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Enhanced Antioxidative Potential by Silymarin Treatment through the Induction of Nrf2/MAPK Mediated HO-1 Signaling Pathway in RAW 264.7 Cells

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Silymarin, which is derived from dried *Silybum marianum* (milk thistle) seeds and fruits, possesses various beneficial properties, such as hepatoprotective, antioxidative, anti-inflammatory, and anticancer activity. This research aimed to explore the antioxidative activity of silymarin against oxidative stress and understand its molecular mechanism in RAW 264.7 cells. The study employed cell viability and reactive oxygen species (ROS) formation assays and western blot analysis. The results demonstrated that silymarin effectively reduced intracellular ROS levels induced by lipopolysaccharide (LPS) in a dose-dependent manner without causing any cytotoxic effects. Moreover, silymarin treatment significantly upregulated the expression of heme oxygenase (HO)-1, a phase II enzyme known for its potent antioxidative activity. Additionally, silymarin treatment significantly induced the expression of nuclear factor-erythroid 2 p45-related factor (Nrf) 2, a transcription factor responsible for regulating antioxidative enzymes, which was consistent with the upregulated HO-1 expression. To investigate the involvement of key signaling pathways in maintaining cellular redox homeostasis against oxidative stress, the phosphorylation status of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) was estimated by western blot analysis. The results showed that silymarin potently induced HO-1 expression, which was mediated by the phosphorylation of p38 MAPK. To further validate the antioxidative potential of silymarin-induced HO-1 expression, tert-butyl hydroperoxide (t-BHP)-induced oxidative damage was employed and attenuated by silymarin treatment, as identified by a selective inhibitor for each signaling molecule. In conclusion, silymarin robustly enhanced antioxidative activity by inducing HO-1 via the Nrf2/p38 MAPK signaling pathway in RAW 264.7 cells.

Key words : Heme oxygenase-1, mitogen activated protein kinase, nuclear factor-erythroid 2 p45-related factor 2, silymarin

Introduction

Inordinate generation of reactive oxygen species (ROS) can lead to damage in cellular components and contribute to promoting various diseases such as atherosclerosis, asthma, and even cancer [11]. Thus, cells have evolved the way to develop their antioxidative mechanisms to cope with oxidative stress [14]. Among various mechanisms to counteract the oxidative damage, one crucial player in maintaining cellular redox homeostasis is the induction of the phase II enzyme including heme oxygenase (HO)-1, NAD(P)H quinone oxidoreductase (NQO)-1 and glutamate–cysteine ligase catalytic subunit (GCLC) [12]. Nuclear factor-erythroid 2 p45-related factor (Nrf) 2, an inducible transcription factor, plays a vital role in the induction of HO-1 and other antioxidative enzymes [12]. Activated Nrf2 moves to the nucleus and binds to the promotor region of Nrf2, the antioxidant response element (ARE), the promoter region of Nrf2, which is possible for the induction of the phase II enzyme expression [18]. The activation of Nrf2 requires the induction of upstream signaling molecules such as mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) [18]. These signaling molecules are stimulated by oxidative stress, and they mediate Nrf2 activation which initiates the expression of antioxidative enzymes and cytoprotective cascades. There-

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fore, Nrf2 acts as a pivotal transcriptional regulator for the induction of antioxidative enzymes as well as MAPKs and PI3K signaling molecules are key components in the Nrf2-mediated signaling cascade.

Silymarin is the polyphenolic flavonoid complex from dried fruits and seeds of milk thistle (*Silybum marianum*) and has been known for its hepatoprotective effect for a long time [1]. In addition, various other biological activities such as anti-inflammatory, antiarthritic, and anti-lipid peroxidative activities [7]. The main component of silymarin is reported as silybin and other constituents are identified as dihydrosibilyn, silydianin, and silychristin [15]. This study attempted to analyze the antioxidative potential of silymarin through the induction of Nrf2-mediated HO-1 expression against t-BHP stimulated oxidative damage in RAW 264.7 cells since the antioxidative activity of silymarin has reported its ROS scavenging activity in RAW 264.7 cells [3].

Materials and Methods

Reagents

Silymarin, DCFH-DA (2', 7'-dichlorofluorescin diacetate), and selective inhibitor (U0126 for ERK, SP600125 for JNK, SB202190 for p38, and LY294002 for PI3K) were procured from Sigma-Aldrich (St. Louis, MO, USA). Tin protoporphyrin (SnPP) and cobalt protoporphyrin (CoPP) were obtained from Frontier Scientific (Logan, UT, USA). Antibodies against HO-1, phosphor-extracellular signal-regulated kinase (ERK), ERK, phosphor-c-Jun NH_2-terminal kinase (JNK), JNK, phosphor-p38, p38, Nrf2, actin, and the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell culture and treatment

The murine macrophage cell line, RAW 264.7 cell, was obtained from Korean Cell Line Bank (KCLB No. 40071; Seoul, Korea), was cultured in Dulbecco's modified Eagle medium (DMEM, Cytiva, Marlborough, MA, USA) with 10% fetal bovine serum (FBS, Cytiva), 100 unit/ml penicillin, and 100 μg/ml streptomycin (Cytiva). Silymarin was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at concentrations of 10, 25, 50, and 100 mg/ml.

Cell viability

The cell viability on RAW 264.7 cells was analyzed by the EZ-Cytox assay kit (Daeil Lab. Service, Seoul, Korea). RAW 264.7 cells were seeded in a 24-well plate and treated with indicated concentrations of silymarin for 24 hr. Then, 10 μl of EZ-Cytox reagent was treated in each well, and the cells were incubated for 1 hr to allow the formation of formazan. The absorbance of the formazan was measured at 480 nm using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

Intracellular ROS formation assay

Intracellular ROS scavenging activity was analyzed by cell-permeable fluorescent dye which was based on the ROS-dependent oxidation from DCFH-DA to DCF. RAW 264.7 cells were stained with 50 μM DCFH-DA for 2 hr. Then, the cells were incubated with indicated concentrations of silymarin for 2 hr, followed by incubation with 1 μg/ml of LPS for 2 hr to generate ROS. The fluorescence was measured by a multi-detection reader (Bio-Tek Instruments Inc.) with excitation and emission wavelengths of 485 nm and 530 nm.

Western blot analysis

The cells were treated with the various indicated concentrations of silymarin for 24 hr to estimate the HO-1 expression level in RAW 264.7 cells. To analyze the expression levels of transcription factor and upstream signaling molecules, the cells were treated with indicated concentrations of silymarin for 4 hr. The crude protein was extracted by ice-cold cell lysis buffer (PRO-PREP, Intron Biotechnology, Seongnam, Korea), and the concentration was determined by Bradford assay. The same amount (50 μg) of each sample was electrophoresed on a 10% SDS-polyacrylamide gel and blotted to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). Then, membranes were incubated with each primary antibody at 4℃ overnight and they were further incubated with the secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG. The membranes were developed by enhanced chemiluminescence and the signal was gained by the ChemiDoc XRS+ system (Bio-Rad Laboratories). Each signal was quantified by the Gel Doc EQ System (Bio-Rad Laboratories).

Statistical analysis

All experimental data were analyzed by the SPSS program (version 26.0, SPSS Inc., Chicago, IL, USA) and exhibited as the mean ± standard deviation of three independent experimental assays. The statistical comparisons were estimated by one-way ANOVA followed by Duncan's multiple-range test and the statistical significance was considered as $p<0.05$.

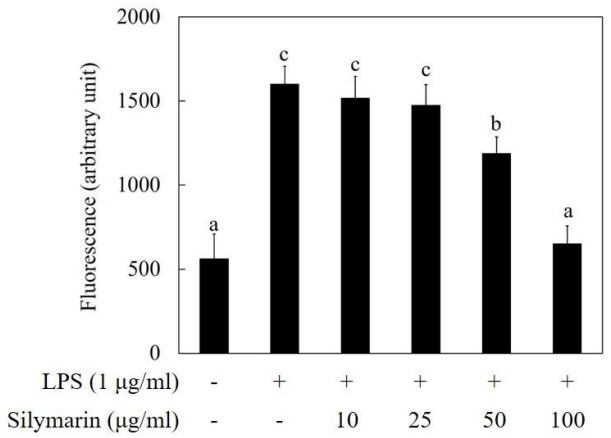

Fig. 1. Significantly scavenged ROS formation by silymarin in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stained with 50 μM of DCFH-DA for 2 hr. Then, the cells were incubated with indicated concentrations of silymarin for 2 hr, followed by incubation with 1 μg/ml of LPS for 2 hr to generate ROS. Data represent the mean±standard deviation of triplicate experiments. Values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test.

Results and Discussion

Potently induced HO-1 expression by silymarin treatment with the Nrf2 activation in RAW 264.7 cells

In this study, the antioxidative potential of silymarin on RAW 264.7 cells was investigated through the induction of the phase II enzyme, HO-1, and its mechanism in RAW 264.7 cells. To evaluate the antioxidative activity of silymarin, a radical scavenging assay was applied to identify the antioxidative activity of silymarin in RAW 264.7 cells. As shown in Fig. 1, silymarin significantly attenuated LPS-induced excessive ROS production in a dose-dependent manner, which indicates silymarin could play a role as an influential candidate as an antioxidant. HO-1, one of the phase II enzymes, plays a crucial role in degrading heme into metabolites, bilirubin/biliverdin, carbon monoxide, and free iron, which have been identified as protective roles against oxidative damage and inflammatory stimuli [4]. Fig. 2A and Fig. 2B show that the condition for potent HO-1 induction was at 100 μg/ml for 12 hr treatment which exhibited no cytotoxicity. Bilirubin has been reported as a potent scavenger of peroxyl radicals *in vitro*, displaying antioxidant activity comparable to that of α-tocopherol. Moreover, it has demonstrated its ability to protect cells from oxidative damage induced by hydrogen peroxide [9]. Additionally, carbon monoxide (CO), a gaseous by-product of heme metabolism, plays a role in reducing pro-inflammatory processes [16]. Furthermore, the induction of ferritin exhibits exceptional antioxidative capacity by efficiently sequestering free cytosolic iron, a critical catalyst of oxygen-oriented radical production [2].

Nrf2, an antioxidative transcription factor, usually plays a role in promoting the antioxidant response elements (ARE) mediated gene expression, which includes the phase II enzymes [13]. Nrf2 primarily exists in an inactive state in the cytoplasm, where it is sequestered by the Kelch-like ECH-as-

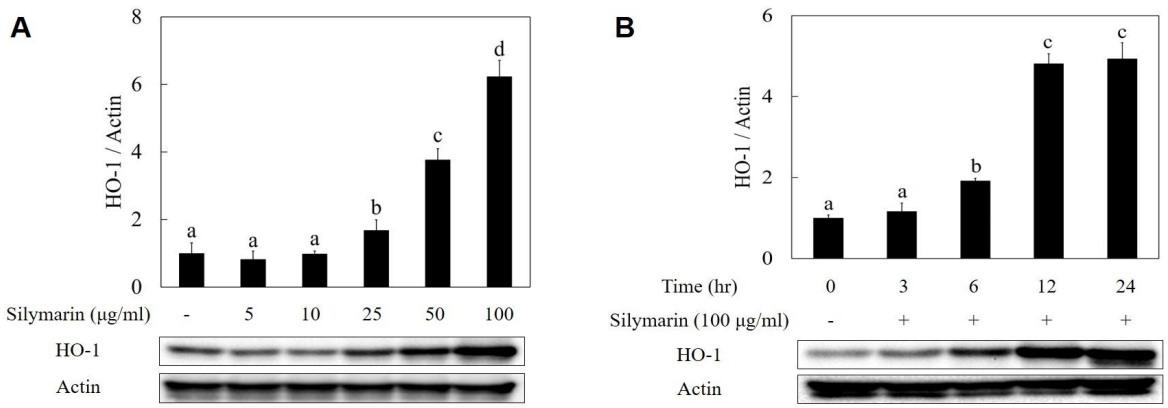

Fig. 2. Potent HO-1 induction by silymarin in RAW 264.7 cells. HO-1 induction by silymarin was analyzed by concentration and exposure time. Panel A shows indicated concentrations of silymarin were treated to induce HO-1 expression for 24 hr. Panel B exhibits indicated that 100 μg/ml of silymarin was exposed as indicated time in RAW 264.7 cells. HO-1 protein expression level was estimated by Western blot analysis. The data are representative of three independent experiments. The protein expression level of HO-1 was quantified by densitometry and actin was used as an internal control. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test.

sociated protein (Keap) 1, the cytoskeleton-associated protein. Nrf2-Keap1 complex can be dissociated by external stimuli such as ROS and electrophiles. Then, freed Nrf2 begins to translocate from the cytoplasm to the nucleus. This translocation leads to the induction of ARE-dependent genes, including HO-1, NQO1, and GCLC [13]. As shown in Fig. 3, Western blot analysis was performed to scrutinize the activated status of Nrf2 and silymarin treatment significantly initiated the Nrf2 activation in RAW 264.7 cells, which was in accordance with the accelerated HO-1 expression.

Overexpressed HO-1 by silymarin treatment attributed to the activation of MAPKs and PI3K/Akt signaling pathways

MAPKs or PI3K/Akt pathways have been reported that they play an important role in regulating the Nrf2-associated HO-1 expression among the various potential upstream signaling molecules in RAW 264.7 cells [19]. In this study, silymarin has been observed whether this agent can trigger the phosphorylation of signaling molecules. Fig. 4 exhibited that silymarin exposure remarkably activated the phosphorylation of p38 MAPK; however, with the exception of p38, ERK, JNK, and PI3K/Akt signaling molecules were not given any discernible effects. In previous research, the role of intracellular kinases related to HO-1 regulation has been reported that several phytochemicals robustly enhanced HO-1 expression through the activation of MAPK and PI3K/Akt pathways in macrophage cells [5, 10].

This study also employed four established selective inhibitors to substantiate the signaling molecule for HO-1 induction by silymarin treatment in RAW 264.7 cells: U0126, SP600125, SB202190, and LY294002 for ERK, JNK, p38, and PI3K, respectively. As shown in Fig. 5, the addition of a selective inhibitor for p38 significantly abolished silymarin-induced HO-1 overexpression while other selective inhibitors did not give any visible effect on HO-1 expression. These results suggest that the upregulated HO-1 expression

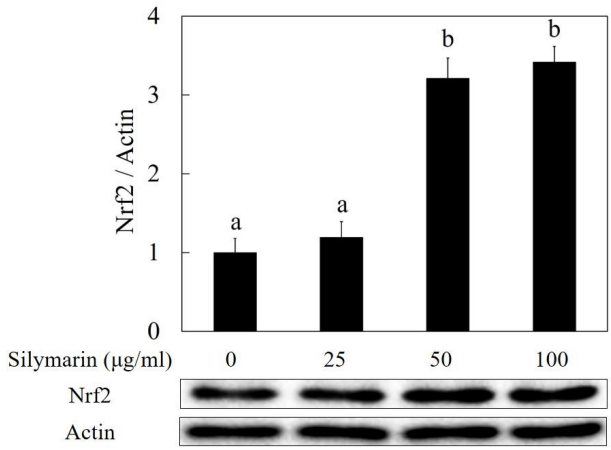

Fig. 3. Nrf2 activation by silymarin treatment in RAW 264.7 cells. Cells were treated with indicated concentrations of silymarin for 4 hr. Activated Nrf2 status was determined by Western blot analysis. The data are representative of three independent experiments. The induction ratio of activated Nrf2 was quantified by densitometry. Actin was used as an internal control. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test. Nrf2, nuclear factor-erythroid 2 p45-related factor 2.

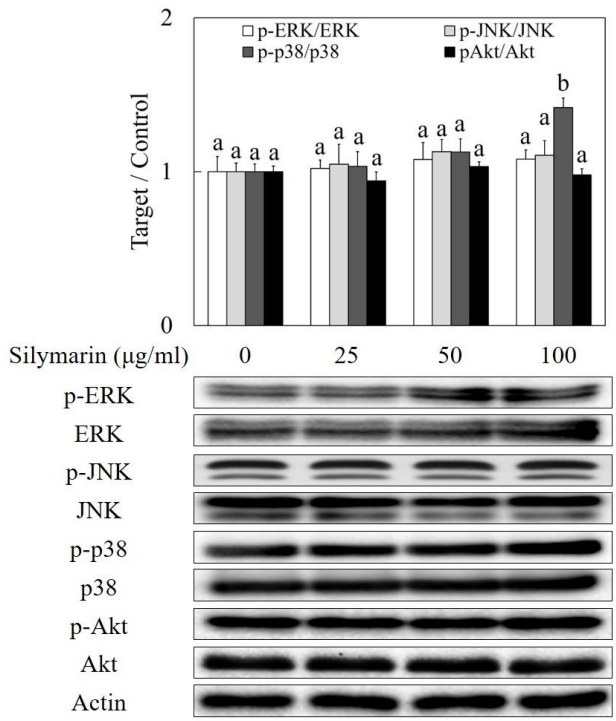

Fig. 4. P38 MAPK phosphorylation by silymarin treatment in RAW 264.7 cells. Indicated various concentrations of silymarin were treated for 4 hr in RAW 264.7 cells. The phosphorylated status of MAPKs and PI3K/Akt were evaluated by Western blot analysis. The data are representative of three independent experiments. The induced ratio of phosphorylated MAPKs and PI3K/Akt was quantified by densitometry. Unphosphorylated MAPKs and PI3K/Akt were used as internal controls. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test. MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.

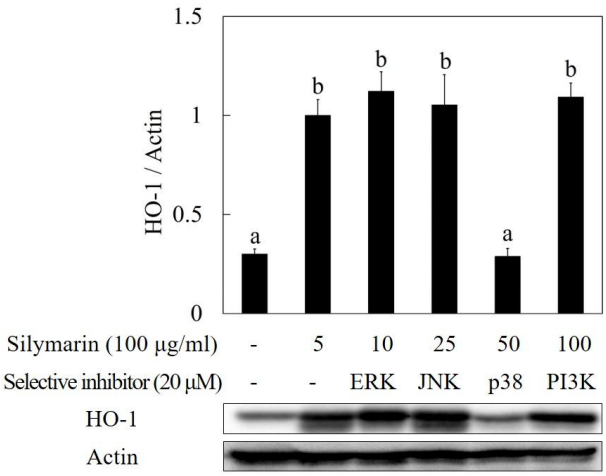

Fig. 5. Silymarin-induced HO-1 expression by the activation of p38 MAPK in RAW 264.7 cells. Silymarin was co-treated with selective inhibitors of each MAPK signaling molecule in RAW 264.7 cells. The indicated concentration of silymarin was treated with 20 μM of each selective inhibitor for ERK, JNK, p38, and PI3K. HO-1 protein expression level was estimated by Western blot analysis. The data are representative of three independent experiments. The relative ratio of HO-1 expression was quantified by densitometry and actin was used as an internal control. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test.

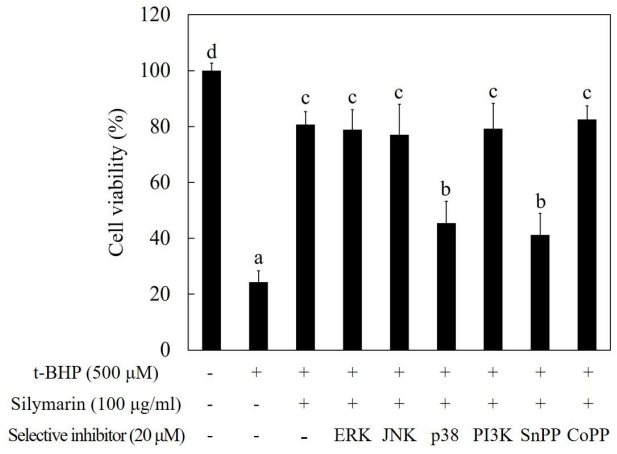

Fig. 6. Strengthened antioxidative potential by silymarin against the t-BHP-induced oxidative damage in RAW 264.7 cells. RAW 264.7 cells were treated with indicated concentrations of silymarin for 12 hr with each selective inhibitor for MAPKs and PI3K/Akt. Then, 500 μM of t-BHP was applied to induce oxidative damage. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test.

by silymarin treatment is regulated by the p38 signaling molecule in RAW 264.7 cells.

Protective effect of silymarin against t-BHP-induced oxidative stress

Exposure to excessively generated ROS can lead to cellular death due to substantial damage inflicted upon cellular lipids, proteins, and DNA. A high dose of t-BHP, one of the organic hydroperoxides, was used to induce fatal oxidative damage in RAW 264.7 cells [17]. The t-BHP is metabolized by cytochrome P450 resulting in the creation of harmful peroxyl and alkoxyl radicals. Consequently, these metabolites trigger lipid peroxidation and ultimately lead to cell death [8]. The optimal condition for t-BHP-induced cellular damage was reported in a previous study [17]. Silymarin was treated at 100 μ/ml for 12 hr to induce the HO-1 expression with or without selective inhibitors as well as SnPP and CoPP (HO-1 inhibitor and inducer). Fig. 6 showed that 500 μM of t-BHP treatment induced a remarkable increase in cytotoxicity as a result of lipid peroxidation, but silymarin treatment significantly mitigated t-BHP-induced cell death. On the other hand, the cytoprotective effect of silymarin against t-BHP-induced oxidative damage was not exhibited in SB202190 (p38 selective inhibitor)- and SnPP (HO-1 selective inhibitor)-treated RAW 264.7 cells due to the abolished HO-1 expression (Fig. 6).

These results suggest that the Nrf2-mediated HO-1 expression by silymarin treatment might strengthen the antioxidative potential through the activation of the p38 MAPK signaling pathway in RAW 264.7 cells.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : RAW 264.7 세포에서 Nrf2/MAPK 의 활성을 통한 HO-1 과발현에 의한 silymarin의 항산화 효과

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Silymarin은 간 보호, 항산화, 항염, 항암 등 다양한 생리 활성을 나타내는 것으로 보고되었고, 본 연구에서는 산화 스트레스에 대한 항산화 잠재력과 그 기전을 세포 생존력 및 활성산소종 생성 분석과 Western blot 분석을 통해 RAW 264.7 세포에서 알아보고자 하였다. Silymarin은 세포 독성 없이 lipopolysaccharide (LPS)에 의해 자극된 세포 내 활성산소종을 농도 의존적으로 소거하였다. 그리고 항산화 효과를 보여주는 것으로 알려진 제2상 효소 중 하나인 heme oxygenase (HO)-1의 발현은 silymarin 처리에 의해 강하게 유도되었다. 또한 silymarin 처리는 항산화 효소의 전사인자인 nuclear factor-erythroid 2 p45-related factor (Nrf)-2의 발현을 유의미하게 유도하였고, 이는 HO-1 발현증가와 일치하였다. 세포내 산화와 환원 항상성 조절과 관련된 신호 전달물질인 mitogen activated protein kinase (MAPK)와 phosphoinositde 3-kinase (PI3K)의 인산화 정도 또한 Western blot으로 분석하였고, 그 결과 silymarin 은 p38 MAPK 인산화에 의해 HO-1 발현을 유도하는 것으로 나타났다. 그리고 tert-butyl hydroperoxide (t-BHP)를 이용하여 세포내 지질 과산화를 유도함으로써 silymarin에 의해 유도된 HO-1의 항산화 효과를 확인하였다. 그 결과 silymarin 처리에 의해 세포사멸이 유의적으로 억제되었고, p38의 선택적 저해제를 처리한 세포군에서는 t-BHP에 의해 유의적인 세포사멸이 발생하였다. 이 결과를 통해 silymarin은 Nrf-2/p38 MAPK 신호 전달 경로를 통해 HO-1의 발현을 유도하고, 이를 통해 항산화 효과를 높이는 것을 확인할 수 있었다.