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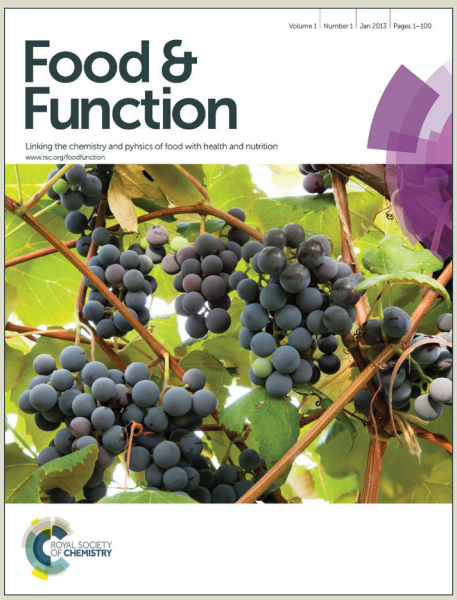

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PAPER

Potent inhibitory effect of silibinin from milk thistle on skin inflammation stimuli by 12-*O*-tetradecanoylphorbol-13-acetate

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Silibinin, a major polyphenol in milk thistle, has been reported to have multiple pharmacological activities which therefore, is urgently needed to well understand how silibinin works on inflammation-associated skin diseases. We herein designed silibinin on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated skin inflammation to test its inhibitory effects. It was demonstrated that silibinin, applied topically onto mouse ears following TPA stimulation, effectively down-regulated the expressions of TPA-induced interleukin-1β (IL-1β), interleukin-6 (IL-6), necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2) in dose-dependent manner respectively. Further mechanistic investigations indicated that silibinin suppressed the expression of IκB kinase (IKK) by inhibiting phosphoinositide 3-kinase/ protein kinase B (PI3K/Akt) signaling pathway, and thereby suppressing TPA-stimulated nuclear factor-κB (NF-κB) activation. Promisingly, silibinin, used for transdermal application, may be a potent naturally occuring anti-inflammatory agent for preventing inflammation-associated skin diseases.

Introduction

It has been established that inflammation, causally linked to the release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2)[1, 2], is a hallmark of many diseases, including arthritis[3], cardiovascular disease[4], and cancers[5]. Clinically, steroids and non steroidal anti-inflammatory drugs have long been used as the effectively therapeutical anti-inflammatory agents. However, long-term oral administration of them was frequently associated with serious side effects in patients[6, 7]. Recently, using naturally occurring compounds, including those derived from vegetables, fruits, and herbs, as potent preventive or therapeutic agents has become a promising strategy in chemoprevention of inflammation-associated diseases[8, 9]. Epidemiological studies indicated that unique agents derived from natural plants have been found to have inhibitory effect on inflammatory diseases[10]. As such, identify functional food components or nutrients as naturally occurring anti-inflammatory agents to treat inflammation-associated skin diseases is urgent and promising for chemotherapeutic patients.

Milk thistle, an annual or biennial plant of the Asteraceae family, is widely distributed in Asia, Europe, United States and South America[11]. The root, young shoots and leaves of *milk thistle* are popularly consumed as food[12]. The seeds of *milk thistle* have been used as a dietary supplement for the treatment of liver and biliary disorders[13]. Furthermore, the seeds of *milk thistle* have been widely using to make tea for daily consumption in Europe and United States[14]. It has been indicated that the extracts from *milk thistle* showed several beneficial biological effects, such as anti-oxidant[15], hepatoprotective[16] and anti-inflammatory activities[17]. Earlier studies confirmed that silibinin (Fig. 1) is one of the major active constituents of silymarin, which is extracted from *milk thistle*[18]. Moreover, silibinin is response for *milk thistle*'s biological activities. However, studies on the anti-inflammatory mechanisms of action for silibinin on skin inflammation are still lacking.

Here, the aim of this study was to gain insight into the mechanisms underlying the potent inhibitory effect of silibinin on skin inflammation. Therefore, firstly, we designed to explore inhibitory effects of silibinin on skin inflammation by using 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema model. Secondly, immunohistochemical analysis revealed that silibinin effectively inhibited TPA-induced IL-1β, IL-6, TNF-α and COX-2 expressions in dose-dependent manner. Furthermore, silibinin suppressed the TPA-stimulated expression of IKK and NF-κB activation by inhibiting PI3K/Akt signaling pathway. Accordingly, silibinin may be a promising anti-inflammatory agent for the treatment of inflammatory skin diseases.

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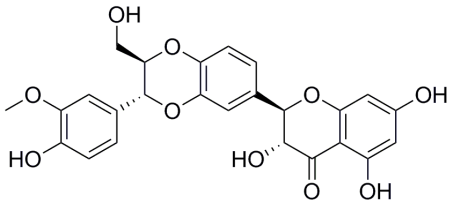


$C_{25}H_{22}O_{10}$
Mol. Wt.: 482.44

Fig. 1 Chemical structure of silibinin.

Experimental Procedures

Material

Silibinin (assay of 98%, PubChem CID 31553) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Silibinin at different concentrations were dissolved in 100 μL vehicle (DMSO : methylene chloride = 20 : 80) respectively. TPA was supplied from Henan Cancer Hospital (Henan, China). TPA (PubChem CID 27924) was dissolved in acetone at 0.008 nM (w. t. = 616.84). DMSO, dichloromethane and other chemicals were used in the purest form available commercially. IL-1β, IL-6, TNF-α, COX-2, anti-PI3K p85 and anti-phospho-PI3K p85 were purchased from Bioss Biotechnology Co. (Beijing, China). Anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-Ser473 Akt, anti-IKK, anti-Ser32 IκB-α, anti-Ser536 p65, anti-phospho-Ser473 Akt, anti-phospho-Ser32 IκB-α, and anti-phospho-Ser536 p65 antibodies were obtained from Beyotime Biotechnology Co. (Beijing, China).

Animals

Female BALB/c mice at 6-week old (approval documents: SCXK/20130002), supplied from Center of Animal Test of SUN Yat-sen University (Guangzhou, China), were divided into six groups: each group consisting of two mice. These experiments performed three times. All animals were group housed (25±1 ^{o}C at 50% relative humidity), tap water ad libitum and fed standard mouse chow diet. For mouse ear edema model, both ears of female BALB/c mice were topically treated with 15 μL vehicle (DMSO : methylene chloride = 20 : 80), 15 μL silibinin in different concentrations respectively, 6 min prior to TPA treatment (0.008 nM in acetone). The mice were then euthanized after 6 h. Two ear punches (6 mm in diameter) from both ears were then taken and weighed. All experiments were performed in compliance with the relevant laws and institutional guidelines, and the Center of Animal Test of SUN Yat-sen University has approved the experiments.

Histological appearance of mouse ears

Female BALB/c mice were all sacrificed after 6 hours. Both ears were removed in toto, fixed in 10% formalin, decalcified in EDTA buffer, subjected in a series progression of dehydration, and then embedded in paraffin. Samples were serially sectioned at 4 μm and processed routinely for hematoxylin and eosin (H&E) staining. The histological changes were obtained under microscope.

Determination of IL-1β, IL-6, TNF-α and COX-2

After sacrifice, both ears of female BALB/c mice were removed and the deparaffinized skin sections (4 μm) were incubated with 1.2% H_2O_2 in PBS to quench the endogenous peroxidase activity. The primary antibody of proliferating cell nuclear antigen was diluted 100 times then applied to each section overnight at 4 ^{o}C. After washing with PBS, the sections were incubated with a biotin-conjugated horseradish peroxidase antibody (1:200) for 1 h at room temperature. Finally, the peroxidase was detected using the 3, 3-diaminobenzidine tetrahydrochloride reaction, which produced the brown label in the epidermal tissue. The numbers of positive staining cells were counted in five different fields at both ends as well as in the middle for each section.

Assay of NF-κB p65, phospho-p65, IκBa, phospho-IκBa, IKK, Akt, phospho- Akt, PI3K p85 and phospho-PI3K p85

NF-κB transcriptional activity and PI3K/Akt signaling pathway were measured by immunohistochemistry analysis respectively. Firstly, mouse ears of female BALB/c mice were removed in toto, fixed in 10% formalin, decalcified in EDTA buffer, subjected in a series progression of dehydration, and then embedded in paraffin. Secondly, samples prepared completely were serially sectioned at 4 μm and processed routinely for staining. Finally, the histological changes were examined under microscope.

Scoring the expression of biomarkers

For each female BALB/c mouse, ≥5 ducts per histologic type of TPA-induced ductal lesion were scored independently by two experienced investigators not aware of the identity of the specimens (×200). For IL-1β, IL-6, TNF-α, COX-2, PI3K p85, p-PI3K p85, Akt, IKK, IκBα, p65, p-Akt, p-IκBα and p-p65, we used integrated optical density (IOD) and the following semi-quantitative scoring system. The intensity of staining was scored as follows: 0, no staining; 1+, faint; 2+, moderate; 3+, strong. 1+, 2+, and 3+ were recorded as 1, 2, and 3 points, respectively. The extent of staining was graded as follows: 0, no staining; 1+, ≤25% of cells positive; 2+, 26% to 50% of cells positive; 3+, ≥51% of cells positive. [19].

Statistical analysis

The results are presented as the mean ± SE. Data are presented as mean ± S.D. Comparison of more than two groups was made with a one-way analysis of variance ANOVA followed by Dunnett *t* test and were considered to be significant at $P < 0.05$.

Table 1 Inhibition of TPA-induced ear edema by silibinin

Preventive effect						
Compounds	Total dose (μmol)	Increase in the average weight of ear punches (mg)				
		3h	6h	9h	12h	15h
Acetone	-	0	0.1±0.2	0.1±0.2	0.1±0.2	0
TPA	-	7.2±0.2	11.1±0.3	11.1±0.1	10.8±0.3	10.6±0.2
Silibinin	5	5.7±0.4*	5.3±0.2*	5.3±0.2*	5.2±0.1	5.0±0.3*
Prednisolone	5	5.1±0.3	4.9±0.4*	4.7±0.1	4.6±0.3*	4.4±0.3*

* $P < 0.1$, significantly different from TPA-treated group in each column by Dunnett *t* test. The data from each group represent the mean ± SE from 3 mice.

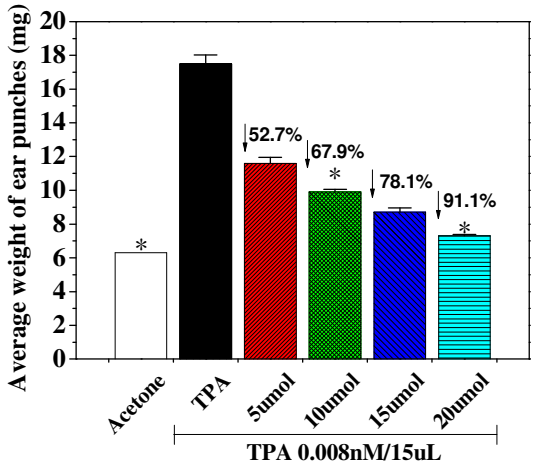


Fig. 2 Effects of silibinin on TPA-induced mouse ear edema. The data from each group represent the mean ± SE from 3 mice. Data are mean ± S.D., *, P < 0.05 (Dunnett t test), compared with TPA-treated mice

Results and discussion

Effects of silibinin on TPA-induced mouse ear edema

To explore how long silibinin effects is persisted in the TPA-stimulated mouse ear edema model, we applied 5 μmol silibinin and 5 μmol prednisolone onto mouse ears to determine the decrease in the average weight of mouse ears at 3 h, 6 h, 9 h, 12 h and 15 h, respectively. As shown in Table 1, silibinin markedly inhibited the average weight of TPA-induced mouse ears at all times up to 15 h. the average weights was decreased by 20.8%, 52.7%, 52.7%, 52.3% and 52.8% for 5 μmol of silibinin onto mouse ear at 3 h, 6 h, 9 h, 12 h and 15 h, respectively. In addition, prednisolone had more potent activity in the same model.

The anti-inflammatory activity of silibinin can be demonstrated by inhibitory effects of silibinin at different concentrations on TPA-stimulated mouse ear edema. Moreover, it has reported that TPA was a well-known promoter of skin tumorigenesis[20]. Herein, upon stimulation with TPA alone, the average weight of ear punches was significantly increased from 6.3 mg to 17.5 mg (Fig. 2). To investigate whether silibinin has any influence on decrease in the average weight of ear punches, we applied silibinin at 5 μmol, 10μmol, 15 μmol or 20 μmol, 6 min prior to treatment with TPA. These results suggested that when silibinin at different concentrations (5 μmol, 10μmol, 15 μmol and 20 μmol) were applied topically onto mouse ears following TPA stimulation, the average weight of ear punches were dose-dependently decreased. Furthermore, significantly highest concentration (20 μmol, P < 0.05) of silibinin caused more than 90% decrease in the average weight of ear punches.

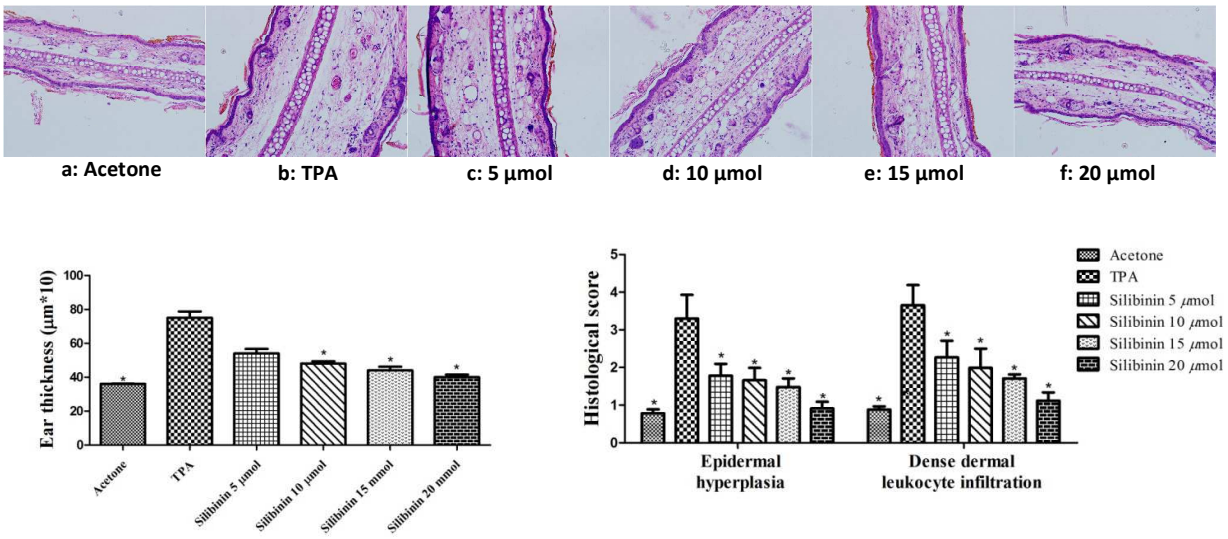
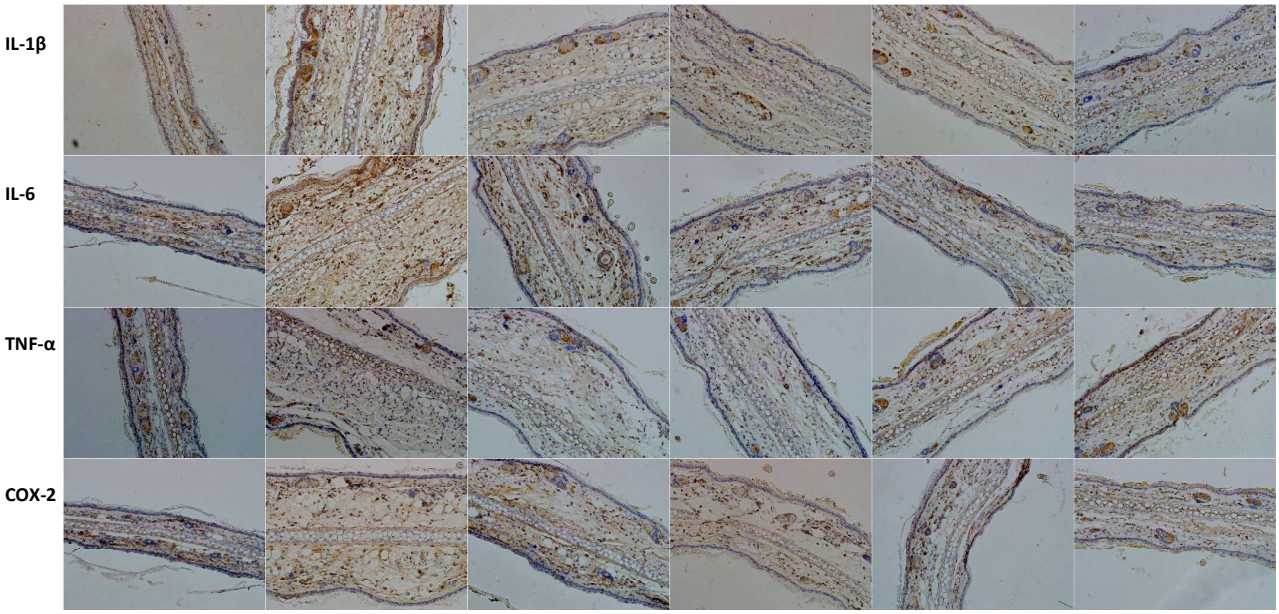


Fig.3 H&E staining for histological changes of TPA-induced mouse ears pretreated with acetone control, TPA and silibinin at different concentrations. Data are expressed as mean ± S.D. (n = 3) *P ＜ 0.05 (Dunnett *t* test), compared with TPA -treated mice. Magnification 200×.

Inhibitory effects of silibinin on histological appearance of mouse ears

To investigate whether or not silibinin plays a role in histological appearance of TPA-induced mouse ear edema, we evaluated the transdermal application of silibinin by using TPA-induced mouse ear edema model. In addition, mouse ears, pretreated with acetone, TPA and silibinin at different concentrations respectively, were stained with H&E stain. Histological appearances of the ear sections indicated that pretreatment with acetone alone onto mouse ears show the normal appearance in the epidermal layer without any significant lesion. However, immunohistochemical analysis showed that mouse ears, treated topically with TPA alone, caused significant swelling assessed by ear thickness (Fig. 3). Moreover, topical application of silibinin following TPA stimulation would effectively suppress the signs of inflammatory responses, such as hyperkeratosis, epidermal hyperplasia and dense dermal leukocyte infiltration (Fig. 3c-f). In comparison with the inhibitory effects of pretreatment with silibinin at different concentrations (5 µmol, 10µmol and 15 µmol) on TPA-induced mouse ear edema, silibinin at 20 µmol, pretreated topically, showed the most markedly morphological alterations. (Fig. 3f).



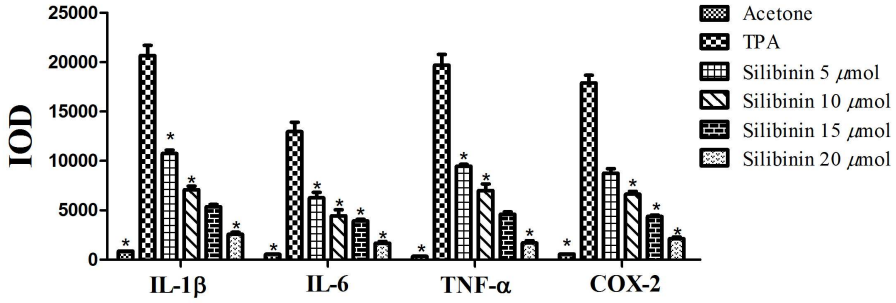
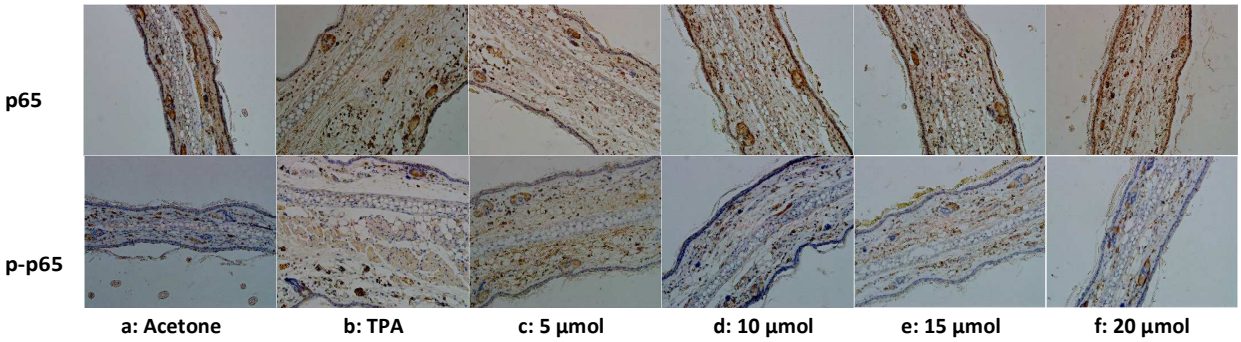


Fig. 4 Immunohistochemical staining on IL-1β, IL-6, TNF-α and COX-2 for mouse ears treated with acetone, TPA and silibinin at different concentrations. Data are expressed as mean ± S.D. (n = 3) *P ＜ 0.05 (Dunnett *t* test), compared with TPA-treated mice. Magnification 200×.

Effects of silibinin on TPA-induced pro-inflammatory mediators

It was indicated that TPA, the prototype tumor promoter, is a potent inducer of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) expression in mouse skin[20, 21]. Thus, we determined the effects of silibinin on the expression of pro-inflammatory cytokines involved in skin inflammation stimuli by TPA. Immunohistochemical analysis indicated that the levels of pro-inflammatory cytokines were dramatically increased following TPA stimulation, which was markedly down-regulated by pretreatment with silibinin in a dose-dependent manner (Fig. 4).

It has been reported that COX-2 is an inducible enzyme that is implicated in inflammation[22]. To investigate whether or not silibinin has any influence on TPA-induced COX-2 expression, we applied silibinin at different concentrations. These results showed that silibinin, applied topically onto mouse ears prior to TPA treatment, dose-dependently inhibited TPA-induced COX-2 expression (Fig. 4).



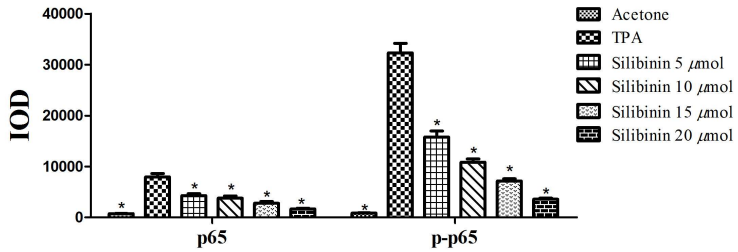


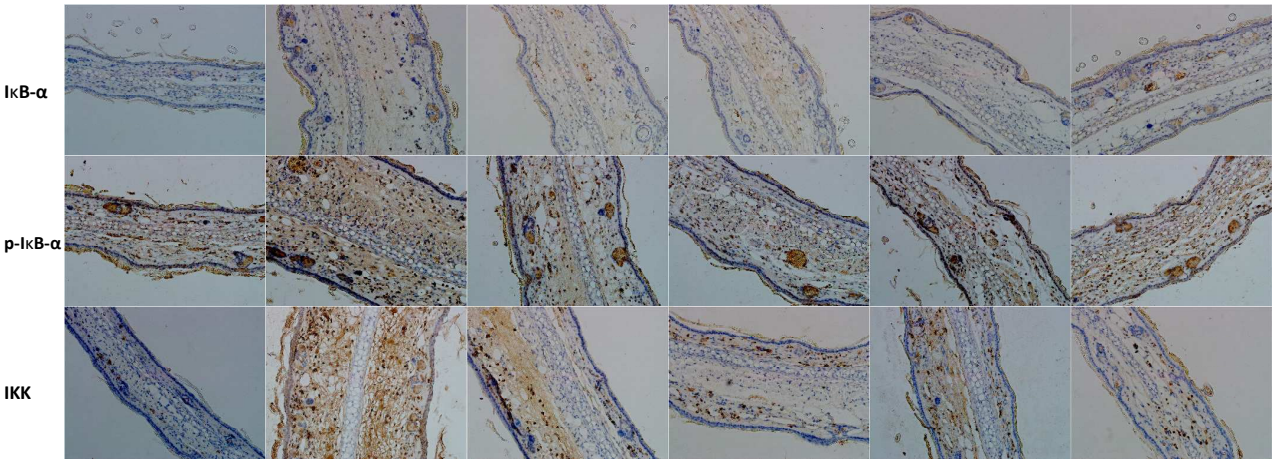
Fig. 5 Immunohistochemical staining on p65 and phospho-p65 for mouse ears treated with acetone control, TPA and silibinin at different concentrations. Data are expressed as mean ± S.D. (n = 3) *P < 0.05 (Dunnett *t* test), compared with TPA-treated mice. Magnification 200×.

Inhibitory effects of silibinin on NF-κB nuclear translocation and activation in TPA-induced mouse ear edema

It has been indicated that NF-κB activation is critical for regulating pro-inflammatory mediators (IL-1β, IL-6, TNF-α and COX-2) expression in TPA-induced inflammatory responses[23]. Therefore, we determined whether silibinin affected NF-κB signaling in TPA-induced mouse ear edema by immunohistochemical analysis. These results indicated that upon treatment with TPA, p65 translocated from cytoplasma to nucleus, which was markedly suppressed by pretreatment with silibinin in a dose-dependent manner. Moreover, silibinin, applied topically onto mouse ears following TPA stimulation, would significantly decrease the nuclear levels of phospholation of p65. It was confirmed that NF-κB transcriptional activity, stimulated by TPA, was markedly increased compared with the control group, but this was effectively inhibited by silibinin at 20 μmol (Fig. 5).

Effects of sulindac-based compound on TPA-induced NF-κB activation by targeting IKK

It is demonstrated that IKK represents the major upstream kinase for IκBα phosphorylation, which is implicated in NF-κB activation. Thus, we determined effects of silibinin on TPA-stimulated IKK/NF-κB signaling pathway and phosphorylation of IκBα by immunohistochemical analysis. As described in Fig. 6, silibinin effectively inhibited degradation of IκBα and the phosphorylation of IκBα in a dose-dependent manner. Consistent with this observation, IKK activity, markedly increased in mouse ears treated with TPA, was effectively down-regulated by silibinin in a dose-dependent manner. These results suggested that silibinin could effectively inhibit TPA-induced NF-κB activation at the level (or upstream) of IKK in vivo, which makes it a promising anti-inflammatory agent.



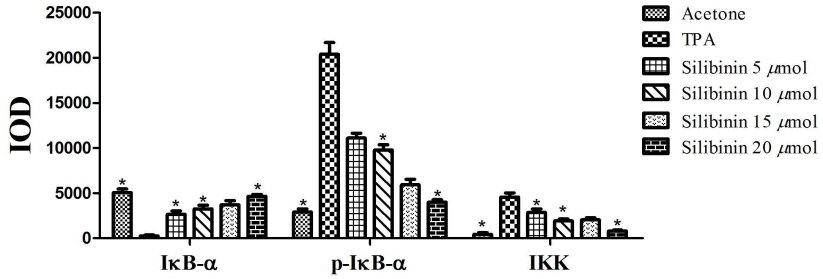
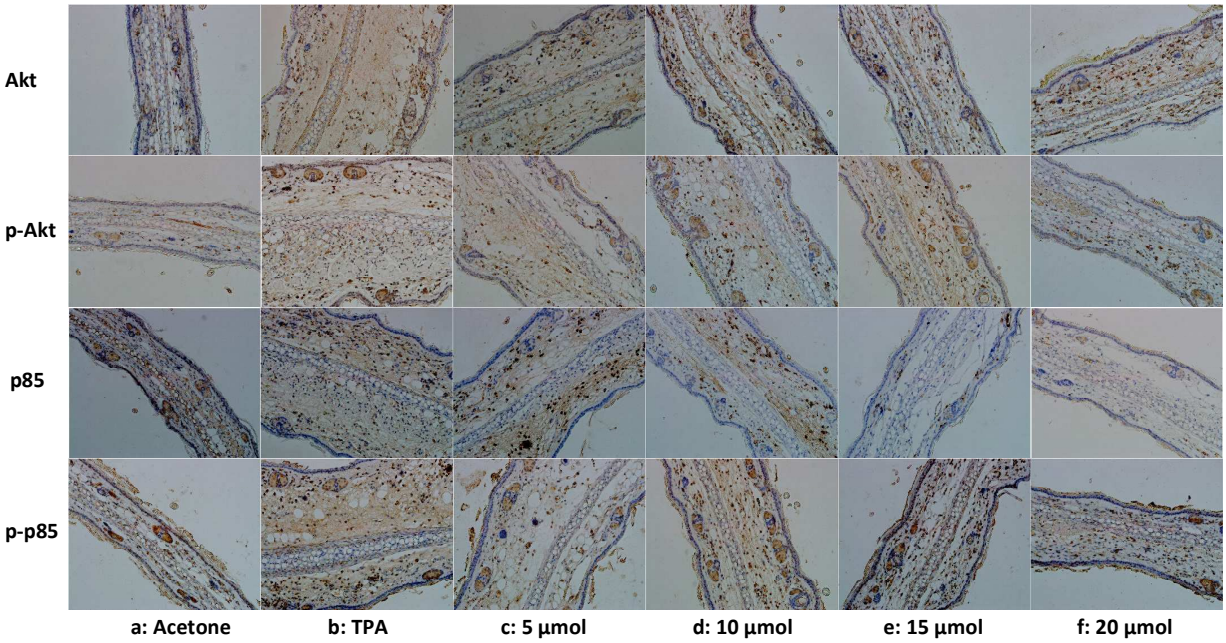


Fig. 6 Immunohistochemical staining on IκBα, p-IκBα and IKK for mouse ears treated with acetone control, TPA and silibinin at different concentrations. Data are expressed as mean ± S.D. (n = 3) *$P < 0.05$ (Dunnett *t* test), compared with TPA-treated mice. Magnification 200×.



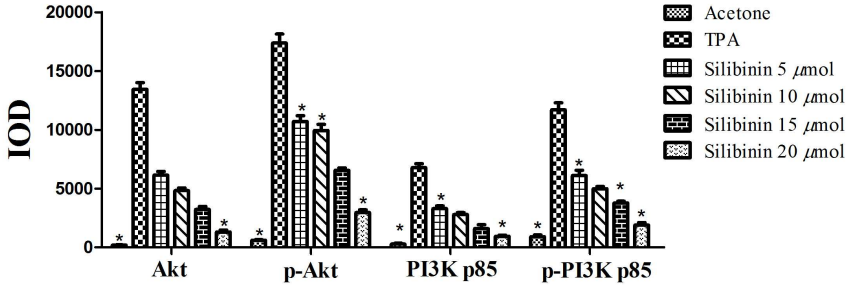


Fig. 7 Immunohistochemical staining on Akt, p-Akt, PI3K p85 and p-PI3K p85 for mouse ears treated with acetone control, TPA and silibinin at different concentrations. Data are expressed as mean ± S.D. (n = 3) *P < 0.05 (Dunnett *t* test), compared with TPA-treated mice. Magnification 200×.

Effects of sulindac-based compound on TPA-induced activation of PI3K p85and Akt

PI3K/Akt signaling pathway is involved in the induction of pro-inflammatory cytokines and COX-2 stimuli by TPA via modulation of NF-κB[24, 25]. Therefore, it is important for us to investigate whether or not silibinin has any influence on the PI3K/Akt signaling pathway. As illustrated in Fig. 7, immunohistochemical analysis revealed that topical application of TPA alone resulted in significant increases in the phosphorylation of PI3K p85 and Akt in mouse skin. Furthermore, pretreatment with silibinin following TPA

stimulation would inhibit TPA-induced phosphorylation of PI3K p85 and Akt in a dose-dependent manner. All these results clearly suggested that silibinin at 20 μmol is more active than silibinin at 5 μmol, 10 μmol and 15 μmol. As indicated, silibinin, used for transdermal application, inhibits the expressions of TPA-induced inflammatory responses (IL-1β, IL-6, TNF-α and COX-2) by blocking NF-κB activation through interference with activation of PI3K/Akt/IKK signaling pathway (Fig. 8)

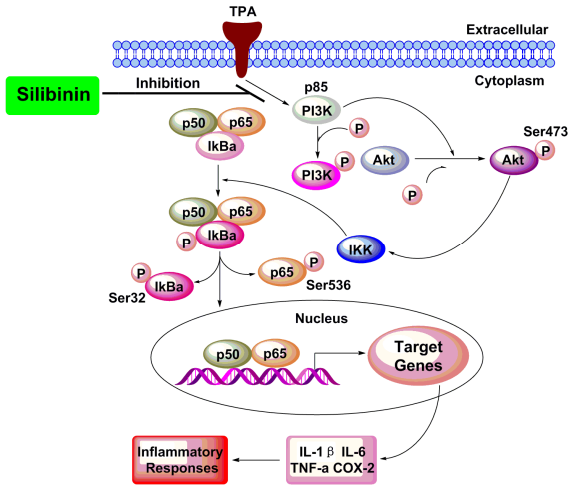


Fig. 8 A schematic representation of suppression of PI3K/Akt signaling pathway, NF-κB activation and IL-1β, IL-6, TNF-α and COX-2 expression by silibinin in TPA-induced mouse ear edema.

Conclusions

In summary, silibinin, derived from an edible plant *milk thistle,* exert inhibtory effects on inhibiting TPA-induced skin inflammation. The further mechanistic studies revealed that silibinin inhibited NF-κB transcriptional activity *via* PI3K/Akt signaling in a dose-dependent manner and identified PI3K as a potential target for silibinin to treat skin inflammation. Significantly, silibinin may serve as a naturally occuring anti-inflammatory candidate for development of effective agent for inflammation-associated diseases prevetion. These results indicate that *milk thistle* may be a beneficial edible plant for further development of inflammation preventive health supplement.

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