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Effect of Silymarin on Different Acute Inflammation Models and on Leukocyte Migration

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Abstract

In-vivo anti-inflammatory activity of silymarin was tested in different acute inflammation experimental models. In carrageenan-induced paw oedema in rats, silymarin given orally reduced in a dose-dependent manner the food-pad abscesses (ED50 = 62·42 mg kg^{-1}). In xylene-induced ear mouse inflammation, silymarin applied topically was more effective than administered intraperitoneally, with effects comparable with those of indomethacin. Silymarin also produced a dose-dependent inhibition of leukocyte accumulation in inflammatory exudates following intraperitoneal injection of carrageenan in mice; silymarin significantly reduced the number of neutrophils. Silymarin was unable to inhibit phospholipase A_2 in an in-vitro assay.

Besides its known anti-oxidative properties and its ability to act as a radical scavenger, these results suggest that silymarin exerts an important anti-inflammatory action in-vivo by reducing oedema with the effect markedly influenced by the inhibition of neutrophil migration into the inflamed site.

Silymarin, a standardized extract of flavonolignans from *Silybum marianum* seeds, is widely used in Europe for the treatment of liver disorders. Silybin is the main component of silymarin but the isomers silychristin and silydianin are also present in the extract.

Silymarin is capable of protecting liver cells directly by stabilizing the membrane structures, including endoplasmic reticulum. This effect is mainly due to the inhibition of lipid peroxidation on biological membranes by its capability of reaction with numerous toxic oxygen free radicals producing more stable and less reactive compounds. Free radicals are recognized to have an important role in pathological processes such as inflammation. In this paper we investigate the in-vivo effect of silymarin on experimental acute inflammation.

Materials and Methods

Carrageenan-induced paw oedema
Oedema was induced in male Wistar rats, 180–200 g, according to the method described by Winter et al (1962). Sterile carrageenan in saline (0·1 mL, 1%) was injected into the plantar side of the right hind paws of groups of eight animals. Oral doses of silymarin were selected to reach the approximate ED50 (25, 50, 75 and 100 mg kg^{-1}; 10 mL kg^{-1}) and were administered 1 h before the injection of carrageenan. Control animals received the vehicle (0·8% Tween 80 in saline). Paw volumes were measured by means of a plethysmometer before the administration of carrageenan and 3 and 5 h later. The increase in volume was taken as the volume of oedema. Results were compared with those obtained in the control group. The reference compound was indomethacin (25 mg kg^{-1}, 10 mL kg^{-1}). ED50 was calculated 3 h after injection of carrageenan.

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Mouse ear oedema
This inflammatory response was induced as described by Tang et al (1984). Swiss mice, 20–25 g, were divided into groups of six. Test products were administered orally and intraperitoneally. In both cases the phlogistic agent, xylene, was applied with an automatic pipette to the right ear in a volume of 30 mL (15 mL to each inner and outer surface). For topical application, drugs were dissolved in acetone and applied in the same volume to the right ear. Left ears received acetone only. For intraperitoneal administration the vehicle was Tween 80 (0·8% in saline) and injection of the test compound followed 30 min after xylene application.

Inflammation was allowed to develop for 2 h, after which the animals were killed by cervical dislocation and a section (6 mm diameter) through the central portion of both ears was obtained and weighed. The swelling induced by xylene was assessed as the increase in the weight of the right ear punch biopsy over that of the left ear.

Mouse carrageenan peritonitis
Inflammation was induced by a modification of the technique of Griswold et al (1987). Drugs or vehicle were administered orally to groups of six Swiss mice. Carrageenan (0·25 mL, 0·75% in saline) was injected intraperitoneally 1 h later, and after 4 h the animals were killed. Two millilitres of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) was then injected into the peritoneal cavity. Following a gentle massage, peritoneal exudates were removed and total leukocytes determined on a Neubauer chamber and the differential cell count determined by microscopic counting of Giemsa- and May-Grünwald-stained slides.

Phospholipase A_2 assay
Phospholipase A_2 was assayed following the method of Ferrandiz et al (1994). Membranes of *Escherichia coli* were labelled with [^{3}H]oleate. *E.coli* strain CECT 101 were seeded in medium containing 1% tryptone, 0·5% NaCl and 0·6%

sodium dihydrogen orthophosphate pH 5·0 and grown for approximately 8 h at 37°C in the presence of 5 mCi mL^{-1} [^{3}H]oleic acid (spec. act. 10 Ci $mmol^{-1}$) until growth approached the end of the logarithmic phase. After centrifugation at 2500 *g* for 10 min, the membranes were washed in buffer (0·7 M Tris-HCl, 10 mM $CaCl_2$, 0·1% bovine serum albumin, pH 8·0), resuspended in saline and autoclaved for 30–45 min. The membranes were then washed, centrifuged again and frozen at −70°C. At least 95% of the radioactivity was incorporated into phospholipids. Two secretory enzymes were assayed, *Naja naja* and bee venom enzymes, which were diluted in 10 mL 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer pH 7·5 and preincubated at 37°C for 10 min with 2·5 mL test compound solution or its vehicle (methanol). Incubation proceeded for 10 min in the presence of 20 mL autoclaved oleate-labelled membranes and was terminated by addition of 100 mL ice-cold 0.25% bovine serum albumin in saline to a final concentration of 0·07% w/v. After centrifugation at 2500 *g* for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting. Control tubes contained enzyme and the inhibitor vehicle.

Materials

Silymarin was a gift from Madaus Cerafarm Laboratory (Spain). The relative amounts of the three constituents of silymarin were 70% silybin, 17% silydianin and 13% silychristin. All doses were refered to the silybin content, the main active flavonolignane of the mixture. Carrageenan type IV, indomethacin, *Naja naja* and bee venom enzymes and mepacrine were purchased from Sigma Chemicals. Tween 80, xylene and acetone were from Panreac Chemical (Spain). [9,10-^{3}H]Oleic acid was obtained from Du Pont (Itisa, Madrid, Spain).

Statistical analysis

Results are presented as means ± s.e.m. from three determinations. Data were analysed for significance using Student's *t*-test and ED50 (the dose which caused 50% inhibition of the inflammatory response in-vivo) was calculated using linear regression analysis.

Results

The acute inflammation induced by carrageenan was inhibited by oral administration of silymarin in a dose-dependent fashion (Table 1). The inhibition values were similar at 3 and 5 h. The ED50 was 62·42 mg kg^{-1} calculated 3 h after carrageenan administration.

The effect of silymarin on xylene-induced ear inflammation was different depending on the route of administration, being more effective applied topically (Table 2) than administered intraperitoneally (Table 3). When the topical route was assessed, silymarin showed a higher inhibition (44·52%) than indomethacin (35·96%) at the same dose (0·5 mg/ear). The ED50 value was 1 mg/ear for the topical silymarin application.

Silymarin inhibited leukocyte infiltration induced by carrageenan in mice in a dose-related manner (Table 4). High doses (75 and 100 mg kg^{-1}) of this compound significantly decreased total leukocyte number (ED50 = 73·34 mg kg^{-1}). Migration of neutrophils was also drastically reduced in this peritonitis model.

Table 1. Effect of silymarin on carrageenan-induced hind-paw oedema in rats (n = 8).

Treatment (mg kg^{-1})	Inhibition (% mean ± s.e.m.)	
	3h	5h
Silymarin		
25	30·20 ± 5·23*	31·25 ± 3·19*
50	45·04 ± 3·25**	43·77 ± 4·11**
75	56·38 ± 3·74**	64·83 ± 7·23***
100	68·95 ± 7·94***	58·11 ± 4·22**
Indomethacin		
25	89·89 ± 8·23***	75·71 ± 9·02***

*P < 0·05; **P < 0·01; ***P < 0·001 compared with control.

Table 2. Effect of topical silymarin on xylene-induced ear oedema in mice (n = 6).

Treatment (mg ear^{-1})	Ear swelling (mg)	Inhibition (%)
Control	4·56 ± 0·12	–
Indomethacin		
0·5	2·92 ± 0·38**	35·96
Silymarin		
0·5	2·53 ± 0·40**	44·52
1·0	2·26 ± 0·25***	50·44
2·0	1·95 ± 0·31***	57·24

**P < 0·01; ***P < 0·001 compared with control.

Table 3. Effect of intraperitoneal silymarin administration on xylene-induced ear oedema in mice (n = 6).

Treatment (mg kg^{-1})	Ear swelling (mg)	Inhibition (%)
Control	4·45 ± 0·07	–
Indomethacin		
50	2·62 ± 0·24***	41·12
Silymarin		
50	3·90 ± 0·19	12·36
75	3·58 ± 0·18*	19·35
100	3·36 ± 0·30**	24·49

*P < 0·05; **P < 0·01; ***P < 0·001 compared with control.

Table 4. Effect of silymarin and indomethacin on carrageenan induced cellular infiltration.

Treatment (mg kg^{-1})	Leukocytes ($\times 10^5$ mL^{-1})	Inhibition (%)	Neutrophils ($\times 10^5$ mL^{-1})	Inhibition (%)
Control	8.67 ± 0.94	–	5.21 ± 0.30	–
Indomethacin 10	5.62 ± 0.82*	35.18	1.94 ± 0.14***	62.76
Silymarin 50	5.93 ± 0.32*	31.60	0.97 ± 0.06***	81.38
Silymarin 75	4.46 ± 0.08***	48.56	0.67 ± 0.04***	87.14
Silymarin 100	3.10 ± 0.34***	64.24	0.52 ± 0.01***	90.02

*P < 0·05; ***P < 0·001 compared with control.

Table 5. Effect of silymarin on the enzymes *Naja naja* and bee venom phospholipase A_2.

Enzyme	Compound (M)	Inhibition (%)
Naja naja PLA_2	Silymarin (10^{-2})	–
	Mepacrine (10^{-3})	68.11 ± 2.40**
Bee venom PLA_2	Silymarin (10^{-2})	–
	Mepacrine (10^{-3})	65.38 ± 2.97**

**$P < 0.01$ compared with control.

As shown in Table 5, silymarin was active on neither *Naja naja* nor bee venom phospholipase A_2.

Discussion

Silymarin was moderately active against oedema induced by carrageenan, while the reference compound indomethacin showed higher anti-oedematous potency. Calculated regression line from these data yielded an ED50 value of 62·49 mg kg^{-1}, which was similar to that obtained for other flavonoids in this type of assay (Villar et al 1984).

Carrageenan-induced oedema is commonly used as an experimental animal model of acute inflammation and is believed to be biphasic. The first phase is due to the release of histamine and 5-hydroxytryptamine; the second phase is caused by the release of bradykinin, protease, prostaglandin and lysosome (Vinegar et al 1987). Silymarin seems to produce an inhibition of the release of mediators involved in both phases of inflammation.

It is not surprising that, in the xylene-induced ear inflammation, silymarin showed a better anti-oedema effect after topical administration because much higher concentration of drug can be attained topically in this experimental model, and drug metabolism and excretion are avoided by this route of administration.

Xylene produces an increase on the capillary permeability due to histamine and 5-hydroxytryptamine release (Creasy et al 1971). It is likely that the anti-oedematous effect of silymarin against this type of irritant is related to the inhibition of production of these inflammatory mediators. Moreover, the reduction of histamine release by silymarin has been demonstrated previously in human basophil leukocytes in an in-vitro assay (Miadonna et al 1987).

Leukocyte aggregation at the site of inflammation is a fundamental event in the inflammatory process. Silymarin exerted a great inhibition on the leukocyte migration induced by carrageenan in mice. In this assay, silymarin was a more potent inhibitor of the infiltration of neutrophils than indomethacin at the assayed dose (10 mg kg^{-1}), as the non-steroidal anti-inflammatory agents such as indomethacin, are far less effective in preventing cell infiltration into inflammatory lesions, than agents that inhibit both enzyme pathways of arachidonate metabolism (cyclooxygenase and lipoxygenase) (Griswold et al 1987). It has also been reported that silymarin is able to inhibit the lipoxygenase (Fiebrich & Koch 1979a) and the cyclooxygenase (Fiebrich & Koch 1979b) enzymes in different in-vitro assays.

The presence of elevated levels of PLA_2 in inflamed tissues and in a variety of experimental models suggests a direct role in inflammation. The release of arachidonic acid from phospholipids is a rate-limiting step for the formation of arachidonic acid metabolites in polymorphonuclear leukocytes and depends mainly on PLA_2 activity (Meade et al 1986). Silymarin was unable to inhibit this enzyme in this type of in-vitro assay.

Our findings suggest that silymarin produces an important in-vivo anti-inflammatory effect. The inhibition of the leukocyte migration into the inflamed site plays a key role in its mechanism of action. The effects of silymarin may depend upon its ability to behave as a free-radical scavenger in the context of the lipid peroxide intermediates which are involved as initiators of the cyclooxygenase and lipoxygenase reactions. These preliminary results indicate that silymarin may offer promise for the therapy of inflammation besides acting as a hepatoprotective agent.

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References

Creasy, N. H., Allenby, A. C., Schock, C. (1971) Mechanism of cutaneous applied penetration accelerants on the skin circulation of the rat. Br. J. Dermatol. 85: 368–380
Ferrandiz, M. L., Sánz, M. J., Bustos, G., Payá, M., Alcaraz, M. J., De Rosa, S. (1994) Avarol and avarone, two new antiinflammatory agents of marine origin. Eur. J. Pharmacol. 253: 75–82
Fiebrich, F., Koch, H. (1979a) Silymarin, an inhibitor of lipoxygenase. Experientia 35: 1548–1550
Fiebrich, F., Koch, H. (1979b) Silymarin, an inhibitor of prostaglandin synthetase. Experientia 35: 1550–1552
Griswold, D. E., Marsh, P. J., Webb, E. F., Godfrey, R., Newton, J., Dimartino, M. J., Sarau, H. M., Gleason, J. G., Poste, G., Hanna, N. (1987) SK&F86002: a structurally novel antiinflammatory agent that inhibits lipoxygenase and cyclooxygenase-mediated metabolism of arachidonic acid. Biochem. Pharmacol. 36: 3463–3470
Meade, C. J., Turner, G. A., Bateman, P. E. (1986) The role of polyphosphoinositides and their breakdown products in A23187-induced release of arachidonic acid from rabbit polymorphonuclear leukocytes. Biochem. J. 238: 425–436
Miadonna, A., Tedeschi, A., Leggieri, E., Lorini, M., Zanussi, C. (1987) Effects of silybin on histamine release from human basophil leukocytes. Br. J. Clin. Pharmacol. 24: 747–752
Tang, X-C., Lin, Z-G., Cai, W., Chen, N., Shen, L. (1984) Antiinflammatory effect of 3-acetylaconitine. Acta Pharmacol. Sinica 5: 85-89
Villar, A., Gasco, M. A., Alcaraz, M. J. (1984) Anti-inflammatory and anti-ulcer properties of hypolaetin-8-glucoside, a novel plant flavonoid. J. Pharm. Pharmacol. 36: 820–823
Vinegar, R., Truax, J. F., Selph, J. L., Johnston, P. R., Venable, A. L., McKenzie, K. K. (1987) Pathway to carrageenan-induced inflammation in the hind limb of the rat. Fed. Proc. 46: 118-126
Winter, C. A., Risley, E. A., Nuss, G. W. (1962) Carrageenan-induced oedema in hind paw of the rats as an assay for anti-inflammatory drugs. Proc. Soc. Biol. Med. 11: 544–547