

# Silybin from *Silybum Marianum* Seeds Inhibits Confluent-Induced Keratinocytes Differentiation as Effectively as Retinoic Acid without Inducing Inflammatory Cytokine

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**Summary** Retinoic acid (RA) has been effective for improving wrinkles. However, it has also been reported that RA induces skin irritation. In this study, we explored new botanical compounds that show RA-like activity, but do not induce inflammation *in vitro*. Keratinocytes were maintained in a confluent condition and induced differentiation. Under this condition keratinocytes were treated with many botanical extracts and their morphological change were observed and compared with RA-treated. We found that silybin, which is a major flavonolignan from *Silybum Marianum* seeds, induced RA-like morphological change and prevented differentiation. We showed that silybin, like RA, reduced the expression of keratinocyte terminal differentiation markers and stimulated the expression of basement membrane component proteins. In contrast, silybin, unlike RA, did not stimulate the secretion of IL-1 $\alpha$ , which is a skin irritation mediator. These results suggest that silybin has RA-like activity on keratinocytes and has the potential to improve wrinkle without inducing skin irritation.

**Key Words:** silybin, flavonoid, keratinocyte, retinoic acid, wrinkle

## Introduction

Signs of cutaneous aging like wrinkling and sagging develop earlier in sun-exposed skin. This is known as photoaging [1]. It is characterized by a decrease in the amount and degeneration of collagen, dermal elastosis, and glycosaminoglycan deposition [2, 3], and damages to the connective tissues of the skin, which is composed primarily of collagen fiber bundles, elastic fibers and basement membranes [4]. Treatment for the changes responsible for fine and deep wrinkles in photoaged skin is therefore an important cosmetic issue.

The topical application of retinoids such as all-trans-retinoic acid (RA) and retinol induce repair of UV-damaged skin *in vivo*, leading to the effacement of wrinkles [5, 6]. Large double-blind, vehicle-controlled studies have provided

incontrovertible evidence that the topical administration of RA improves wrinkles considerably [7]. At the molecular level, retinoids exert their effects through two families of nuclear receptors, namely the retinoid A receptor (RAR) and retinoid X receptor (RXR), and change the expression of various genes, including the enhancement of collagen synthesis [8], keratinocyte proliferation [9] and inhibition of keratinocyte differentiation [10]. In human skin, RAR $\gamma$  and RXR $\alpha$ , whose heterodimeric partner is RXR $\alpha$  and RXR $\gamma$ , are the predominant species. RA promotes the production activities of extra cellular matrix (ECM) and reduces the collagenase level by the inhibition of activator protein-1 (AP-1) transcriptional activities [11, 12]. Recent studies have shown that RA exerts its effects through heparin-binding epidermal growth factor (HB-EGF) and epidermal growth factor receptor (EGFR) [13, 14]. Many studies have reported that these changes influence skin, causing epidermal hyperproliferation, compaction of the stratum corneum, and the improvement of wrinkling [15]. However, it has also been reported that RA cause skin irritation, including burning, itching, and erythema [16, 17].

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In this study, we investigated a new functional compound with RA-like activity, but which does not induce skin irritation. To find this compound, we searched for compounds that have the same inhibitory effect on confluent-induced keratinocytes differentiation that retinoic acid induces, without inducing interleukin-1 $\alpha$ , a skin irritation marker. We identified silybin as a new active component. Silybin is a plant-derived flavonoid from *Silybum marianum* and it is one of the oldest known and most widely used traditional European medicine, and has mainly been used for liver disorders [18]. We investigated the effects of silybin treatment on keratinocytes, and demonstrated that silybin modulated the expression of keratinocyte differentiation-associated markers like RA, and stimulated the expression of basement membrane component proteins such as laminin-5 and laminin-5 receptor, integrin  $\beta$ 4, effectively as RA. Unlike retinoic acid, however, silybin does not stimulate IL-1 $\alpha$  production. These results suggest that silybin may have a functional component that improves wrinkling without inducing skin irritation.

## Materials and Methods

### *Materials and cell culture*

RA, silybin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Various botanical extracts, used in screening study, were originally extracted from each plant in our laboratory or purchased from each supplying company. Our extracts were obtained by using Accelerated Solvent Extractor (Dionex, Sunnyvale, CA), after mixing 40 ml 95% ethanol and each 1 g dry plants, purchased from each supplying company. Obtained extracts were concentrated under reduced pressure. Silymarin, which is an extract from *Silybum Marianum* seeds, and involve silybin as major consists, were purchased from Indena S.P.A. (Milano, Italy). RA, silybin and each various botanical extracts were dissolved in DMSO, and redissolved in KGM (Cambrex Bioscience, East Rutherford, NJ). Normal human epidermal keratinocytes isolated from adult breast skin were purchased from Cambrex Bioscience.

### *Confluent-induced keratinocyte differentiation assay*

The keratinocytes were seeded at  $5 \times 10^4$  cells per well in 6-well plates and cultured in KGM allowing the cells to attach overnight. For the succeeding culture days, these cells were cultured in KGM with various compounds and botanical extracts by changing the growth medium every two days. When the cells were grown to confluence six days later, morphological changes were observed by using a microscope.

### *Western blotting*

For western blotting of cellular proteins, keratinocytes

were lysed using protein extraction reagent (Pierce Biotechnology, Rockford, IL). The lysates were centrifuged at 12,000 g for 15 min and the supernatants were collected. For western blotting of laminin- $\gamma$ 2, cellular media were collected and concentrated by centrifugal filter device (Millipore, Billerica, MA). The protein concentration was determined by BCA (bicinchoninic acid) assay according to the supplier's protocol. Equal amounts of protein (20  $\mu$ g) were separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes. The membranes were subsequently blocked with 5% skim milk in TBS/T (20 mM Tris-HCl at pH 7.6, 137 mM NaCl, 0.1% Tween 20) and incubated with primary antibody for 2 h. After several washes, the HRP-conjugated secondary antibody was added (final dilution 1:10000) for 2 h at room temperature. The signals were detected using the enhanced ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ).

### *Interleukin-1 $\alpha$ production assay*

For detection of Interleukin-1 $\alpha$  (IL-1 $\alpha$ ), keratinocytes were seeded at  $3 \times 10^5$  cells per well in a 10 cm dish and cultured in KGM, allowing the cells to attach overnight. After these cells were incubated in KGM with RA or silybin for 4 days, the cellular media were collected and examined the amount of IL-1 $\alpha$  examined by using a human IL-1 $\alpha$  ELISA kit (Pierce).

### *RXR $\alpha$ , RAR $\alpha$ protein interaction*

To investigate whether silybin could interact and translocate both the RXR $\alpha$  and RAR $\alpha$ , we used the PathHunter profiling system from DiscoverX Corporation (Fremont, CA) [19]. The protein interaction assay is based on detection of protein-protein interactions between an activated, full length NHR protein and a nuclear fusion protein containing Steroid Receptor Coactivator Peptide (SRCP) domains with one or more canonical LXXLL interaction motifs. The NHR is tagged with the ProLink<sup>TM</sup> component of our EFC assay system, and the SRCP domain is fused to the enzyme acceptor component (EA) expressed in the nucleus. When bound by ligands, the NHR will recruit the SRCP domain, and complementation will occur, generating an active  $\beta$ -Gal enzyme and production of a chemiluminescent signal. The PathHunter NHRPro Cell lines which express each RXR $\alpha$  and RAR $\alpha$  protein, were expanded from freezer stocks in flasks according to standard procedures and maintained. For profiling, the cells were seeded into 384 well microplates at a density of 10000 cells per well in a total volume of 50  $\mu$ l and were allowed to adhere overnight. An intermediate dilution of compound stocks was generated such that 5.5  $\mu$ l of 10X compound could be added to each well with a final DMSO concentration of 1% of total volume. For profiling the compound in the agonist mode, the cells were

incubated in the presence of compound at 37°C for 5 h. After appropriate compound incubation, an assay signal was generated through a single addition of PathHunter Detection reagent cocktail (DiscoverRx) for the agonist assays followed by one hour incubation at room temperature. Microplates were read following signal generation with a PerkinElmer (Waltham, MA) ViewLux™ instrument for chemiluminescent signal detection.

## Results

### Screening of compounds with retinoid-like activities in keratinocytes

First, we explored the screening methods that could easily detect retinoic acid (RA)-like activities in keratinocytes. We found that keratinocytes treated with RA showed morphological changes in the confluent condition. In confluent conditions, DMSO-treated control keratinocytes showed a flatter cell shape and observed like differentiated cells (Fig. 1Ab). These keratinocytes did not maintain proliferation activities (data not shown). However, keratinocytes incubated with RA did not show the flattened changes that were observed like proliferating keratinocytes (Fig. 1Aa, c). To look them like profiling cells, we also examined whether RA-treated keratinocytes maintain proliferation activity by measuring the growth rate after reseeding the confluence

cells, and detected proliferation of these cells (data not shown). These data indicate RA-treatment maintained proliferation activity, and inhibited confluent-induced morphological changes like differentiated keratinocytes. Next we explored new RA-like compounds by observing the morphological changes of the keratinocytes in the confluent and differentiated conditions. Using this confluent-induced differentiation assay, we examined 300 compounds and botanical extracts. We found that treatment with silymarin and silybin, which is a major biologically active component of silymarin complex, isolated from seeds of the milk thistle (*Silybum marianum* L. Gaertn.) induced morphological change and show phenotype like differentiation cells like RA (Fig. 1Ad). Treatment with silybin also preserved the proliferation of keratinocyte (data not shown). Silybin is a flavonolignan (Fig. 1B), and the major biologically active constituent of silymarin. Silymarin, which has antioxidant activities, is one of the oldest known herbal extracts and is a widely used traditional European medicine for liver disorders.

### Modulation of expression on keratinocyte differentiation-associated markers by silybin

It has been shown that keratinocytes change their gene expression by differentiation stage, and that also RA acts predominantly on the latter process to inhibit the expression

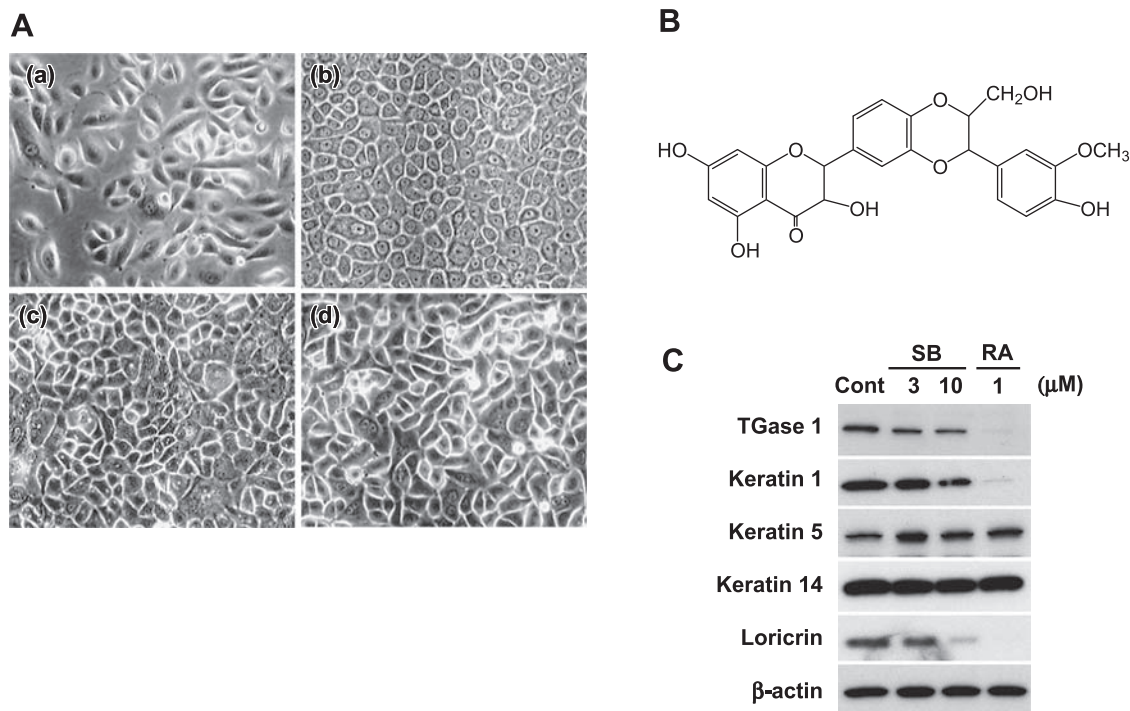


Fig. 1. Phase-contrast image of keratinocytes treated with RA or silybin on confluent-induced differentiation (A). (a), not confluent proliferative keratinocyte. Keratinocytes were incubated in KGM with DMSO (b), RA 1 μM (c), silybin 10 μM (d) for six days and inhibitory effects of RA and silybin on morphological and cell-differentiation were observed. Structure of silybin (B). Western blot analysis keratinocytes differentiation-associated markers (C).

of differentiation-associated markers [20–24]. Therefore, we examined the effect of silybin on the expression of keratinocyte differentiation-associated markers. Under confluent conditions, silybin treatment reduced the expression of tarannglutaminase-1 (TGase1), keratin 1, and loricrin dose-dependently and also did not change the expression of keratin 5 like RA (Fig. 1C). As determined these result, silybin shows RA-like activities in the keratinocytes confluent condition, reduced the expression of differentiation-associated markers, and inhibited differentiation.

#### Effect of silybin on the secretion of interleukin 1 $\alpha$

We examined the cytotoxicity of silybin by LDH (lactate dehydrogenase)-cytotoxic test, and compared with RA. LDH-cytotoxicity assay reveals that both SB (Sigma) 10  $\mu$ M- and RA 1  $\mu$ M-treatment did not affect cytotoxicity (data not shown). However it has been reported that RA stimulate the secretion of IL-1 $\alpha$  [25, 26], which is well known as an inflammatory mediator in acute inflammation of the skin [27] and which induces skin irritation. Therefore, we next examined the effect of silybin on the secretion of IL-1 $\alpha$  and compared it with RA. RA increased the secretion of IL-1 $\alpha$  from keratinocytes, but silybin did not (Fig. 2). The concentration of IL-1 $\alpha$  secreted from RA 1  $\mu$ M-treated cells was five-fold higher than SB 10  $\mu$ M-treated cells, as these show the same effect on the inhibition of confluent-induced morphological change. It was suggested that silybin had the capacity to induce lower inflammation and irritation to the skin than RA and retinoids.

#### Effect of silybin on the interaction and translocation of RXR $\alpha$ , RAR $\alpha$ protein

It was shown that RA interacts and translocates RXR and RAR protein to nuclear and activates transcription of their receptor's targets. To examine this effect of silybin, we used

an enzyme fragment complementation technology system provided by the DiscoverRx Corporation and compared it with activities of RA. The concentration of silybin used 3 or 10  $\mu$ M, which was used in both experiments on confluence-induced keratinocyte differentiation (Fig. 1A) and on the secretion of IL-1 $\alpha$  (Fig. 2). As shown in Fig. 3, RA significantly interacts and translocates both RXR $\alpha$  and RAR $\alpha$ , and exhibited agonist activities to these receptors. In contrast, silybin did not exhibit these activities at 10  $\mu$ M.

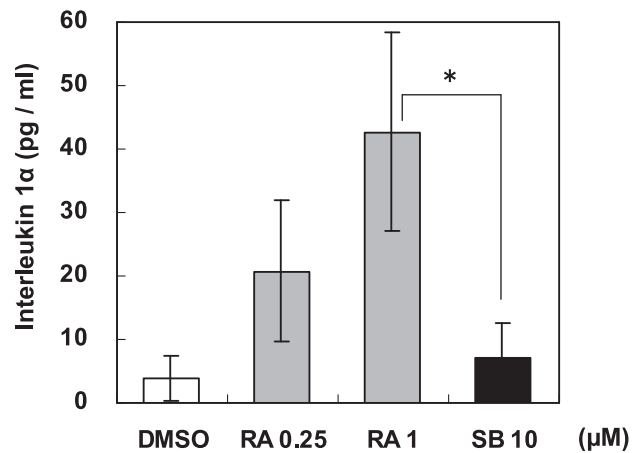


Fig. 2. Effect of silybin and retinoic acid on the secretion of interleukin 1 $\alpha$ . The effect of silybin and retinoic acid-treatment on the secretion of interleukin 1 $\alpha$ , which is well known as an inflammatory mediator in acute inflammation of the skin, were examined. The secretion of interleukin-1 $\alpha$  in KC culture media incubated with RA 0.25 and 1  $\mu$ M, but not silybin 10  $\mu$ M, were increased. Each value represents the mean  $\pm$  SD (bar) for triplicate assays. \* $p$ <0.05.

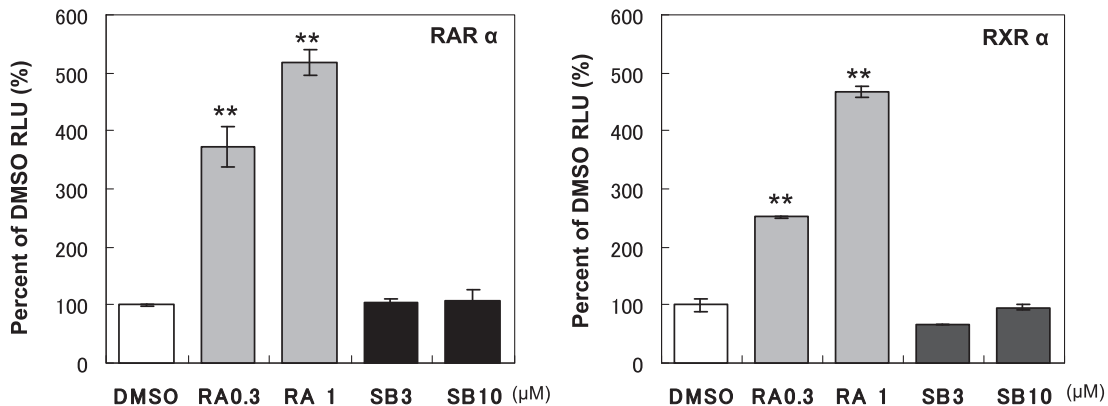


Fig. 3. Modulation of RAR $\alpha$  and RXR $\alpha$  agonist activity after RA and silybin treatment. RA exhibited strong agonist activity with RAR $\alpha$  and RXR $\alpha$ , no activity was observed with silybin. Each value represents the mean  $\pm$  SD (bar) for triplicate assays. \*\* $p$ <0.001 versus DMSO.

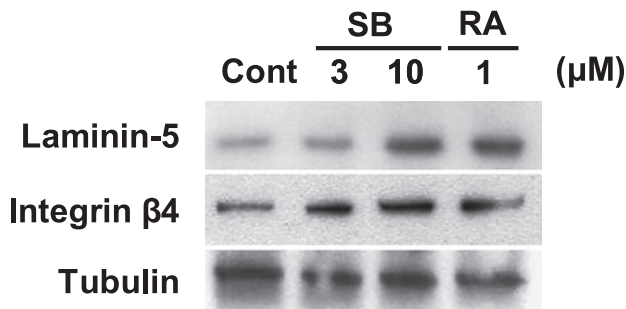


Fig. 4. Effect of SB treatment on laminin-5, laminin receptor, integrin  $\beta$ 4. Keratinocytes were treated with RA or silybin and induced differentiation. After six days cell and medium were collected, and used for western blot.

#### *Effect of silybin on the production of basement membrane proteins such as laminin 5 and integrin $\beta$ 4*

It has been reported that the increase of laminin 5 production in epidermal cells enhanced the repair of damaged basement membrane of skin and this may be an effective approach to prevent the wrinkling and sagging that occurs [28]. Our previous study has shown that RA increased the production of laminin 5 and integrin  $\alpha$ 6 and  $\beta$ 4, heterodimers which are receptors of laminin 5, in keratinocytes and inhibited cells damaged by UV irradiation. Therefore, we investigated the effects of silybin-treatment on the production of laminin 5 and integrin  $\beta$ 4. Treatment with silybin enhanced the production of laminin 5 and integrin  $\beta$ 4 in keratinocytes (Fig. 4). These data suggest that both RA and silybin stimulated the production of basement membrane proteins.

#### **Discussion**

In this study, we investigated a new functional compound which showed activities like RA without its side effects, such as not inducing IL-1 in keratinocytes. RA and retinoids have been considered among the most beneficial compounds for the improvement of wrinkles [29]. Many studies have presented functional evidence of these effects by amassing clinical data on skin [30]. These reports show that RA stimulates cell proliferation in the basal and spinous layers of the skin, leading to thickening. In addition, RA further modulates ECM proteins synthesis and inhibits collagenase production through retinoid receptors and AP-1 activation *in vivo* [10]. Recently, some studies have suggested that RA induced HB-EGF production and EGER signaling [13, 14]. However *in vitro*, meaning in monolayer keratinocytes, the effect of RA has not been clarified and a useful method for screening RA-like compounds has not existed. Therefore, we established an easy screening method to observe morphological changes of differentiated cells under the

confluent conditions. Using this method, we investigated new compounds like RA. As a result, we determined that silybin shows cell shape changes similar to RA under a differentiated condition (Fig. 1A). Silybin treatment does not cause flattened cell shape changes, and show cell shapes like proliferative cells. Furthermore we examined the effect of silybin on the expression of differentiation-associated markers, and showed that silybin reduced the expression level of the terminal differentiation markers transglutaminase1, loricrin and keratin1 (Fig. 1C). These results demonstrate that both silybin and RA inhibit confluent-induced keratinocyte differentiation.

Silybin has traditionally been used to treat liver disorders [18]. It has also been known that silybin exerts its effect by its strong anti-oxidant activities [31]. A number of studies also have shown that silybin suppress UV-induced photocarcinogenesis and cell damage by its antioxidant effects [32, 33]. Because we used a confluent-induced differentiation condition, it can be assumed that the effect of silybin in this study was independent of silybin's strong anti-oxidant activities. Furthermore silybin did not bind and activate retinoid receptors such as RAR $\alpha$  and RXR $\alpha$  (Fig. 3). These data may indicate that silybin shows RA-like cellular morphological changes through other mechanism and signal cascade. Our studies also revealed that RA stimulated IL-1 $\alpha$  production in keratinocytes, but silybin did not (Fig. 2). This data may support that silybin modulate through the different pathway with RA.

However, our results showed that both RA and silybin stimulated the expression basement membrane proteins, laminin-5 and integrin  $\beta$ 4 (Fig. 4). In addition our finding, it has been observed that silybin reduces MMPs (matrix metalloproteinase) activation in tumors [34] and also inhibits UVB- and EGF-induced signaling involving AP-1 and nuclear factor- $\kappa$ B (NF- $\kappa$ B) in JB6 cells [35]. It has been known that RA also influenced these molecules [6]. Although there are various difference in cell types and stimulating inducers that used between these studies and our study, these results indicate that silybin and RA show both different and similar effect, and their control mechanism may be partly different. It might be suggested that silybin directly targets downstream of RA signaling pathway or modulate through non-classical routes of RA, such as activation of HB-EGF and unknown mechanism. Although further studies will be necessary to understand the mechanism that silybin affect in this confluent-induced condition, these studies in silybin may be useful tool for particular understanding of RA signaling cascade.

We demonstrated that RA stimulated IL-1 $\alpha$  production in keratinocytes, but silybin did not (Fig. 2). This difference results in IL-1 $\alpha$  induction has led us to conclude that silybin is a more useful and safe compound in keratinocytes and skin, because silybin shows RA-like activities without

inducing inflammatory mediators. It seems that silybin does not appear to have a negative RA effect in keratinocytes and skin. Recently it has been shown that silymarin, is composed primarily of silybin, has the ability to protect mice from UVB-induced immunosuppression and that this protective effect is mediated, at least in part, through IL-12 [36]. This activity which regulates the secretion of cytokines may contribute to the inhibition of IL-1 $\alpha$  secretion directly.

We also found that both silybin and RA induced laminin-5 and the laminin receptor integrin  $\beta$ 4 (Fig. 4). Laminin-5 is a major basement membrane component. Several studies indicate that basement membrane components are affected by photoaging, but that these effects are modulated by RA. In this study, we revealed that laminin-5 protein synthesis was induced by silybin and RA treatment. Another laminin-5 receptor integrin  $\beta$ 4, was also induced by silybin and RA treatment. These effects on the production of these two proteins may explain that both silybin and RA induced the similar cell phenotype changes, although RA shows these activities through retinoid receptors while silybin does not. A method that measures the production of these proteins might be useful for the screening of RA-like compounds. In addition, silybin might modulate basement membrane components, and might affect photoaging, inducing an improvement in wrinkling.

In summary, the results presented in this report demonstrate that silybin inhibited confluent-induced keratinocyte differentiation and modulates the production of basement membrane components like RA. However, unlike RA, silybin did not induce inflammatory changes such as the stimulation of interleukin-1 $\alpha$  in keratinocytes. These results strongly suggest that silybin might be a potential agent for the prevention and safe treatment of skin aging and wrinkling.

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