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# Selective inhibition of NF-κB activation by the flavonoid hepatoprotector silymarin in HepG2

## Evidence for different activating pathways

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**Abstract The bioflavonoid silymarin is found to potently suppress both nuclear factor kappa-B (NF-κB)-DNA binding activity and its dependent gene expression induced by okadaic acid in the hepatoma cell line HepG2. Surprisingly, tumor necrosis factor-α-induced NF-κB activation was not affected by silymarin, thus demonstrating a pathway-dependent inhibition by silymarin. Many genes encoding the proteins of the hepatic acute phase response are under the control of the transcription factor NF-κB, a key regulator in the inflammatory and immune reactions. Thus, the inhibitory effect of silymarin on NF-κB activation could be involved in its hepatoprotective property.**


*Key words:* Nuclear factor kappa-B; Antioxidant; Hepatocyte; Flavonoid; Silymarin; Inflammation

### 1. Introduction

Inflammatory reactions are triggered in many liver diseases, as the consequence of the introduction of a toxin, drug or infectious agent, to induce a repair process and to reestablish the original functions of the hepatic tissue. However, a failure to eliminate the noxious agent, in addition to the disruption of regulatory mechanisms, such as the ones controlling the resolution of the acute phase response, may lead to the development of chronic liver inflammation.

An inflammatory response depends on the de novo synthesis of many mediators, including regulatory proteins, which are produced upon an inducible gene expression. This gene expression is controlled by transcription factors which are activated by external inflammatory stimuli. The transcription factor nuclear factor kappa-B (NF-κB) has been suggested to play a key role in these reactions. The activation of NF-κB is itself induced by a variety of stimuli such as proinflammatory cytokines, phorbol esters, bacterial or viral products, phosphatase inhibitors, oxidants and ultraviolet radiation [1]. Evidence of the involvement of reactive oxygen intermediates in NF-κB activation has been presented as well [2]. Upon activation, the inhibitory protein IκB, sequestrating NF-κB in the cytosol, is phosphorylated and degraded. The inducible phosphorylation of IκB proteins generally occurs on two serines in their $NH_2$-terminal domain [3]. However, in certain cells under certain conditions, tyrosine phosphorylation of IκB has been demonstrated [4,5]. Following the IκB release, NF-κB translocates into the nucleus and binds to specific DNA motifs in the promoter region of genes whose product is implicated in inflammatory and immune responses [1]. Accordingly, controlling NF-κB activation has become a pharmacological challenge, particularly in the chronic inflammatory disorders [6].

Silymarin is a flavonoid blend extracted from the seeds of Lady's thistle (*Silybum marianum* (Gaertn.)). Its pharmacologically active components are the flavonolignans silibinin and its derivatives with silibinin as the primary element of the blend (Fig. 1). Silymarin has been clinically used for its beneficial effects on various liver diseases such as alcohol or drug intoxication, mushroom poisoning and viral hepatitis [7], whose pathogenesis involves an inflammatory response. The properties underlying its hepatoprotective effects are thought to be multiple: free radical scavenging activity, prevention of glutathione oxidation and depletion, membrane stabilizing effect, inhibition of arachidonic acid metabolism and increased protein synthesis by activation of RNA polymerase I [8–11].

The objective of the present study was to investigate whether silymarin can block NF-κB activation and its dependent gene expression induced by various stimuli in the human hepatoblastoma cell line HepG2 and to show its potential to inhibit the inflammatory response in liver disorders.

### 2. Materials and methods

#### 2.1. Materials

Eagle's Minimum Essential Medium and non-essential amino acids were obtained from Life Technologies (Gaithersburg, MD, USA). L-Glutamine, sodium pyruvate, penicillin and streptomycin were obtained from UCSF Cell Culture Facilities (San Francisco, CA, USA). Okadaic acid (ammonium salt) (OA) was obtained from Alexis (San Diego, CA, USA). Recombinant human tumor necrosis factor (TNFα) was generously provided by Genentech (South San Francisco, CA, USA). Silymarin and lipopolysaccharide from *Escherichia coli* serotype 055:B5 (LPS) were obtained from Sigma (St. Louis, MO, USA) as were all other chemicals unless specified. Silymarin was dissolved in dimethylsulfoxide (DMSO) at concentrations a thousand times the final concentrations, so that DMSO final concentration was equal to or less than 0.1%.

#### 2.2. Cell culture

The HepG2 cell line (HB 8065; American Type Culture Collection,

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*Abbreviations:* EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; LPS, lipopolysaccharide; OA, okadaic acid; PMA, phorbol myristate acetate; TNFα, tumor necrosis factor


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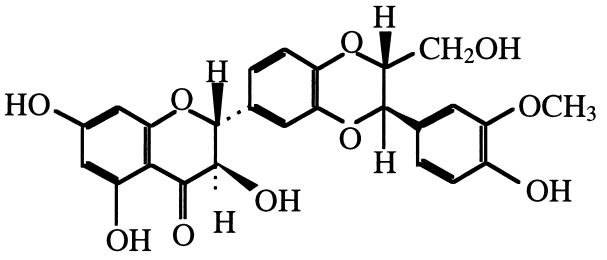

Fig. 1. Chemical structure of silibinin, the main constituent found in silymarin extract. $C_{25}H_{22}O_{10}$, FW 482.4, 2-[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (CAS # 22888-70-6).

Rockville, MD, USA), a human hepatoblastoma-derived cell line, was cultured in Eagle's Minimum Essential Medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, Earle's salts, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin and supplemented with 10% defined fetal bovine serum (Hyclone, Logan, UT, USA). Cells were seeded at a density of 40–100 000 cells/cm$^2$ in 6-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA), containing 3 ml of medium and grown in a humidified air atmosphere with 5% $CO_2$ at 37°C.

*2.3. Preparation of nuclear extracts from HepG2*

HepG2 cells (500 000 cells/cm$^2$) were treated separately with 25 ng/ml TNFα and 100 ng/ml LPS for 1 h or with 0.6 μM OA for 30 min. Silymarin (0.5–25 μg/ml) was added to the medium 24 h earlier. Nuclear extracts were then prepared according to Olnes and Kurl [12] with slight modifications. In brief, cells were washed with ice-cold phosphate buffered saline (PBS), harvested, centrifuged and resuspended in 400 μl of a freshly prepared buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 8.0), 5% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, 1 mM benzamidine and 1% (w/v) aprotinin) and kept on ice for 15 min before the addition of 25 μl of 10% (v/v) NP-40. Incubation was continued on ice for an additional 5 min, followed by centrifugation for 30 s at 15 000×*g* at 4°C. The pellet was suspended in 100 μl of buffer C (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 20% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM benzamidine and 1% (v/v) aprotinin), incubated at 4°C for 15 min, vortexed for 15 min and finally centrifuged for 20 min (15 000×*g* at 4°C). The supernatant (nuclear extract) was collected and frozen at −80°C. Protein concentration was measured using the Bio-Rad Protein Assay I (Bio-Rad, Richmond, CA, USA).

*2.4. Electrophoretic mobility shift assay (EMSA)*

EMSAs were performed as previously described [13]. Equal amounts of the nuclear protein extracts (7.5–10 μg) were incubated with the NF-κB specific $^{32}$P-labeled double-stranded oligonucleotide (Promega, Madison, WI, USA). Oligonucleotides were labelled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and then purified on Chroma-Spin-10-TE (Clontech, Palo Alto, CA, USA). Binding reactions were carried out at room temperature for 30 min, in a 20-μl volume containing the nuclear extract, 4 μl of 5× binding buffer (125 mM HEPES (pH 7.9), 5 mM EDTA (pH 8.0), 2.5 M NaCl, 5 mM DTT and 50% (v/v) glycerol), 2 μg poly(dI-dC)·poly(dI-dC) (Pharmacia, Piscataway, NJ, USA) and about 0.05 pmol of the labeled oligonucleotide (50–100 000 cpm). The binding specificity was determined using the unlabeled wild-type probe (100-fold in excess) to compete with the labeled oligonucleotide. A cold mutant oligonucleotide (100-fold in excess) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added in some experiments to the reaction to further determine the binding specificity. Next, the samples were loaded on a 6% non-denaturing polyacrylamide gel and run with a 0.5× TBE buffer, pH 8.0. Dried gels were autoradiographed overnight at room temperature.

*2.5. Cell transfection and reporter assay*

HepG2 were plated at 40 000 cells per cm$^2$ in 12-well plates, and 24 h later transiently co-transfected with the plasmids pGL3-4κB-Luc [14] and pRL-TK (plasmid reference containing a *Renilla* luciferase gene driven by a minimal thymidine kinase promoter) using Superfect reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.3 μg of both plasmids was mixed with the Superfect reagent (10 μl/μg of plasmid DNA) and subsequently added to the cell culture. The medium was changed after 2 h and cells were allowed to recover for 24 h prior to medium supplementation with silymarin. Afterwards, transiently co-transfected HepG2 cells were separately treated with different concentrations of OA and TNFα. Cell lysis was performed 8 h after the

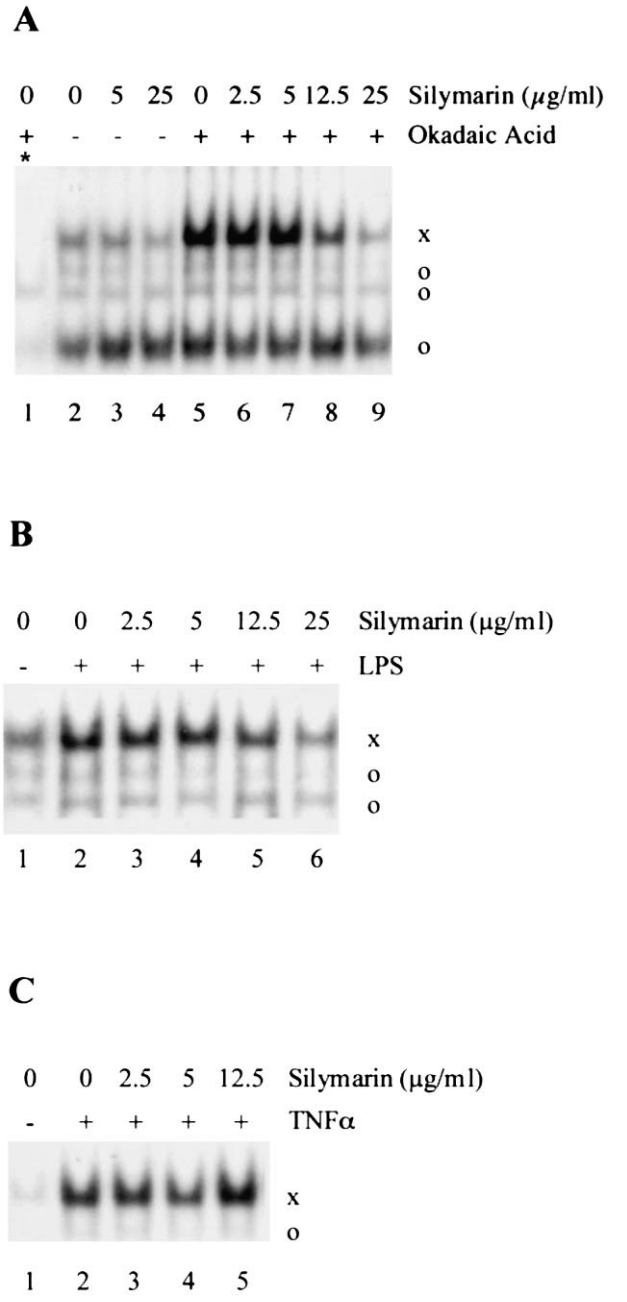


Fig. 2. Silymarin inhibits OA- and LPS- but not TNFα-induced NF-κB activation in HepG2 cells. EMSA analysis of HepG2 nuclear extracts after stimulation with OA (0.6 μM) for 30 min (A), LPS (100 ng/ml) (B) and TNFα (25 ng/ml) (C) for 2 h. Silymarin (0.5–25 μg/ml) was added to the culture medium 24 h before the treatments. Representative experiments are shown. A: In lane 1, '*' represents the addition of a large excess of unlabelled κB-specific oligonucleotide for competition purposes. A, B and C: 'x' represents NF-κB complex and 'o' non-specific complexes.

treatments and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) with an LKB/Wallac luminometer 1250 (Wallac, Gaithersburg, MD, USA). The Dual-Luciferase system is based on the subsequent measurement of firefly (from pGL3-4κB-Luc) and *Renilla* (from pRL-TK) luciferase activities in the same tube with the same extract. The firefly luciferase activity was normalized in that system with the *Renilla* luciferase activity to correct for differences in transfection efficiency.

## 3. Results

### 3.1. Silymarin inhibits OA- and LPS- but not TNFα-induced NF-κB binding activity in HepG2 cells

The HepG2 cell line was chosen to study NF-κB activation in response to various stimuli. This cell line has been intensively investigated; it exhibits morphological and biochemical characteristics of normal human hepatocytes [15,16]. The flavonoid silymarin was added to the culture medium of HepG2 cells for 24 h. Then, the cells were challenged with various stimuli inducing NF-κB activation. After stimulation with OA (0.6 μM) for 30 min, nuclear extracts were prepared and NF-κB DNA binding activity was assessed by EMSA (Fig. 2A). The addition of 25 μg/ml of silymarin to unchallenged cells caused a diminution of the basal or constitutive NF-κB DNA binding activity. A significant inhibitory effect was observed with silymarin concentrations above 12.5 μg/ml, while a silymarin concentration of 25 μg/ml completely abolished OA-induced NF-κB activation. In addition, the inhibitory effect of silymarin was specific on the OA-induced NF-κB DNA binding activity. In other words, the AP-1, SRF and C/EBPβ DNA binding activities were not affected by silymarin (data not shown).

Treatment of HepG2 cells with LPS (100 ng/ml) for 1 h also induced NF-κB activation and was completely inhibited when the cells were pre-incubated with 25 μg/ml of silymarin (Fig. 2B).

In contrast, TNFα-induced NF-κB DNA binding activity was not altered by silymarin (Fig. 2C). Silymarin was inefficient as well with respect to phorbol myristate acetate (PMA)-induced NF-κB activation (data not shown).

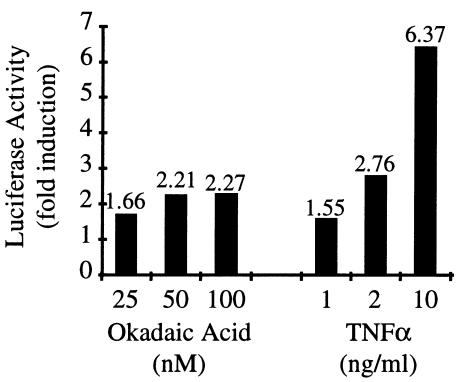


Fig. 3. Okadaic acid and tumor necrosis factor-α induce NF-κB-dependent gene expression in HepG2. OA, TNFα and LPS were added to the culture medium for 8 h prior to the cell lysis. The Dual-Luciferase assay was performed and luciferase activity expressed as relative to that of the control (untreated cells). The data are presented as means (with values on top of the bars) from at least 2 independent experiments, each performed in duplicate.

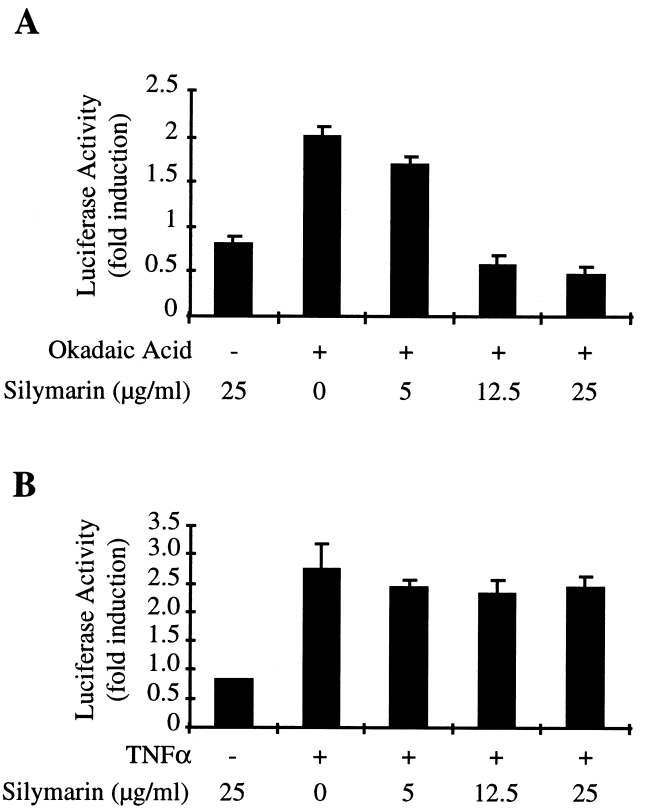


Fig. 4. Silymarin inhibits OA- but not TNFα-induced NF-κB-dependent gene expression in HepG2 cells. HepG2 cells were transiently co-transfected, the medium supplemented with silymarin (5–25 μg/ml) for 24 h and then treated separately with OA (50 nM) (A) and TNFα (2 ng/ml) (B) for 8 h. The cells were subsequently harvested and luciferase activity reported as relative to that of the control and values are the mean ± S.E.M. from at least 3 independent experiments, each carried out in duplicate (A and B).

### 3.2. Okadaic acid, tumor necrosis factor-α and lipopolysaccharide induce NF-κB-dependent gene expression in HepG2 cells

Transiently co-transfected HepG2 cells were exposed to OA (25–100 nM) or TNFα (1–10 ng/ml) for 8 h. Dual-Luciferase assays performed after these treatments demonstrated the inducing capacity of these stimuli to activate NF-κB (Fig. 3). While TNFα induced NF-κB-dependent gene expression in a concentration-dependent manner, OA (50 nM) caused a nearly two-fold increase which was not enhanced with higher concentrations. Treatments with the PMA induced NF-κB-dependent gene expression in a similar pattern to TNFα, whereas LPS responded in the same manner as OA (data not shown). The specificity of the induction was tested in a separate set of experiments using a plasmid containing mutated κB motifs (data not shown).

### 3.3. Silymarin potently inhibits OA- and but not TNFα-induced NF-κB-dependent gene expression in HepG2 cells

To confirm the previous results that silymarin not only inhibits NF-κB DNA binding activity but also suppresses the induction of NF-κB-dependent gene expression, HepG2 cells were transiently co-transfected with both pGL3-4κB-Luc and pRL-TK plasmids. After a period of 24 h with varying concentrations of silymarin, HepG2 cells were challenged for 8 h

using OA (50 nM) or TNFα (2 ng/ml) independently (Fig. 4). The extent of the inductions achieved with these inducers were between two and three times higher than that of the control.

As observed with OA-induced NF-κB DNA binding activity, silymarin completely suppressed NF-κB-dependent gene expression with concentrations as low as 12.5 μg/ml (Fig. 4A). The basal luciferase activity was also notably reduced (Fig. 4A and B). Further, silymarin (25 μg/ml) efficiently inhibited LPS-induced NF-κB-dependent gene expression (data not shown). However, silymarin had no significant effect on NF-κB-dependent gene expression induced by TNFα or PMA (Fig. 4B and data not shown).

## 4. Discussion

NF-κB-dependent gene expression patterns were found to be different between the two groups of inducers evaluated: OA and LPS in one group, TNFα and PMA in another. The same distinction was also noticed in the ability of silymarin to suppress both NF-κB DNA-binding activity and its dependent gene expression. Thus, we demonstrate the co-existence of two activating pathways in HepG2 cells: one that is highly sensitive to silymarin and one that is non-responsive to this inhibitor.

### *4.1. NF-κB activating pathways*

The inducers used in the present study belong to distinct classes of NF-κB stimuli. OA is a serine/threonine phosphatase (PP1 and PP2A) inhibitor which has been shown to activate NF-κB [17], possibly by blocking PP2A-induced IκB kinase (IKK) inactivation in HeLa cells [18]. TNFα-induced NF-κB activation has been extensively studied and revealed several proteins mediating its effect from the membrane receptor to the newly identified IKK kinase complex ([19] and references therein). Accordingly, their signaling pathways appear to converge at the IκB phosphorylation step.

At least two lines of evidence are found in the literature demonstrating that, in a given cell line, NF-κB is activated via different pathways. First, two defective mutant cell lines have been shown as responsive only to a subset of stimuli [20,21]. In addition, some, but not all, of these stimuli induce NF-κB activation using a pathway that is insensitive to the action of the antioxidant and metal chelator pyrrolidine dithiocarbamate (PDTC) [20,21]. Second, in Jurkat T-cells, OA was found to induce NF-κB activation via an antioxidant-insensitive pathway [22,23]. Similarly, the processing of p105, NF-κB precursor, induced by PMA/ionomycin was not affected by PDTC in the same cell line [24].

In HepG2 cells, silymarin was unable to block TNFα-induced NF-κB activation. However, in the Würzburg subclone of Jurkat T-cells treated with TNFα, silymarin exhibited a potent inhibitory effect (C. Saliou, unpublished observations) at concentrations similar to those blocking OA- or LPS-induced NF-κB activation in HepG2. Comparable observations have been made where the inhibition by PDTC is cell-specific [25].

While IκBα appears to be the main regulator of TNFα-induced NF-κB activation in most of the cells, Han and Brasier demonstrated a key role for IκBβ in the second phase of NF-κB activation by TNFα [26]. This particularity could explain the non-responsiveness of TNFα-induced NF-κB activation to silymarin in HepG2 but not in Würzburg cells.

### *4.2. Potential silymarin-sensitive steps in NF-κB activating cascades*

Reactive oxygen intermediates are proposed to be involved in NF-κB activation, though their target is still unknown (see [2] and references therein). Likewise, many antioxidants exert an inhibitory action on NF-κB activation [27]. Silymarin, like most antioxidants, is a reducing agent due to its hydrogen and electron donating properties. Concentrations of silymarin similar to those used herein have been shown to efficiently prevent GSH depletion in HepG2 cells challenged by an oxidative stress induced by high concentrations of acetaminophen [9]. This preventive effect could maintain the cellular reducing potential, thus rendering them less sensitive to the action of reactive oxygen intermediates. Whether the antioxidant activity of silymarin is responsible for the action reported herein is challenged by conflicting reports. First, greater concentrations of silibinin, the most active component of silymarin extract, than the concentrations used in the present study may be necessary to scavenge free radicals such as superoxide [11]. Second, the AP-1 DNA binding activity, not affected by silymarin (data not shown), has been suggested to be induced by antioxidants [28]. Third, in Jurkat T-cells [22,23] and certain mutant cell lines [20,21] but not in HeLa cells [29], OA-induced NF-κB activation is insensitive to antioxidants.

Other targets of silymarin may include kinases and/or phosphatases that regulate NF-κB activity. In that respect, silymarin has been shown to block the epidermal growth factor (EGF)-receptor-associated tyrosine kinase activity in the epidermoid carcinoma cell line A431 [30]. Nevertheless, to date, there is no indication that EGF-receptor signal transduction is involved in NF-κB activation. Other flavonoids, genistein and quercetin, have been reported to inhibit NF-κB activation by blocking the tyrosine phosphorylation [4,31]. In another study, evidence was presented where okadaic acid induced the tyrosine phosphorylation of a cellular protein thought to have a role in NF-κB activation [32]. Ultraviolet radiation has also been reported to increase tyrosine phosphorylation [33] and in a previous study [34], silymarin was also found to be particularly effective at blocking UV-induced NF-κB DNA binding activity and its dependent gene expression in a human keratinocyte cell line as well. However, whether silymarin blocks a tyrosine phosphorylation-dependent step in NF-κB activation or acts through its antioxidant properties are only the possible explanations of the mechanisms of the inhibition that are currently being investigated.

### *4.3. Conclusion*

The present investigation demonstrates that silymarin exerts a selective, but specific, inhibitory action on NF-κB activation according to the stimulus, thus confirming that different stimuli induce NF-κB activation via distinct pathways.

The results of this study also contribute to the understanding of how silymarin protects against liver intoxication since NF-κB activation is its target.

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