg and L. Ernster, *Eur. J. Biochem.* **61**, 43 (1976).
24. W. H. Down, in *Symposium on the Pharmacodynamics of Silymarin* (Eds R. Braatz and C. C. Schneider) p. 119. Urban and Schwarzenberg, Munchen-Berlin-Wien (1976).
25. A. M. Gressner and H. Greiling, *FEBS Lett.* **74**, 77 (1977).