ORIGINAL ARTICLE

Action mechanism of anti-wrinkle effect of *Rhamnus yoshinoi* methanol extract in human dermal fibroblast and keratinocyte cell lines

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Abstract

Rhamnus yoshinoi is a deciduous broad-leaf bush and endemic species widely found in Korea. Recently, we reported that *R. yoshinoi* methanol extract (RYME) had excellent antioxidant activity and inhibition of collagenase and elastase activity in cell-free system. In this study, we investigated the ability of RYME to control the mRNA and protein expression levels of the known skin wrinkle-related factors in cultured human dermal fibroblast and keratinocyte cell lines. Treatment with 100 or 200 µg/mL RYME strongly blocked the UVB-induced downregulation of type I collagen mRNA expression ($p < 0.001$) and partially blocked the UVB-induced upregulation of MMP-3 mRNA expression in HaCaT human keratinocytes ($p < 0.05$ or $p < 0.001$). Treatment with RYME at 100 µg/mL considerably decreased MMP-1 mRNA expression in UVB-exposed HaCaT cells ($p < 0.01$). In HaCaT cells, RYME exhibited the potential to improve UV light-induced skin wrinkles. Moreover, RYME selectively inhibited the UVB-induced ERK-1/2 protein phosphorylation in CCD-986sk human dermal fibroblasts at 80 and 160 µg/mL. UV-induced ERK-1/2 protein phosphorylation is one of the major mechanisms of the generation of UV-induced skin wrinkles. Therefore, it is likely that the anti-skin wrinkling effect of RYME could be attributable to selective inhibition of UV induced ERK-1/2 protein phosphorylation.

Keywords Anti-wrinkle effect · CCD-986sk cells · ERK-1/2 protein phosphorylation · HaCaT cells · MMP-3 · *Rhamnus yoshinoi*

Introduction

The epidermis is the most exposed part of the skin that is in direct contact with the external environment. It is composed of multiple superposed cell layers that form an effective protection barrier. Keratinocytes are the most abundant cell type, constituting 80–95% of epidermal cells [1], and are essentially required at the edge of wounds for re-epithelialization by facilitating cell migration, proliferation, and differentiation to close a wound gap [2].

Type I collagen is the most abundant of the various types of collagen, comprising between 85 and 90% of the total skin

collagen and, thus, its severe loss or damage by ultraviolet B (UVB) prematurely ages the skin [3]. In cells, type I collagen is synthesized as a soluble precursor, type I procollagen, which is secreted from fibroblasts and proteolytically processed to form insoluble collagen fibers [4].

Matrix metalloproteinases (MMPs) are a large family of zinc-containing endopeptidases that degrade collagen and other extracellular matrix (ECM) proteins [5]. They play important roles in matrix remodeling in many physiological and pathological processes, and their expression is regulated by diverse signaling pathways [6]. MMPs produced by keratinocytes can translocate to the dermis to exert collagenolytic effect by diffusion through direct binding to the collagenous ECM [7].

UV exposure to diverse cell surface receptors in epidermal and dermal cells concomitantly activates numerous receptor-mediated signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) family including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK [8]. The UV-induced

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activation of MAPKs, especially JNKs and ERKs, stimulates heterodimerization of c-Jun and c-Fos, members of the Jun and Fos family of transcription factors, respectively, to form the activator protein-1 (AP-1) complex, which binds AP-1 sites in the promoter and enhancer regions of target genes [9]. It is known that AP-1 is the major driving force of MMPs expression in keratinocytes and skin fibroblasts [10].

Crude plant extracts that are rich in phenolic content are of interest to the cosmetic industry because of their anti-oxidation and anti-photoaging activities, mediated by inhibition of MMP activation [11]. *Rhamnus yoshinoi* (family Rhamnaceae) is a deciduous broad-leaf bush and endemic species widely found in Korea except Gyeonggi and Chungnam provinces. Recently, we reported that *R. yoshinoi* methanol extract (RYME) had excellent antioxidant activity and inhibition of collagenase and elastase activity in cell-free system [12]. In this study, the action mechanism of anti-wrinkle effect of *R. yoshinoi* were investigated using human dermal fibroblast and keratinocyte cell lines.

Materials and methods

Reagents and apparatus

Following reagents were obtained from the Lonza Company (Cascade, MD, USA): Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). UV irradiation was performed with an UVB sunlamp (Sankyo Denki, Tokyo, Japan). Inverted microscope (CKX41, Olympus, Tokyo, Japan) was used for morphological observation of the cells used, including human keratinocytes and dermal fibroblasts. A CO₂ incubator (MCO-17AC, Sanyo Electric, Osaka, Japan) was used for the cell culture.

Plant material

Methanol extract from plant of *R. yoshinoi* was obtained from the Korea Plant Extract Bank (Daejeon, Korea). The leaves and flowers of *R. yoshinoi* were collected at March, 2002 from Baekun Mountain, Jeollanam-do, Korea. Pulverized samples (100 g) were added to a flask and subjected to extraction thrice for 24 h each time with 1 L of 100% methanol at 45 °C. The extract was filtered, concentrated, and dried. The yield of the RYME was 20.0%. This specimen was dissolved in DMSO before use.

Cell culture

Human dermal fibroblast cell line CCD-986sk cells (ATCC, CRL-1947, VA, USA) and a immortalized human

keratinocyte cell line HaCaT cells [Korean Cell Line Bank (KCLB), Seoul, Korea] maintained at 37 °C in a humidified condition of 95% air and 5% CO₂ in DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin.

Cell viability assay

CCD-986sk or HaCaT cells were grown in 96-well plates (1×10^4 cells/100 μ L/well) overnight. Cells were then treated without or with RYME or vitamin C at the indicated concentrations and/or times in the absence or presence of UVB (10 or 30 mJ/cm²). At each time point, cells were incubated with MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium)] (20 μ L/well) for 1 h at 37 °C. The absorbance was then measured at 490 nm using a microplate reader (Bio-rad 680, Bio-rad, CA, USA).

Cell morphology analysis

After treatments, phase contrast images of the conditioned cells were taken with a phase contrast microscope equipped with a digital camera (Leica DMI1, Leica Microsystems Inc, Wetzlar, Germany)

Preparation of whole cell lysates

HaCaT, CCD-986sk or HDF cells were treated without or with RYME or vitamin C at the indicated concentrations and/or times in the absence or presence of UVB (10 or 30 mJ/cm²). After treatments, respective cells were washed twice with PBS supplemented with 1 mM Na₃VO₄ and 1 mM NaF and exposed to cell lysis buffer [20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/mL leupeptin, 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma, MO, USA)]. Cells were then harvested and centrifuged for 15 min at 4 °C at 13,000 rpm. The supernatant was saved and protein concentrations were determined with bichinchoninic acid (BCA) assay at 560 nm using a microplate reader (Bio-rad 680, Bio-rad, CA, USA).

Western blot analysis

Proteins (10 μ g) were separated by SDS-PAGE (10%) and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore Co, MA, USA). The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (vol/vol) Tween 20 (TBST) followed by blocking with TBST containing 5% (wt/vol) non-fat

dried milk. The membranes were incubated overnight with primary antibodies specific for type 1 procollagen (1:2000, Abcam, Cambridge, UK), MMP-1 (1:1000, Abcam), MMP-3 (1:1000, Abcam), p-ERK-1/2 (1:2000, Cell Signaling Technology, Danvers, MA, USA), T-ERK-1/2 (1:2000, Cell Signaling Technology), p-p38 MAPK (1:1000, Cell Signaling Technology), T-p38 MAPK (1:1000, Cell Signaling Technology), p-JNK-1/2 (1:1000, Cell Signaling Technology), T-JNK-1/2 (1:1000, Cell Signaling Technology) or β -actin (1:10,000, Sigma). The membranes were then exposed to appropriate secondary antibody coupled to horseradish peroxidase (1:2000, Sigma) specific for respective primary antibody for 2 h and washed with TBST. Immunoreactivities were detected by enhanced chemiluminescence (ECL) reagents (Advansta, CA, USA). Equal protein loading was assessed by the level of β -actin protein.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

After treatments, total cellular RNA from HaCaT cells was isolated with the RNeasy-B (Tel-Test). Three micrograms of total RNA were reverse transcribed using a random hexadeoxynucleotide primer and reverse transcriptase. Single stranded cDNA was amplified by PCR with the following primers. The PCR conditions applied were: type I collagen, 30 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 50 s, and extension at 72 °C for 70 s; MMP-1, 30 cycles of denaturation at 95 °C for 60 s, annealing at 56 °C for 60 s, and extension at 72 °C for 60 s; MMP-3, 30 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 120 s, and extension at 72 °C for 180 s; β -actin, 25 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. β -Actin was used as an internal control to evaluate the relative expression of type 1 collagen, MMP-1, and MMP-3. PCR products were visualized after ethidium bromide staining on a 1.2% agarose gel. Band intensity was semi-quantified using the Image J software (Image J version 1.8.0, NIH, Bethesda, MD, USA).

Primer sequences of target genes used in the PCR are listed in Table 1.

Statistical analysis

Data are presented as the mean $\pm$ standard deviation of three independent experiments. Differences between groups were evaluated by one-way analysis of variance followed by a Duncan multiple range test for post-hoc comparison using SPSS 21.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The effect of RYME or vitamin C treatment on viability of control or UVB-irradiated CCD-986sk cells As shown in Fig. 1, compared with control, treatment with RYME or vitamin C at the concentrations (40, 80, 160 or 320 $\mu\text{g/mL}$) and times (4 or 8 h) tested did not largely affect viability of control or UVB (30 mJ/cm^2)-irradiated CCD-986sk cells. Each cell viability under these experimental conditions was over 89%.

The effect of RYME or vitamin C treatment on phosphorylation and expression levels of ERK-1/2, p38 MAPK, and JNK-1/2 in control or UVB-irradiated CCD-986sk cells As shown in Fig. 2, compared with control (lane 1), UVB exposure (lane 2) largely increased phosphorylation levels of ERK-1/2 (Fig. 2a), p38 MAPK (Fig. 2b), and JNK-1/2 (Fig. 2c) in CCD-986sk cells without altering total protein expression levels of each kinase. Notably, treatment with RYME at 80 or 160 $\mu\text{g/mL}$ considerably blocked the UVB-induced ERK-1/2 phosphorylation without changing their total protein expression levels (lanes 3 and 4). However, treatment with RYME at 80 or 160 $\mu\text{g/mL}$ did not interfere with the UVB-induced p-p38 MAPK phosphorylation and did not influence the protein's total expression levels. Although treatment with RYME at 80 or 160 $\mu\text{g/mL}$ strongly blocked the UVB-induced JNK-1/2 phosphorylation, it also

Table 1 Primer sequences of target genes used in the PCR

Items	Primers		Expected size (bp)
Type I collagen	Forward (5' $\rightarrow$ 3')	CTGGTCCCAAGGGTAACAG	285
	Reverse (5' $\rightarrow$ 3')	GCCAGGAGAACCACGTTC	
MMP-1	Forward (5' $\rightarrow$ 3')	CTGAAGGTGATGAAGCAGCC	420
	Reverse (5' $\rightarrow$ 3')	AGTCCAAGAGAATGGCCGAG	
MMP-3	Forward (5' $\rightarrow$ 3')	CCTCTGATGGCCCAAGATTGA	440
	Reverse (5' $\rightarrow$ 3')	GAAATTGGCCACTCCCTGGGT	
β -actin	Forward (5' $\rightarrow$ 3')	GGTGAAGGTCGGTGTGAACG	99
	Reverse (5' $\rightarrow$ 3')	GGTAGGAACACGGAAGGCCA	

MMP-1 matrix metalloproteinase-1, bp basepair

Fig. 1 Effect of RYME or vitamin C treatment at the indicated concentrations and/or times on viability of control or UVB-irradiated CCD-986sk cells. RYME: *R. yoshinoi* methanol extract. Vit. C: vitamin C. Values are mean $\pm$ SD of three independent measurements

led to strong downregulation of total protein expression of JNK-1/2. Vitamin C treatment at 80 $\mu\text{g/mL}$ had no effect on phosphorylation and total expression levels of ERK-1/2, p-38 MAPK, and JNK-1/2 in UVB-exposed CCD-986sk cells (lane 5).

The effect of RYME or vitamin C treatment on morphology of control or UVB-irradiated CCD-986sk cells As shown in Fig. 3, treatment with RYME (80 or 160 $\mu\text{g/mL}$) or vitamin C (80 $\mu\text{g/mL}$) for 4 h did not largely alter the morphology of control or UVB (30 mJ/cm²)-irradiated CCD-986sk cells.

The effect of RYME or vitamin C treatment at the indicated concentrations on viability of control or UVB-irradiated HaCaT human keratinocytes As shown in Fig. 4, compared with control, treatment with RYME (50, 100 or 200 $\mu\text{g/mL}$) or vitamin C (100 $\mu\text{g/mL}$) for 6 h did not largely affect viability of control or UVB (10 mJ/cm²)-irradiated HaCaT cells. Each cell viability under these experimental conditions was over 91%.

The effect of RYME or vitamin C treatment at the indicated concentrations on morphology of control or UVB-irradiated HaCaT human keratinocytes As shown in Fig. 5, compared with control (no RYME or vitamin C), treatment with RYME (100 or 200 $\mu\text{g/mL}$) or vitamin C (100 $\mu\text{g/mL}$) for 6 h did not change the morphology of control or UVB (10 mJ/cm²)-exposed HaCaT cells.

The effect of RYME treatment on mRNA expression of type 1 collagen, MMP-1, and MMP-3 in control or UVB-irradiated HaCaT cells As shown in Fig. 6, in the absence of UVB, treatment with RYME at 100 or 200 $\mu\text{g/mL}$ did not influence mRNA expression of type I collagen in HaCaT cells compared with control. Interestingly, treatment with RYME at 100 or 200 $\mu\text{g/mL}$ considerably down-regulated MMP-1 mRNA expression ($p < 0.01$). Further strikingly, treatment with RYME at 100 or 200 $\mu\text{g/mL}$ strongly repressed MMP-3 mRNA expression ($p < 0.001$). In comparison, treatment with vitamin C at 100 $\mu\text{g/mL}$ strongly repressed MMP-1 mRNA expression ($p < 0.001$), but it had no effect on type I collagen mRNA expression and even upregulated MMP-3 mRNA expression ($p < 0.001$). Remarkably, there was strong downregulation of type I collagen mRNA in UVB (10 mJ/cm²)-irradiated HaCaT cells ($p < 0.001$). Distinctly, there was slight increase in MMP-1 mRNA expression but a large increase in MMP-3 mRNA expression in UVB (10 mJ/cm²)-irradiated HaCaT cells ($p < 0.001$). Importantly, treatment with RYME at 100 or 200 $\mu\text{g/mL}$ strongly blocked the UVB-induced reduction of type 1 collagen mRNA expression in HaCaT cells ($p < 0.001$). Treatment with RYME at 100 $\mu\text{g/mL}$ considerably decreased MMP-1 mRNA expression in UVB-exposed HaCaT cells ($p < 0.01$), with no effect at 200 $\mu\text{g/mL}$. Treatment with RYME at 100 or 200 $\mu\text{g/mL}$ was also able to block the UVB-induced upregulation of MMP-3 mRNA expression in HaCaT cells ($p < 0.05$ or $p < 0.001$). Notably, vitamin C treatment at 100 $\mu\text{g/mL}$ also

Fig. 2 Effect of RYME or vitamin C treatment on phosphorylation and expression levels of ERK-1/2, p38 MAPK, and JNK-1/2 in UVB-irradiated CCD-986sk cells. RYME: *R. yoshinoi* methanol extract. **a** p-ERK-1/2, phosphorylated ERK-1/2; T-ERK-1/2, total ERK-1/2. **b** p-p38 MAPK, phosphorylated p38 MAPK; T-p38 MAPK, total p38 MAPK. **c** p-JNK-1/2, phosphorylated JNK-1/2; T-JNK-1/2, total JNK-1/2

strongly blocked the UVB-induced reduction of type I collagen mRNA expression in HaCaT cells ($p < 0.001$). However, vitamin C treatment at 100 μg/mL had no effect on mRNA expressions of MMP-1 and MMP-3 in UVB-exposed HaCaT cells.

Discussion

Impaired collagen homeostasis is assumed to be due to both a decrease in collagen biosynthesis and an increase in collagen fibril fragmentation and degradation, which are largely influenced by increased expression and activity of MMPs or by the decreased expression and activity of tissue inhibitor of metalloproteinases or both [13]. UVB is known to induce the expression of MMP-1, -3, and -9 in the normal human epidermis in vivo [14]. Further, MMP-1 and -3 are considered as the major contributors to photoaging [15]. MMP-1 (interstitial collagenase) initiates the cleavage of types I and III fibrillar collagen in human skin, while MMP-3 (stromelysin-1) activates proMMP-1 by its partial proteolysis and further degrades the collagen fragments [16,

17]. MMP-3 exhibits the broadest substrate specificity for proteins such as collagen type IV, proteoglycans, fibronectin, and laminin [18].

The HaCaT cell line of immortalized human epidermal keratinocytes was used instead of normal keratinocytes [19]. The multipotent stem cells from the hair follicle bulge can contribute to repairing of the damaged epithelium, as well as contributing to growth of hair follicles and sebaceous glands [20]. Thus, the immortalized adult epithelial HaCaT stem cell line derived from bulge stem cells is a good model of epidermal keratinocytes for studying skin photodamage in vitro because of its reparative potential, and the significant effect UV damage could have on precursor cell lineages. In fact, Moon et al. [21] and Kim et al. [22] reported the anti-photoaging effects of natural compounds on HaCaT cells by evaluating collagen production and MMP expression.

Several reports have shown that natural compounds that protect against UV-induced skin damages could up-regulate collagen biosynthesis by modifying related signals [23]. Collagenase inhibitors have been identified as potential therapeutic candidates for anti-photoaging and wrinkle prevention [24]. *R. yoshinoi* is a deciduous broad-leaf bush

Fig. 3 Morphological observation in control or UVB-irradiated CCD-986sk cells treated with RYME or vitamin C for 4 h. RYME: *R. yoshinoi* methanol extract

Fig. 4 Effect of RYME or vitamin C treatment at the indicated concentrations on viability of control or UVB (10 mJ/cm²)-irradiated HaCaT cells. RYME: *R. yoshinoi* methanol extract. Values are mean $\pm$ SD of three independent measurements

readily found in Korea. Considering the excellent inhibition of collagenase activity of RYME which was superior to that of vitamin C [12], we investigated the ability of RYME to control the mRNA expression levels of the known skin wrinkle-related factors, type I collagen, MMP-1, and MMP-3, in cultured human HaCaT keratinocytes.

In this study, treatment with RYME at 100 or 200 µg/mL strongly blocked the UVB-induced reduction of type I collagen mRNA expression. In addition, it

considerably suppressed the UVB-induced upregulation of MMP-3 mRNA expression and weakly decreased MMP-1 mRNA expression in UVB-irradiated HaCaT cells. In contrast, although treatment with 100 µg/mL vitamin C effectively suppressed the UVB-induced downregulation of type I collagen mRNA expression, it did not suppress UVB-induced upregulation of MMP-3 mRNA expression in HaCaT cells and it also did not decrease MMP-1 mRNA in UVB-irradiated HaCaT cells. Collagen deficiency

Fig. 5 Morphological observation in control or UVB-irradiated HaCaT cells treated with RYME or vitamin C. RYME: *R. yoshinoi* methanol extract

in chronically photodamaged skin may result from the increased degradation of collagen by UV-induced MMPs. Therefore, the present findings suggest that RYME may prevent the UVB-induced skin connective tissue damage by negatively regulating UVB-induced MMP-1/3 expression in skin cells, especially epidermal keratinocytes. In a previous study, Moon et al. [21] reported that pretreatment with *Cos-taria costata* fucoidan significantly inhibited MMP-1 mRNA and protein expression compared to UVB irradiation alone in HaCaT cells. Kim et al. [22] also reported that fucosterol inhibited MMP expression and promoted type I procollagen production in UVB-induced HaCaT cells.

MAPK signal transduction pathways play an important role in regulating a variety of cellular functions [25], including cell growth [26], MMPs expression [27], and type I procollagen synthesis [28]. Although a “cross-talk” exists among the MAPK pathways, a large body of evidence suggests that ERKs primarily mediate cellular responses to mitogenic growth factors [29], while JNKs and p38 MAPK preferentially regulate cellular responses to inflammatory cytokines and physical or mechanical stress [30].

In this study, RYME strongly blocked the UV-induced JNK-1/2 protein phosphorylation in cultured human CCD-986sk fibroblasts at 80 and 160 µg/mL concentrations, and it also induced downregulation of total JNK-1/2 expression. It is likely to be that RYME might reduce mRNA expression of JNK-1/2 at the transcriptional levels in UVB-irradiated cells, which would lead to down-regulation of total

JNK-1/2 protein expression. Another explanation would be that RYME might enhance proteolysis of total JNK-1/2 protein at the post-translational levels under these experimental conditions by influencing the JNK-1/2 protein stability. A previous study also reported that oxidative stress due to high reactive oxygen species (ROS) accumulation initiates MAPK signaling by phosphorylating these kinases [31]. Recently, we reported that RYME inhibited ROS generation [12]. The present study revealed that RYME specifically inhibited the UV-induced ERK-1/2 protein phosphorylation in cultured human CCD-986sk fibroblasts at 80 and 160 µg/mL concentrations. In contrast, vitamin C treatment at 80 µg/mL did not affect UVB-induced ERK-1/2 protein phosphorylation in CCD-986sk cells. ERK-1/2 activity is crucial for AP-1-dependent transcriptional upregulation of MMPs that mediate the degradation of type I collagen, AP-1-dependent transcriptional downregulation of type I collagen, or both. Therefore, it is also conceivable that the blockage of ERK-1/2 activation may be linked to the RYME-induced enhancement of type I collagen and MMP-1/3 suppressant effects on UVB-exposed HaCaT cells.

In conclusion, this study showed, for the first time, that RYME blocked the UVB-induced decrease in type I collagen expression and suppressed the UVB-induced increase in MMP-1/3 expression in HaCaT human keratinocytes. In addition, RYME specifically inhibited the UV-induced ERK-1/2 protein phosphorylation in HDFs. UV-induced ERK-1/2 protein phosphorylation is one of the major mechanisms of

Fig. 6 a Effect of RYME on mRNA transcript levels of type I collagen, MMP-1, and MMP-3 in control or UVB-irradiated HaCaT cells. RYME: *R. yoshinoi* methanol extract.

b Quantification of mRNA expression levels of type I collagen, MMP-1, and MMP-3 normalized to those of β -actin in (a). Values are presented the mean $\pm$ standard deviation of three independent experiments. $**p < 0.01$, $***p < 0.001$ versus control HaCaT cells (no UVB irradiation), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus UVB-irradiated HaCaT cells

the generation of UV-induced skin wrinkles. Therefore, it is likely that the anti-skin wrinkling effect of RYME could be attributable to selective inhibition of UV induced ERK-1/2 protein phosphorylation. Although, recently, we reported that RYME had excellent antioxidant activity and inhibition of collagenase and elastase activity in cell-free system [12], important issues remain unresolved including identification of anti-skin wrinkling active components of RYME and evaluation of its anti-skin wrinkling effect on animal or skin equivalent models.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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