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Full Length Research Paper

Dose-dependent anti-inflammatory effect of silymarin in experimental animal model of chronic inflammation

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Silymarin is a polyphenolic flavonoid derived from milk thistle (*Silybum marianum*) that has anti-inflammatory, cytoprotective, and anticarcinogenic effects. It has been used medicinally to treat liver disorders including acute and chronic viral hepatitis, toxin/drug induced hepatitis and alcoholic liver disease. The efficacy and dose-response effect of *silymarin* (125, 250 and 500 mg/kg) were assessed against negative and positive control using formalin-induced paw edema in rats as a model of chronic inflammation. In this model, all doses of *silymarin* were given intraperitonealy (IP) 30 min before induction of inflammation and continued for 7 consecutive days. Paw edema was measured before and 6 days after induction of inflammation using vernier caliper method and balance method. *Silymarin* in 250 and 500 mg/kg significantly lowered paw edema (P < 0.05) in both methods and found to be comparable with that produced by the reference drug dexamethazone and significantly different from that produced by acetyl salicylic acid and *silymarin* 125 mg/kg (P < 0.05). Therefore, silymarin exerts anti-inflammatory activity in rat model of chronic inflammation which was significantly increased as the dose increased up to 500 mg/kg.

Keywords: *Silymarin*, chronic inflammation.

INTRODUCTION

Inflammation is an important physiological reaction, which occurs in response to a wide variety of injurious agents (bacterial infection or physical trauma) ultimately aiming to perform the dual function of limiting damage and promoting tissue repair (Nathan, 2002). It requires the participation of various cell types expressing and reacting to diverse mediators along a very precise sequence (Gouwy et al., 2005). Although inflammation is beneficial in the setting of defense of the host against infectious invaders, it may become unchecked contributing to the pathogenesis of common chronic inflammatory diseases such as atherosclerosis, obesity induced insulin resistance, arthritis, inflammatory bowel disease and multiple sclerosis (Wellen and Hotamisligil, 2005; Hanauer, 2006). Chronic inflammation begins 2 -4 days after the onset of acute response and can last for weeks to months or years due to the persistence of the initiating stimulus, interference of the normal healing process, repeated bouts of acute inflamemation or low-grade smoldering due to continued production of immune response mediators (Whicher and Chambers, 1984). Silymarin is a group of plant-derived flavornoids extracted from the fruits and seeds of the milk thistle (*Silybum marianum* L. Gaertn, F. Asteraceae). Chemically, six main active constituents of silymarin have been separated by using a combination of liquid chromatography and electrospray ionization mass spectrometry, and include silybin A, silybin B, isosilybin A, isosilybin B, silychristin and silydianin as shown in Figure 1 (Lee et al., 2007). Silymarin has been primarily used in liver disorders including hepatitis, alcoholic liver diseases (ALD) and cirrhosis (Ferenci et al., 1989; Saller et al., 2001) and is also useful for toxin-induced liver toxicity including poisoning from a fungus called death cap mushroom (Amanita Phalloides) (Pepping, 1999).

The *in vivo* anti-inflammatory activity of silymarin was tested in different experimental models of inflammation, and the results suggested that an important anti-inflammatory action was achieved by inhibition of neutrophils migration into the

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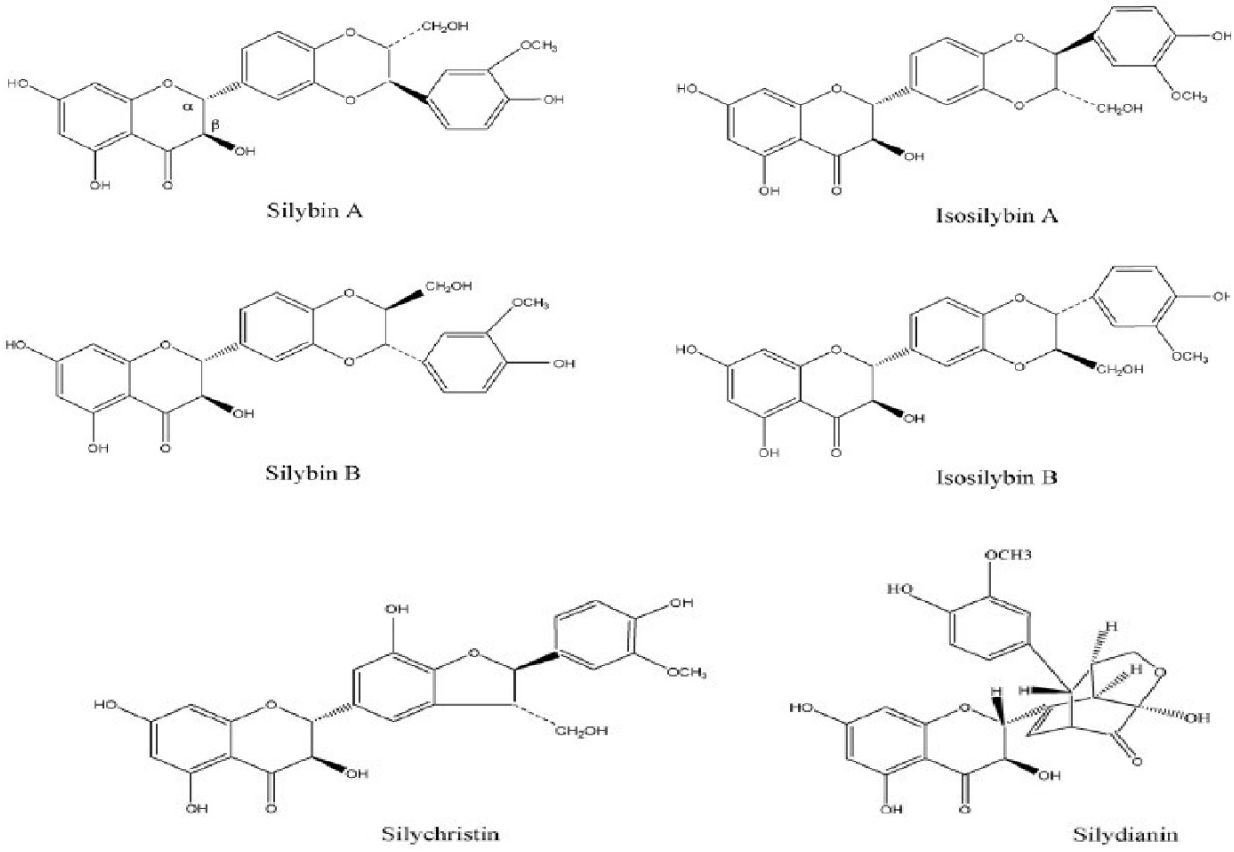

Figure 1. Structures of the main active constituents of silymarin, diastereomers of silybin (silybin A and B), diastereomers of isosilybin (isosilybin A and B), silychristin and silydianin (Lee et al., 2007).

inflamed site, which lead to the release of ROS, RNS and proteolytic enzymes resulting in microvascular endothelial injury, increase endothelial barrier permeability and edema (De la Puerta et al., 1996).

Most of these effects have been attributed to direct and/or indirect antioxidant capacity of silymarin, such as being a scavenger of reactive oxygen species (ROS), phenylglyoxylic ketyl radicals and a chain breaking antioxidant (Luper, 1998). The present study was designed to evaluate the efficacy and dose-response effect of silymarin in experimental animal model of chronic inflammation.

MATERIALS AND METHODS

Silymarin, as a crude powder (Luna Co, Egypt) extracted from seeds of the Milk Thistle, was dissolved in 98% dimethyl sulfoxide (Merk Co, Germany) to produce a stock solution of 250 mg/ml, from which different doses prepared according to the body weight of the animals. Sprague- Dawley rats weighing 180-220 g of both sexes (24 female and 12 male) were purchased from the National Centre for Drug Research and Quality Control, Baghdad. They were kept in the animal house of the department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad at 25 ± 2°C, relative humidity 60-70%, and light: dark cycle of 12: 12 h for 1 week before starting experiments. Animals were housed in groups of 6 in stainless steel cages and provided with standard rodent pellet diet (GAFCO, Tema) and the food was withdrawn 12 h before the experiment, water was allowed ad libitum. All experiments were performed according to the guidelines of laboratory animals' care and the ethical guidelines for the investigations on experimental animals. In the present study, 36 rats were allocated into 6 groups (6 rats each) and treated as follow: First group treated with dimethyl sulfoxide 2 ml/kg served as control; second and third groups were treated with dexamethazone (American reagent, USA) 1 mg/kg and acetyl salicylic acid (Sanofi, France) 100 mg/kg respectively, served as standard comparators; the other three groups were treated with 125, 250 and 500 mg/kg silymarin respectively, given by IP injection.

Formalin-induced chronic inflammation

The effect of silymarin in chronic inflammation was evaluated byformalin-induced paw edema (Chau, 1989). In this model, chronic inflammation was induced by injecting 0.1 ml of 2% formalin (Al-Jubail, Saudi Arabia) into the sub planter area of the right hind paw of ether anaesthetized rats. All drugs including silymarin (125, 250 and 500 mg/kg), acetyl salicylic acid 100 mg/kg, dexamethazone 1 mg/kg and the vehicle dimethyl sulfoxide 2 ml/kg were given 30 min prior to formalin injection and continued for 7 consecutive days. Dexamethazone and dimethyl sulfoxide doses were given once daily while silymarin and acetyl salicylic acid were administered in two equal doses every 12 h. In this model, the increase in paw edema was measured by 2 methods; vernier caliper method (Joseph et al., 2005)

Table 1. Effect of different doses of silymarin evaluated by vernier caliper method on formalin-induced chronic inflammation in rats.

Treatment Groups	Mean increase in paw thickness (mm) after 6 days	% inhibition
Dimethyl sulfoxide 2 ml/kg	3.34 ± 0.08	_
Acetyl salicylic acid 100 mg/kg	3.01 ± 0.07*[a]	10
Dexamethazone 1 mg/kg	2.15 ± 0.07*[b]	36
Silymarin 125 mg/kg	2.91 ± 0.09*[a]	13
Silymarin 250 mg/kg	2.20 ± 0.05*[b]	34
Silymarin 500 mg/kg	2.23 ± 0.06*[b]	33

Data were expressed as mean ± SEM, Number of animals = 6 in each group. *P < 0.05 with respect to control group. Values with non-identical subscription (a, b) are considered significantly differ-rent (*P* < 0.05).

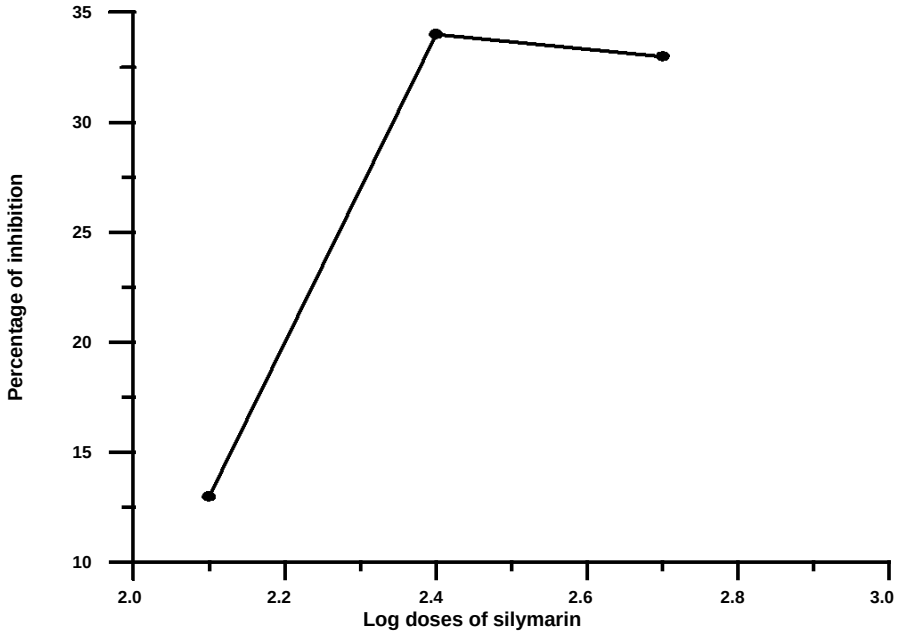

Figure 2. Dose-response relationship of different doses of silymarin evaluated by vernier caliper method on formalin-induced chronic inflammation in rats.

and balance method (Andreadou et al., 1992).

In vernier caliper method, the paw thickness was measured before and 6 days after induction of inflammation by using vernier caliper. The difference in paw thickness after and before induction of inflammation was calculated and presented as mean increase in paw thickness (mm). The ability of anti -inflammatory drug to suppress paw inflammation was expressed as a percentage of inhibittion of paw edema (Duffy et al., 2001) and this percentage can be calculated according to the following equation:

Percentage of inhibition (%) = 100 × (1 - X / Y)
Where X= mean increase in paw volume, thickness or weight of treated rats and Y= mean increase in paw volume, thickness or weight of control rats.

In balance method, the rats were killed by anesthetic ether and the two limbs (left and right paw) were excised by a cutter. The weight of the two limbs then recorded by the balance (where the left paw act as a control) and the difference between them represent mean increase in paw weight (mg). The percentage of inhibition then calculated as mentioned previously.

All results were expressed as mean ± SEM. The significance of differences between treated groups was determined using Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). P-values < 0.05 were considered significant

RESULTS

Evaluation using vernier caliper method

Using vernier caliper method, the suppressive effect of different doses of silymarin on formalin-induced chronic inflammation was shown in Table 1 and Figure 2. Silymarin (125, 250 and 500 mg/kg), acetyl salicylic acid and dexamethazone significantly attenuated progression of chronic inflamemation induced by formalin (P < 0.05) compared to control. Dexamethazone and silymarin (250 and 500 mg/kg) showed the same effect in this model, while 125 mg/kg silymarin produced less anti-inflammatory effect compared to dexamethazone. However, silymarin (125 mg/kg) showed better effect than acetyl salicylic acid in this model although statistically

Table 2. Effect of different doses of silymarin evaluated by balance method on formalin-induced chronic inflammation in rats.

Treatment Groups	Mean increase in paw weight (mg) after 6 days	% of inhibition
Dimethyl sulfoxide 2 ml/kg	260 ± 9.8	_
Acetyl salicylic acid 100 mg/kg	240 ± 18.2 [a]	8
Dexamethazone 1 mg/kg	168 ± 9.4* [b]	35
Silymarin 125 mg/kg	215 ± 9.7 [c]	17
Silymarin 250 mg/kg	190 ± 9.0* [d]	27
Silymarin 500 mg/kg	170 ± 8.9* [b]	35

Data were expressed as mean ± SEM.
Number of animals = 6 in each group.
*P < 0.05 with respect to control group.
Values with non-identical subscripts (a, b, c, d) are considered significantly different (P <0.05).

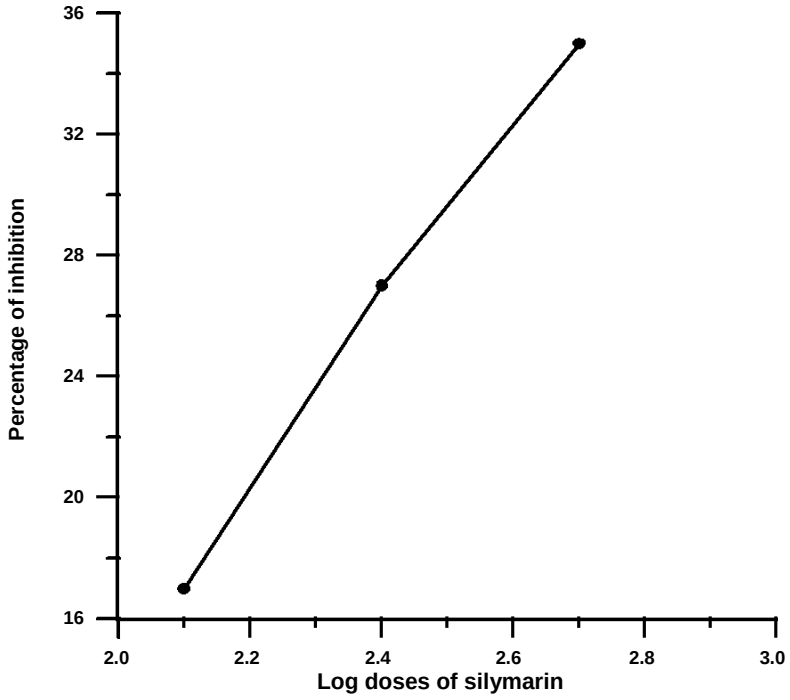

Figure 3. Dose-response relationship of different doses of silymarin evaluated by balance method on formalin-induced chronic inflammation in rats.

non significant. Dexametha-zone also produced better effect than acetyl Salicylic acid with highly significant differences. The percentage of inhibittion of paw edema was significantly increased (P < 0.05) as the dose of silymarin doubled from 125 to 250 mg/kg, while the dose 500 mg/kg results in an effect comparable to that produced by 250 mg/kg along assessment period.

Evaluation using the balance method

Using the balance method, suppressive effect of silimarin on paw edema in formalin-induced chronic inflammation was illustrated in Table 2 and Figure 3. Silymarin (125, 250 and 500 mg/kg) and dexamethazone significantly decreased the chronic inflammation induced by formalin (P<0.05), while acetyl salicylic acid produced non-signifi-cant changes in this model. Dexamethazone produced bet-ter effect than 125 and 250 mg/kg silymarin but comparable to that produced by 500 mg/kg silymarin. The dose-response relationship of silymarin in formalin-induced chronic inflammation model indicated that increasing the dose of silymarin from 125 to 500 mg/kg was associated with an increase in the percentage of inhibition of chronic inflammation.

DISCUSSION

In chronic inflammatory model, inhibition of formalin- induced pedal edema in rats is considered as one of the most suitable test procedures to screen chronic anti-inflamma-

tory agents as it closely resembles human arthritis (Greenwald, 1991). Inflammation induced by formalin is biphasic, an early neurogenic component is mediated by substance P and bradykinin followed by a later tissue-mediated response where histamine, 5-hydroxytryptamine, prostaglandins (PGs) and bradykinin are known to be involved (Wheeler-Aceto and Cowan, 1991).

Silymarin exerts a dose-dependent effect up to 250 mg /kg in vernier caliper method, while in balance method, its effect increased as the dose increased creating a linear relationship. This difference in activity of silymarin between two methods may be related to the nature of the method used, where in balance method cutting of rats paw may vary from one to another depending on the site of cutting; while in vernier caliper method the site of measurement mainly the same for all animals. Silymarin, at doses 6.25, 12.5 and 25 mg/kg when administered orally, reported to produce antiarthritic activity in Mycobacterial adjuvant-induced arthritis model in rats in a dose-dependent manner (Gupta et al, 2000). This effect was mediated through inhibition of the enzyme, 5- lipoxygenase (5-LOX), which is involved in inflammation. Meanwhile, silymarin prevents the development of arthritis by inhibiting enzymatic peroxidation, which involved in the arachidonic acid cascade and leads to the formation of PGs and leukotrienes involved in the development of arthritis. Application of this concept (dual inhibitory effect of both cyclooxygenase and 5-LOX) in the treatment of osteoarthritis resulted in a promising effects marking silymarin as a good candidate for the treatment of such disease (Numan et al., 2006). Recently, three main pathways have been implicated in inflammation induced cell death: inducible nitric oxide synthase (iNOS) and derivatives reactive nitrogen species (RNS), the phagocytic NAD (P) H oxidase (NOX) and derivatives ROS and arachidonate and derivatives prostanoids (Bosca et al., 2005; Cross and Segal, 2004). Maximal activation of each system alone causes little cell death, at least to the macrophages. On the other hand, if iNOS together with either NOX or arachidonate are activated at the same time peroxynitrite is produced, resulting in extensive peroxynitrite- mediated cell death (Borutaite et al., 2006). Peroxynitrite can kill cells by causing DNA damage that activates poly (ADP ribose) polymerase (PARP), and consistent with this they found that PARP inhibitors partially blocked cell death (Szabo, 2003). Therefore, the antioxidants, including silymarin, have an important role in reversing oxidative stress mediated cell death and may constitute a new strategy for the treat-ment of inflammatory and degenerative diseases.

The molecular bases of the anti-inflammatory and anti-carcinogenic effect of silibinin/silymarin might be related to the inhibition of the transcription factor nuclear factor kappa B (NF - B), which regulates and coordinates the expression of various genes involved in the inflammatory process, in cytoprotection and carcinogenesis (Monna et al., 1999; Polyak et al., 2007). In particular, NF- B contributes to the production of interleukin-1 (IL-1) and IL-6, tumor necrosis factor alpha (TNF-), lymphotoxin, granulocyte-macrophage colony-stimulating factor (GM-CS F) and interferon gamma (IFN-) (Barnes and Karin, 19 97). Furthermore, some of these cytokines, e.g., IL-1 and TNF- , activate NF- B themselves, thus creating positive feedback loop and interruption of this loop by silymarin might be responsible for its chronic anti-inflammatory activity (Monna et al., 1999). Like silymarin, the anti-inflam-matory drugs sodium salicylate and dexamethazone were also known to block the activation of NF- B by inhibiting I B phosphorylation and enhancing I B gene expression, respectively (Handel and Girgis, 2001). However, silymarin was effective at a 100-fold lower concentration than salicylate, suggesting that it is a potent inhibitor without substantial toxicity (Walterova and Kren, 2005). Silymarin also inhibited the TNF- -induced activation of mitogen activated protein kinase (MAPK) and Janus kinase (JNK) and abrogated TNF- -induced cytotoxicity and caspase activation. Blocking the activation of NF- B and the kinases may provide, in part, the molecular bases for the anticarcinogenic and anti-inflammatory effects of silymarin, and its effect on caspases may explain its role in cytoprotection (Monna et al., 1999).

Silymarin also has an important immunomodulatory function through inhibition of dendritic cell (DC) maturation under both *in vitro* and *in vivo* conditions (Lee et al., 2007) and also inhibit TNF- secretion by human T-cells tested freshly *in vitro* (Polyak et al., 2007). Thus, immunosuppression considered as a common feature between glucocorticoids, disease modifying antirheumatic drugs including methotrexate and silymarin, and this could explain the similarity in action between dexamethazone and silymarin especially at doses 250 and 500 mg/kg in chronic inflammatory model.

The inhibitory effect of silymarin on formalin-induced arthritis could support its anti-proliferative effect because this model also used to evaluate agents with the probable anti-proliferative activity. The anti-proliferative actions of silymarin converge on inhibition of signaling pathways that regulate the cell cycle including protein kinase B (Akt) and cyclin-dependent kinases (Singh and Agarwal, 2005). However, it is important to emphasize that the anti-proliferative action of silymarin has been described chiefly with one component of silymarin, silibinin, at relatively high doses (100-300 µmol/l or ca. 50-150 µg/ml) (Varghese et al., 2005). Moreover, silymarin is known to contain silybin A, silybin B, isosilybin A and isosilybin B (Lee and Liu, 2003). Thus it is possible that the anti-proliferative, anti-inflammatory, immunomodulatory and antiviral effects of silymarin may be dependent on the dose and the molecular form of silymarin and other molecules present in silymarin extract (MK-001) (Polyak et al., 2007). In conclusion, silymarin in a dose dependent pattern was effective in decreasing chronic inflammatory cascade in experimental animal models of chronic inflammation. Further work is required to evaluate the possible synergistic effect of silymarin doses, lower than 250 mg

/kg/day, on the anti-inflammatory activities of NSADs or steroids in animal models of chronic inflammation.

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