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Clerodendrum trichotomum Extract Attenuates UV-B-Induced Skin Impairment in Hairless Mice by Inhibiting MAPK Signaling

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ABSTRACT

Background: Exposure to solar ultraviolet-B (UV-B) radiation significantly accelerates skin aging by inducing the expression of matrix metalloproteinases (MMPs) such as MMP-1, leading to alterations in the extracellular matrix and consequent photoaging. Some plant components, renowned for their UV-absorbing and antioxidative properties, show potential for mitigating photoaging by reducing UV-B-induced MMP levels. In this context, we explored the inhibitory effects of *Clerodendrum trichotomum* extract (CTE) on UV-B-induced skin damage.

Methods: The mechanism of CTE was predicted using network pharmacology approach. Also, antiaging efficacy was evaluated by mouse model and cellular system using human epidermal keratinocytes (HEKa), including its modulation of mitogen-activated protein kinase (MAPK) signaling pathways.

Results: CTE effectively counters UV-B-induced skin damage, as evidenced by the suppression of MMP-9 and MMP-1 expression in mice. We found that each fraction and chemical constituents of CTE suppressed UV-B-induced MMP-1 secretion in HEKa cells.

Conclusion: CTE inhibits UV-B-induced skin aging by partially suppressing MMP-1 and MMP-9 secretion via the modulation of MAPK signaling pathways.

Ki Mo Kim and A-Rang Im contributed equally to this work.

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1 | Introduction

Skin aging is a complex procedure influenced by various reasons and can be categorized as substantial or external aging. Essential, or chronological, aging is a natural procedure that includes a gradual decay in physiological roles of the skin [1, 2]. Sunlight is related with photoaging and skin cancer [3, 4]. Photoaging manifests as abnormal elastic fibers, chronic inflammation, cellular aging, oxidative stress, and alterations in skin pigmentation [5]. Continuous sunlight exposure leads to the progressive degradation of extracellular matrix (ECM) proteins, notably collagen and elastin. Type I collagen, the predominant type in skin connective tissue, is synthesized primarily by dermal fibroblasts and ensures the strength and resilience of skin. MMP-1, secreted primarily by epidermal keratinocytes and dermal fibroblasts, degrades collagen types I and III, compromising dermal integrity [6–9]. MMP-1, or collagenase, degrades collagen types I and III by cleaving them within the triple helix, producing characteristic fragments. MMP-9, a gelatinase, further hydrolyzes these fragments, thereby collectively compromising the integrity and function of the collagen-rich dermis [10–12]. UV radiation increases MMP-1 synthesis by skin fibroblasts, contributing to photoaging. This process is driven by excessive reactive oxygen species (ROS), which activate mitogen-activated protein kinases (MAPKs), as well as extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK). ERK stimulates c-Fos expression, while p38 and JNK promote c-Jun expression. These proteins combine to form AP-1, which regulates MMP-1, MMP-9, and MMP-3 transcription, leading to collagen degradation [13–17]. Therefore, increased MMP activity plays a crucial role in wrinkle development, a key characteristic of photoaging. Therefore, the identification of novel MMP inhibitors is promising for counteracting the effects of UV radiation-induced aging.

In current years, there has been growing interest in utilize plant extracts to protect against skin damage from solar UV rays. Recent report suggests that consuming herbal phytochemicals orally can shield the skin from the harmful effects of UV-B radiation [18]. *Clerodendrum trichotomum* Thunb, a member of the Verbenaceae family, is native to East Asia and is extensively distributed in regions such as Korea, Japan, Taiwan, and China. Various parts of *C. trichotomum*, including flowers, leaves, and twigs, have traditionally been used as widespread remedies for treating inflammatory conditions such as headaches and high blood pressure. These traditional uses reflect the diverse pharmacological properties associated with this plant [19]. In natural product research, network pharmacology approach is most suitable for multicomponents and multitarget treatment, and has been widely used in this field [20, 21].

Clerodendrum trichotomum, a plant used in traditional medicine, shows various pharmacological effects, such as antihypertensive [22] and anti-inflammatory effects [23]. It contains several phytochemicals, including phenylpropanoid compounds [24–26], flavonoids [27], diterpenoids [28–30], triterpenes [31], and steroids [32], and exhibits antioxidant [19, 33], antiviral [34], and antibacterial properties [35]. However, its potential antiaging effects on skins have not been explored. In this study, we analyzed the mechanism of this plant using network pharmacology and investigated the impact of *C. trichotomum* extract on UV-B-induced skin aging.

2 | Materials and Methods

2.1 | Extraction and Isolation

Clerodendrum trichotomum extract (CTE) and chemical components are prepared as previous paper [19].

2.2 | High-Performance Liquid Chromatography Investigation

The resulting extract (CTE) was subjected to filtration using Whatman filter paper and subsequent concentration under reduced pressure at 45°C by employing a YELA N-1200A rotary evaporator (manufactured by Rikakikai, Japan) to obtain a standardized extract sample. Compounds in methanol were used for HPLC analysis (Waters Alliance 2695 Separations Module, USA, integrated with a PDA detector). The injection volume and flow rate were 10 µL and 0 mL/min, each. The PDA detection was performed from 200 to 420 nm, and compounds were detected at 270 nm. The elution process involved 0.1% TFA (A) and acetonitrile (B). The information of solvent and time are described in Table 1.

2.3 | Chemical Components and Potential Target Genes

Structural information of chemical components was confirmed using PubChem (<https://pubchem.ncbi.nlm.nih.gov/>, accessed on April 03, 2024), ChemSpider (<https://www.chemspider.com/>, accessed on April 03, 2024), TCMSP (<https://old.tcmspe.com/tcmsp.php>, accessed on April 03, 2024), and SwissADME (<http://www.swissadme.ch/>, accessed on April 03, 2024). Genes related to the four chemical components mentioned in the previous report were collected from the Search Tool in the Interactions of Chemicals and Proteins (STITCH) database (<http://stitch.embl.de/>, version 5.0, accessed on April 04, 2024) with “Homo sapiens” and score ≥ 0.15 (as low confidence), and SwissTargetPrediction (<http://www.swiss.targetprediction.ch/>, accessed on April 04, 2024) with “Homo sapiens” species and probability > 0 [36, 37]. Potential target genes were defined as the intersection of genes derived from active components and disease-related genes obtained from the GeneCards: The Human Gene Database (<https://www.genecards.org/>, updated April 01, 2024, accessed on April 04, 2024) with “skin aging.” Potential target genes were shown in a Venn diagram using Venny 2.1 (<https://>

TABLE 1 | Instruction of HPLC time information.

Time (min)	Solvent	
	A (%)	B (%)
0	93	7
10	93	7
40	40	60
41	0	100
45	0	100
50	93	7
60	93	7

csbg.cnib.csic.es/BioinfoGP/venny.html, accessed on April 04, 2024), and the network of components–genes were constructed using a Cytoscape 3.10.1 (<https://cytoscape.org/>, accessed on April 04, 2024).

2.4 | Protein–Protein Interactions (PPIs)

Protein–protein interactions (PPIs) analyses were searched for using STRING version 12.0 (<https://string-db.org/>, accessed on April 04, 2024) with “Homo sapiens” as the biological species set, and confidence score ≥ 0.400 (medium confidence). The topology of PPIs, which is a node connectivity analysis to identify key target, was analyzed and visualized using a Cytoscape 3.10.1 (<https://cytoscape.org/>, accessed on April 04, 2024) [38]. In the PPIs network, the importance of nodes is depicted by color and size [36].

2.5 | Signaling Pathway Analysis

Signaling pathways were evaluated using the DAVID Functional Annotation Bioinformatics Microarray Analysis (<https://david.ncifcrf.gov/>, updated November 29, 2023, accessed on April 08, 2024) and KEGG: Kyoto Encyclopedia of Genes and Genomes (<https://www.genome.jp/kegg/updated> April 04, 2024, accessed on April 09, 2024) with $p > 0.05$. Main pathway and genes were presented using KEGG Mapper (<https://www.genome.jp/kegg/mapper/>, updated April 01, 2024, accessed on April 09, 2024). Gene Ontology analyses, biological process (BP), cellular component (CC), and molecular function (MF), were performed using an Enrichr database (<https://maayanlab.cloud/Enrichr/enrichr>, accessed on April 11, 2024) with adjusted $p > 0.05$, and the network was visualized using Cytoscape 3.10.1 program.

2.6 | Cell Culture

Adult human epidermal keratinocytes (HEKa cells) were obtained from Invitrogen (Carlsbad, CA, USA). These cells were cultivated in EpiLife medium (Gibco-BRL, Burlington, VT, USA), supplement kit, along with antibiotics. The cells were cultured at 37°C, 5% CO₂, and 100% humidity.

2.7 | Cell Viability

Cell viability was evaluated by WST-1 assay, according to the manufacturer's manual.

2.8 | UV Irradiation of the Animal Model and Treatment Regimen

Six-week-old male HR-1 hairless mice were acquired in Japan SLC Inc. (Shizuoka, Japan). The animals were housed under measured environment maintained at 23°C $\pm$ 1°C, humidity 50% following a light/dark cycle 12h. All the experimental procedures were conducted under the guidelines approved by the Animal Care and Use Committee of the Korea Institute of Oriental Medicine (approval number: 16–119) (date of approval: December 22, 2016). Following random assignment into three groups: the control group ($n=6$), UV-B-treated group receiving the vehicle ($n=6$), and UV-B-treated group receiving CTE ($n=6$). Mice in the CTE group were given 0.1 mL of water containing 100 mg/kg body weight CTE once daily for a total of 12 weeks, with administration occurring 5 days a week with a break of 2 days on weekends. UVB irradiation was performed using a UVM-225D Mineralight UV display lamp (UVP, Phoenix,

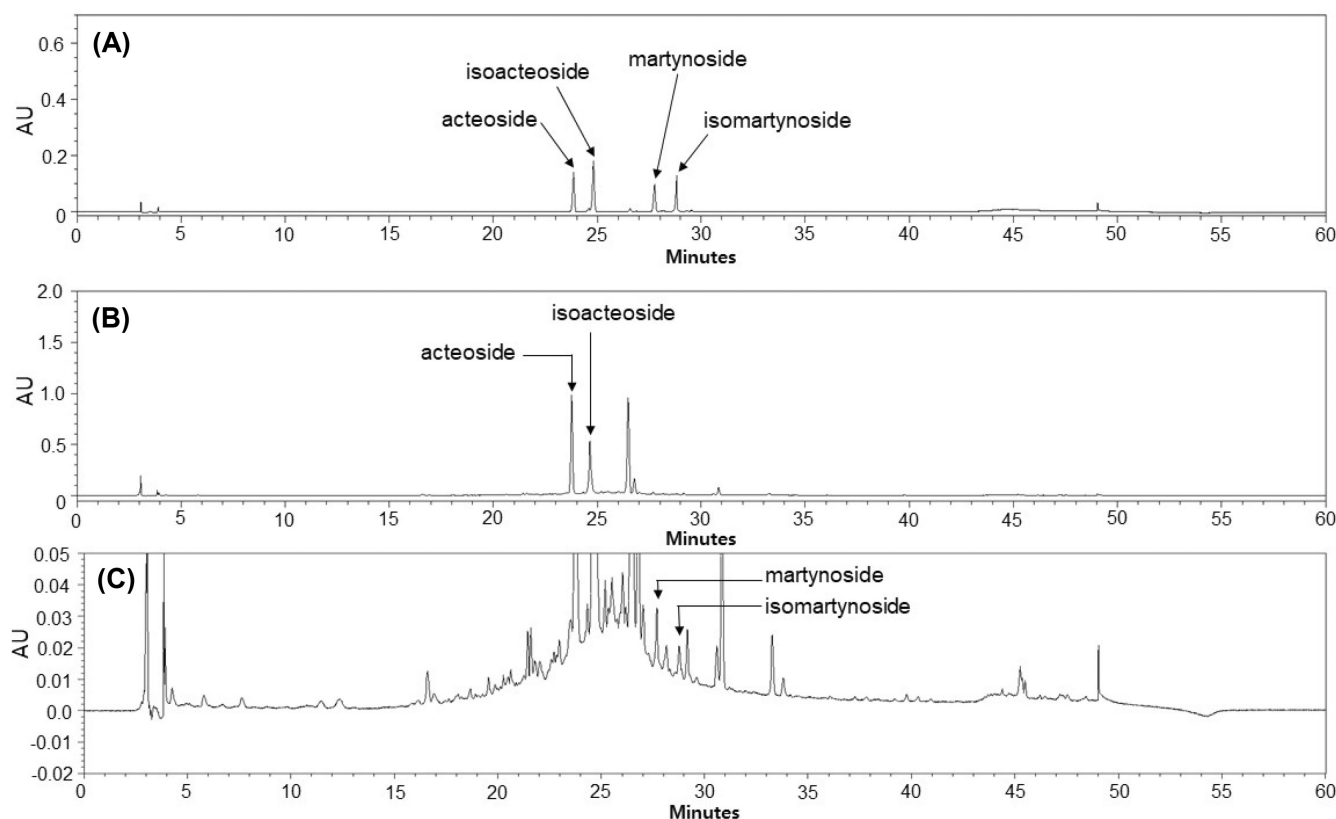
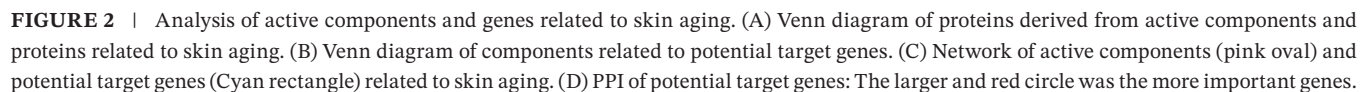


FIGURE 1 | HPLC chromatograms of *Clerodendrum trichotomum* extract (CTE) and reference standards.

backs of the mice three times a week, with the minimum erythema dose (MED) set at 1 MED = 60 mJ/cm² [39]. Mice were killed the day after final UVB exposure. And then, histologic experiments were performed. The UVB radiation was applied to the backs of the mice three times per week for 12 weeks and was progressively increased from 60 mJ/cm² per exposure during weeks 1–3 (one



(D)

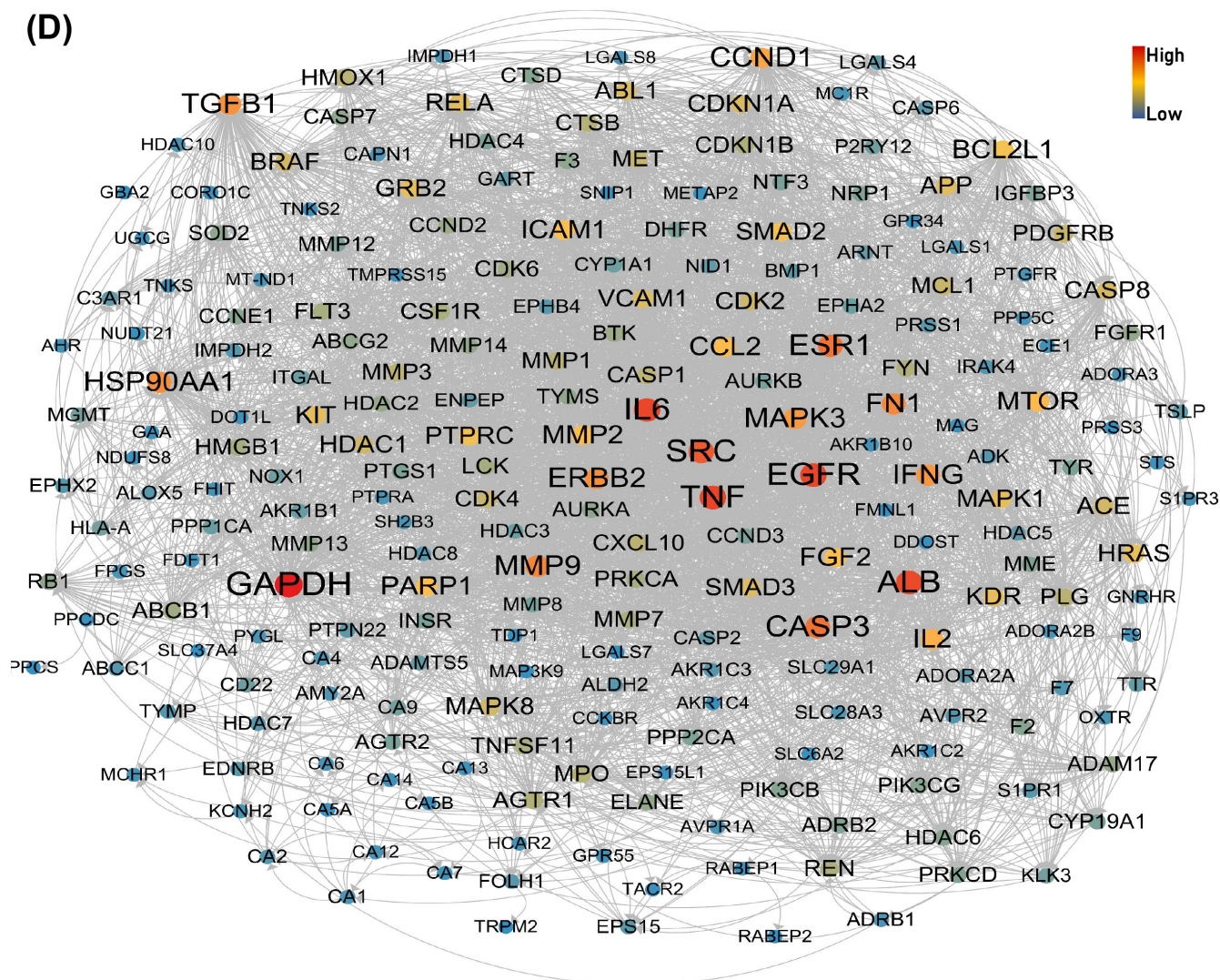


FIGURE 2 | (Continued)

minimal erythematous dose = 60 mJ/cm² to 90 mJ/cm² per exposure until weeks 4–6, and 120 mJ/cm² weeks 7–12.

2.9 | TEWL and Capacitance Evaluation and Histological Investigation

Transepidermal water loss (TEWL) and capacitance were measured by previous paper [13].

2.10 | Western Blotting and Immunological Assays

Protein was extracted from the skin tissue samples using detergent-based lysis buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA), following tissue homogenization (Precellys 24; Bertin Technologies, Montigny-le Bretonneux, France). Total protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). Protein lysates (20 µg) from each sample were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene fluoride membranes

under the semi-dry condition. The membranes were blocked with a 5% skimmed milk solution for 1 h at room temperature. Following blocking, the blots were incubated overnight at 4°C with the primary antibodies (Cell Signaling Technology, Boston, MA, USA) at a dilution of 1:1000. The blots were washed three times for 10 min each in Tris-buffered saline (Bio-Rad Laboratories Inc.). The membranes were then incubated for 2 h with HRP-conjugated anti-rabbit and anti-goat secondary antibodies (Cell Signaling Technology, Boston, MA, USA) at a dilution of 1:5000. Protein bands were visualized using an enhanced chemiluminescence immunoblotting detection kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom), ensuring differentiation from existing methodologies. The level of secreted MMP-1 in the cell supernatants was quantitated according to the manufacturer's protocol (Invitrogen, USA).

2.11 | Statistical Analysis

In vitro experiments were conducted in triplicate, and each experiment was recurrent three times. The data are attending as

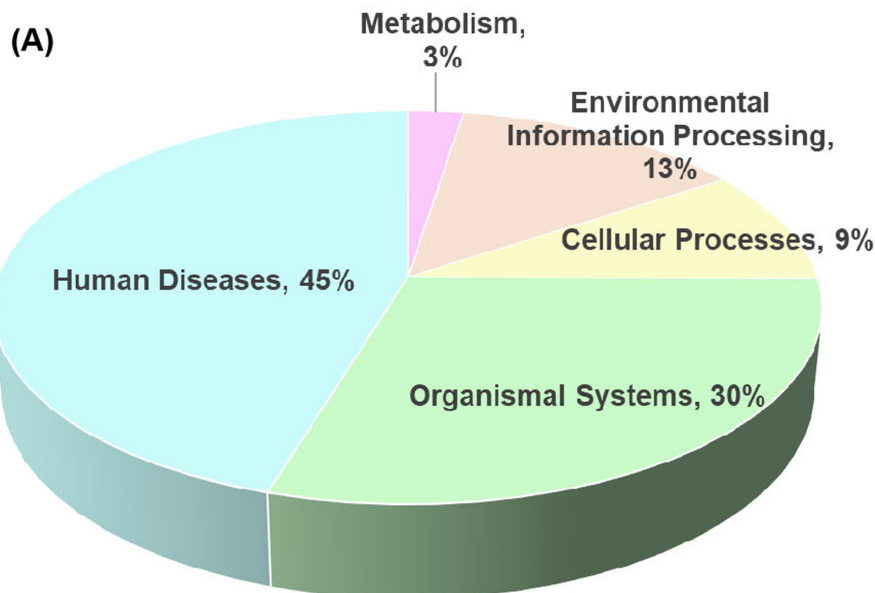


FIGURE 3 | KEGG pathway analysis. (A) Classification of KEGG pathways by categories. (B) Gene ontology analysis of biological process (cyan), cellular components (red), and molecular function (yellow). (C) Network between pathways (orange triangle) related to skin aging and involved genes (cyan rectangle). (D) Schematic diagram of MAPK signaling pathway using KEGG mapper and proteins derived from components (pink) involved in it.

the mean $\pm$ standard error of the mean (SEM). Statistical analysis was accomplished using examination of variance (ANOVA) followed by Tukey's test to determine differences between groups. A significance level of $p < 0.05$ was applied.

3 | Results

3.1 | HPLC Fingerprint of the Extract of *Clerodendrum trichotomum*

HPLC chromatogram of *C. trichotomum* extract (CTE) is shown in Figure 1, including four reference compounds (acteoside, isoacteoside, martynoside, and isomartynoside) [19, 25]. The identification was accomplished by analysis of the UV spectrum, retention order, and retention times of each peak, as depicted in Figure 1A–C.

3.2 | Active Components and Potential Target Genes of CTE

Aforementioned four active components were linked to 265 genes according to STITCH and Swiss Target Prediction DBs, and 19,261 skin aging-derived genes were searched for Gene Cards DB (Data not shown). There was total 237 genes that were the overlap of active component-linked genes and disease-related genes (Figure 2A). Acteoside, isoacteoside, martynoside, and isomartynoside were associated with 147, 88, 90, and 92 skin aging-derived genes, respectively (Figure 2B). The network was visualized in Figure 2C between active components and potential target genes.

3.3 | Protein–Protein Interactions (PPIs)

The protein interaction analysis was performed using STRING database and its topological characteristics were calculated

using “network analyzer” function of Cytoscape software [37]. In PPIs analysis, the top 5% important genes in node, betweenness centrality, and closeness centrality are marked with large red circles in Figure 2B.

3.4 | Signaling Pathways Analysis

As a result of KEGG pathway analysis of genes related to skin aging, KEGG categories were classified with the following ratios: 45% of human disease, 30% of organismal systems, 13% of environmental information processing, 9% of cellular process, and 3% of metabolism (Figure 3A). Gene ontology (GO) analysis, biological process (BP), cellular component (CC), and molecular function (MF) were present in Figure 3B. Among a total of 159 pathways were connected to a total of 73 genes (Figure 3C). Among these seven pathways, the MAPK signaling pathway was most closely related to skin aging, and a total of 32 genes were involved in it. This pathway and its associated genes were represented in (Figure 3D).

3.5 | Effect of CTE Upon UV-B Induced Skin Histology and Signal Transduction Pathways Associated With MAPKs and MMPs

H&E-stained revealed that CTE expend a protective effect compared to UV-B-induced epidermal alterations, as shown in Figure 4A. Masson's trichrome staining results the CTE-treated group exhibited increased collagen fiber abundance and density (Figure 4B). Additionally, CTE on skin hydration was significant, with the TEWL and epidermal thickness being notably decrease in the CTE-treated group shown in Figure 4C,D. CTE administration effectively preserves epidermal thickness and collagen integrity, suggesting its potential as a photoprotective agent. Furthermore, the skin capacitance in the

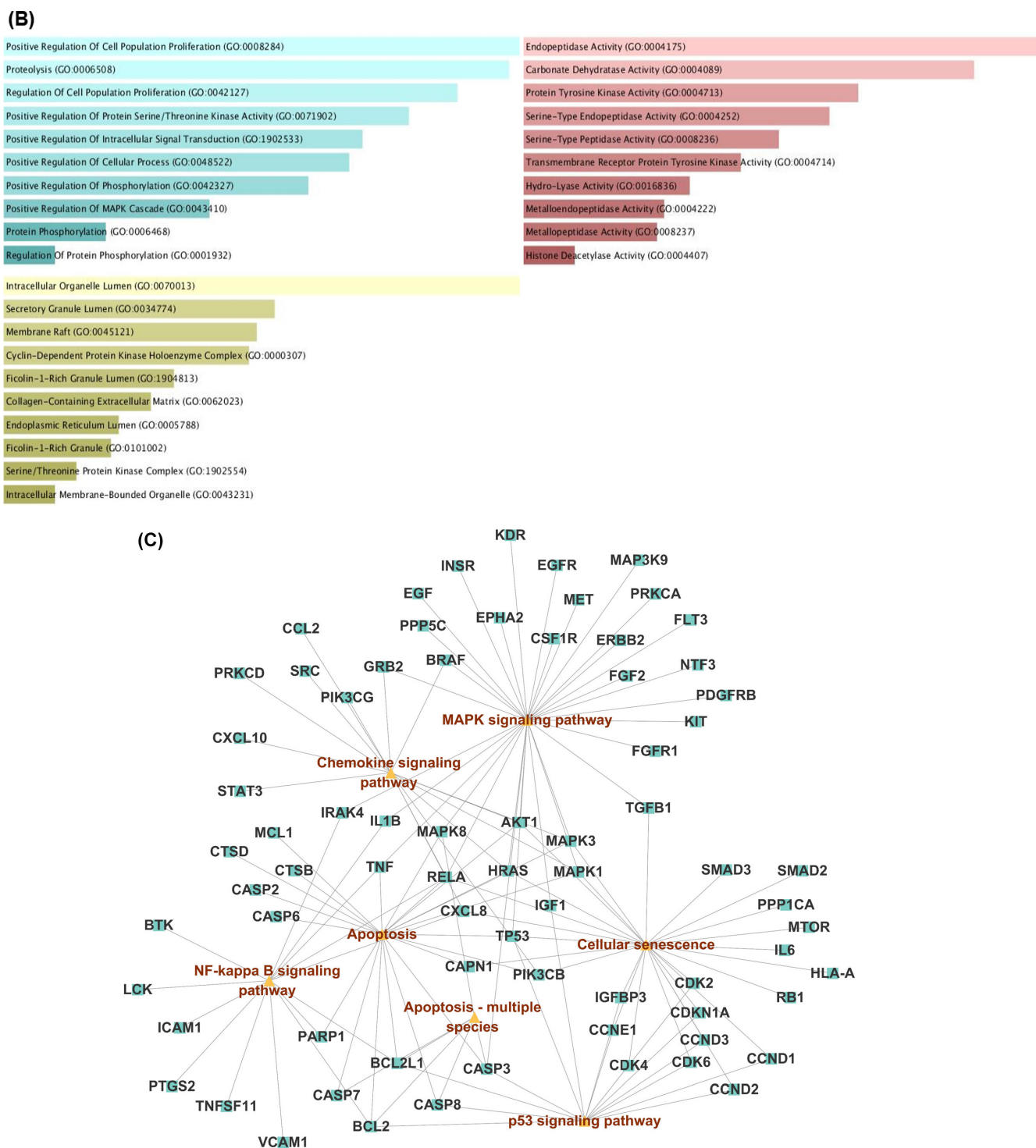


FIGURE 3 | (Continued)

CTE-treated UV-B-exposed group was greater than that in the vehicle-treated UV-B-exposed group, as shown in Figure 4E. For 12 weeks, UV-B exposure of the skin of hairless mice led to an increase in the levels of MMP-1 and MMP-9 by quantifying the protein levels of MMP-1 and MMP-9 in dorsal skin tissue samples using ELISA kits (Figure 4F). CTE administration effectively decreased MMP-1 and MMP-9 levels, which were increased by UV-B irradiation. The effects of CTE modulating MMP expression was assessed by the activation of the MAPKs

signaling pathway. MAPK signaling pathway in regulating MMP expression during UV-B-induced skin photoaging is well established [16]. Western blot analysis revealed a significant reduction in the protein expression of MMP-1 and MMP-9 in the group administered CTE compared to the group treated with vehicle alone after UV-B exposure (Figure 4G). UV-B irradiation led to a notable increase in the phosphorylation levels of MAPK signaling components, CTE significantly attenuated the UV-B-induced phosphorylation of MEK, ERK, JNK, and p38 MAPK,

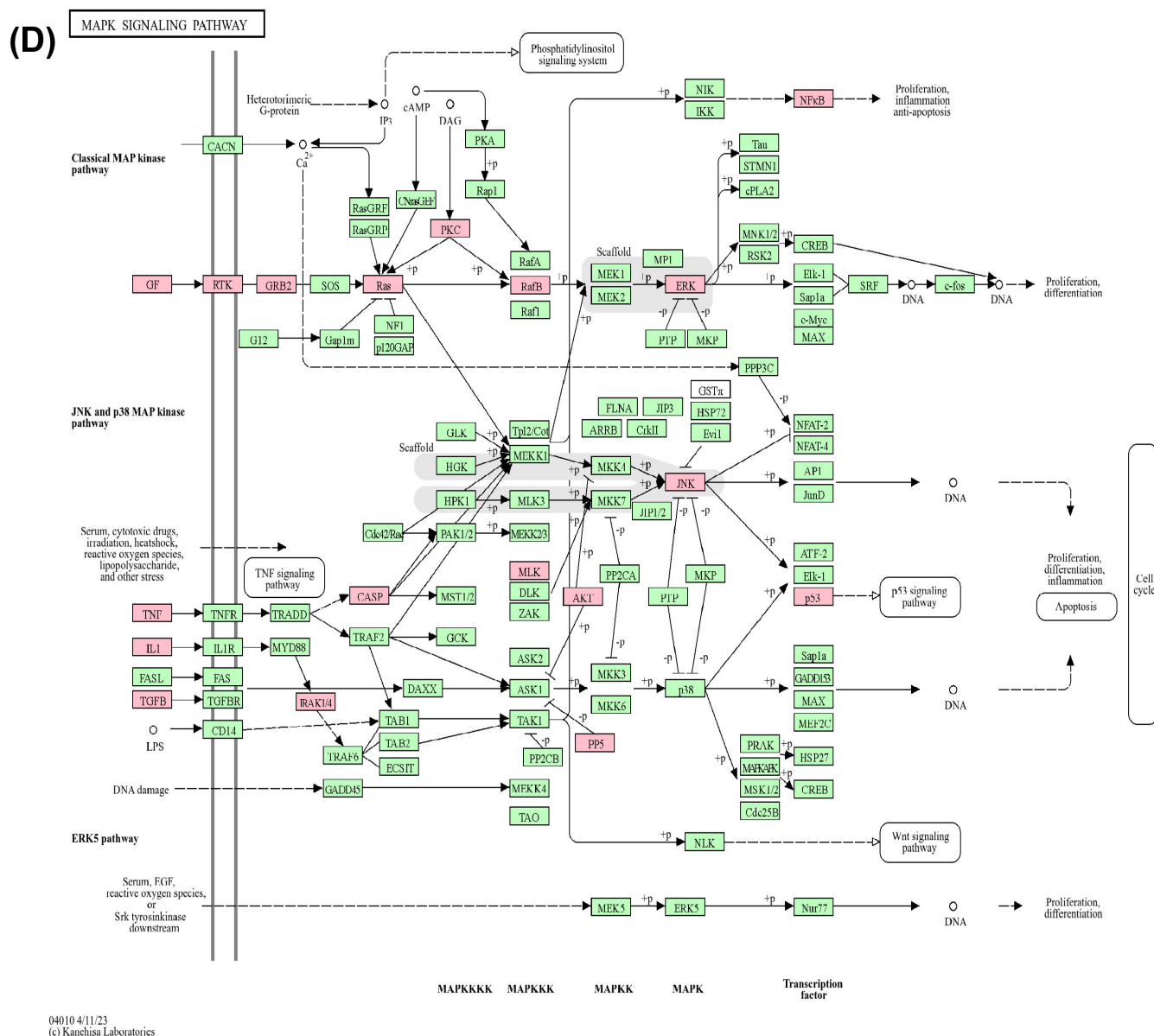


FIGURE 3 | (Continued)

as shown in Figure 4C. These findings elucidated the potential mechanisms by which CTE exerts its inhibitory effects on MMP expression, implicating the modulation of the MAPK signaling pathway.

3.6 | Effects of CTE and Solvent Fractions and Four Compounds Against UV-B-Induced Cell Viability and MMP-1 Secretion in HEKa Cells

CTE exhibited no notable cytotoxicity to cells, as shown in Figure 5A. To explore the protecting effects of CTE and its solvent fractions compared to UV-B-induced cell damage, HEKa cells were treated with varying concentrations of CTE and solvent fractions. Exposure to UV-B resulted in a significant decrease in cell viability (Figure 5B). However, treatment with CTE and its solvent fractions led to an increase in cell viability in a concentration-dependent manner. Besides, UV-B irradiation

of HEKa cells significantly augmented the expression level of MMP-1 in the culture medium (Figure 5C). Four compounds in CTE, exhibited efficacy in inhibiting UV-B-induced MMP-1 secretion, as shown in Figure 5D. Consequently, it can be inferred that the primary components acteoside and isoacteoside are at least more actively involved in mitigating UV-B-induced skin aging in hairless mice.

4 | Discussion

Exposure to UV-B radiation triggers the degradation of ECM apparatuses in the skin by promoting the production of MMPs, indicating that the regulation of MMP activity can be a potential strategy for preventing UV-B induced skin damage. Studies have highlighted the beneficial effects of extracts from plants such as *Aegopodium podagraria* on skin aging [40]. Similarly, extracts from *Eleutherococcus divaricatus*, *Chlorella minutissima*,

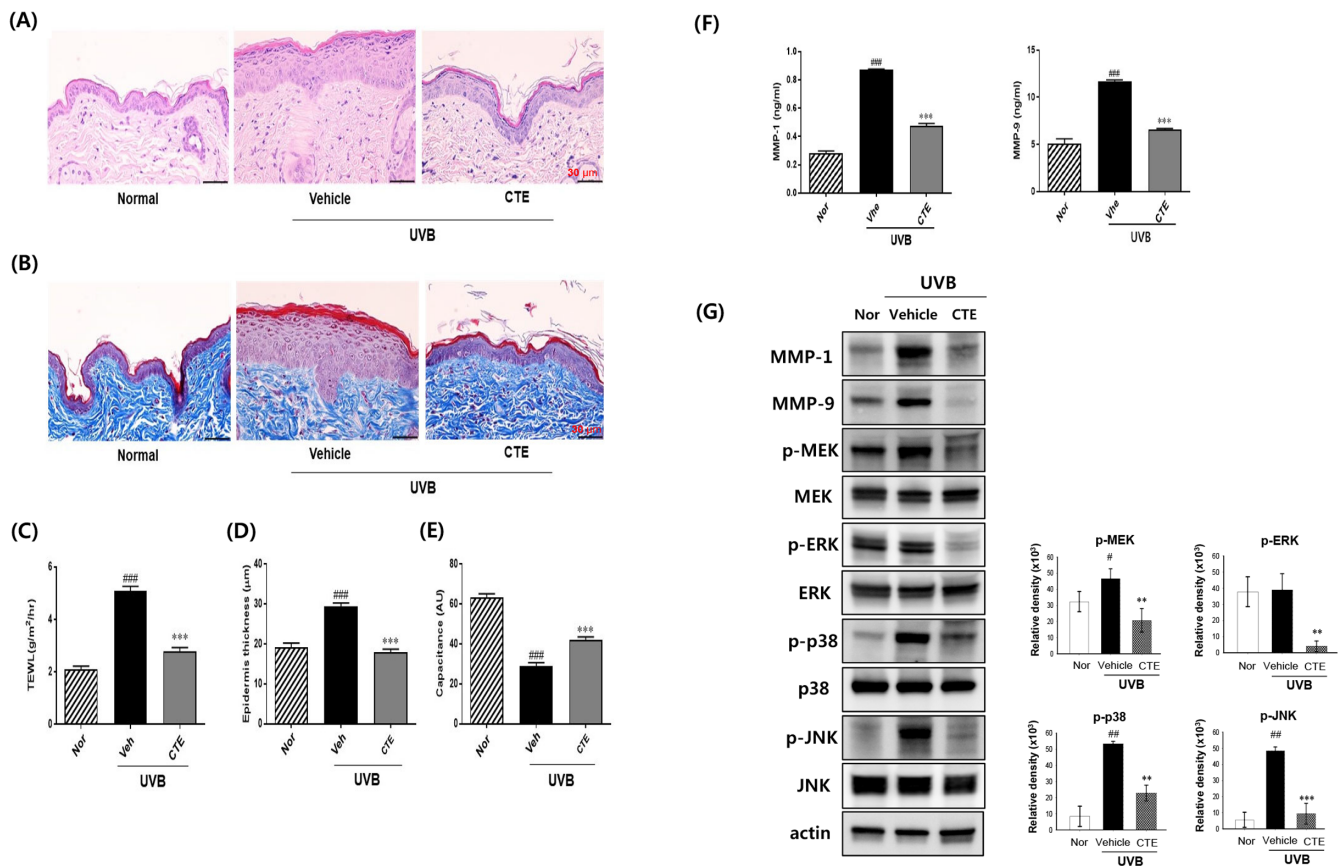


FIGURE 4 | Effect of *Clerodendrum trichotomum* extract (CTE) administration on UV-B-induced skin thickness in hairless mice after 12 weeks of treatment. (A) H&E staining of UV-B-irradiated hairless mouse skin. (B) Masson's trichrome staining demonstrated the protective effect of CTE on collagen fibers (blue). (C) Transepidermal water loss and (D) epidermal thickness of dorsal skin. (E) Skin capacitance of hairless mice. Original magnification, $\times 200$. ### $p < 0.001$ compared to the normal group, and *** $p < 0.001$ compared to the vehicle group. (F) ELISA analysis of MMP-1 and MMP-9 levels in the dorsal skin mice. (G) CTE inhibits MMP-1 and MMP-9, UV-B-induced phosphorylation of ERK, MEK, JNK, and p38 MAP kinases. β -Actin was used as an internal control. Comparison with the normal group is # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, and comparison with the vehicle group is * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar: 30 μm .

Selaginella rossii, and *Vaccinium macrocarpon* have been shown to inhibit MMP-1 and MMP-9 [41–43]. Our results show that CTE treatment notably prevented the expression of MMP-9 and MMP-1. In a previous paper, acteoside and isoacteoside in CTE, which are phenylpropanoids, effectively reduced MMP-1 expression induced by UVB [44]. Martynoside removed free radicals through its antioxidant effects including intracellular DPPH scavenging ability [45] and inhibition of lipid peroxidation [46]. Skin sensitivity and damages epidermal barrier function are connected with aging and are at least partly due to increased production of reactive oxygen species (ROS) [47].

As a result of a network pharmacology analysis based on the aforementioned four active components, it was analyzed that they were related to 237 skin aging genes. Acteoside, isoacteoside, isomartynoside, and martynoside were associated with 147, 88, 92, and 90 skin aging genes, respectively. In the protein–protein interaction analysis of these genes, the genes with elevates grade, nearness significance, and betweenness centrality are central gene in this network. Degree represents the number of connected nodes, so a large value means that it is associated with many genes, and betweenness centrality determines the frequency with which shortest paths between any pair of nodes pass through that node [48]. Nearness centrality

can also be viewed as a measure of the time it takes to sequentially spread information from node to all other accessible nodes [49]. The genes with closeness centrality were GAPDH, AKT1, EGFR, ALB, and TP53. The high betweenness centrality ALB, GAPDH, EGFR, SRC, ESR1, and the genes with high degree were GAPDH, AKT1, TNF, EGFR, and IL6. It has been reported in various papers that these factors are related to skin aging.

By conducting pathway analysis with genes derived from four active components, seven pathways closely related to skin aging were selected: cellular senescence, MAPK signaling pathway, apoptosis, p53, NF- κ B, and chemokine signaling pathway. Several previous studies have reported that increasing procollagen production through the TGF- β /Smad pathway and inhibiting the MAPK, AP-1, and NF- κ B pathways can have an anti-photoaging effect [42, 43, 50]. Phosphorylation of the MAPK signaling pathway mainly involves p38 MAPK, JNK1/2, and ERK1/2 subunits, which participate in the regulation of upstream NF- κ B and p53, leading to the expression and activity of inflammatory factors or UV-induced apoptosis of keratinocyte [51–53]. Previous studies have extensively characterized three MAPK pathways: the mitogen-activated kinase JNK pathway activated by inflammatory cytokines, and the p38 pathway, ERK1/2 pathway activated by cellular stress [54]. Given that MMP-1 expression is

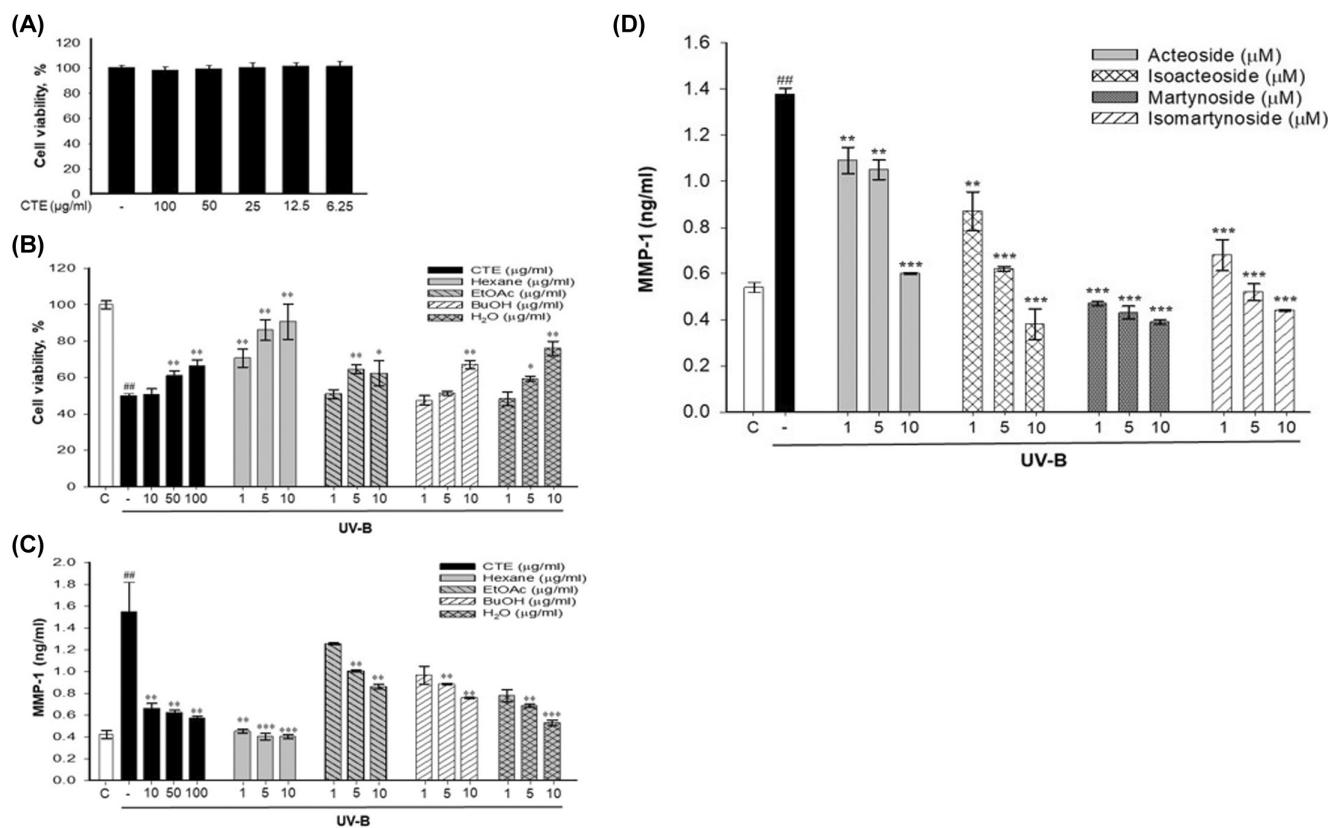


FIGURE 5 | Effects of *Clerodendrum trichotomum* extract (CTE) and solvent fractions (hexane, EtOAc, BuOH, and H₂O) on cell viability and MMP-1 secretion in UV-B-treated HEKa cells. (A) Cytotoxicity of CTE in HEKa cells. (B) CTE on cell viability according to the WST-1 assay. (C) HEKa cells were irradiated or not irradiated with UV-B and treated with various concentrations of CTE and solvent fractions for 48 h. The culture supernatants were collected for MMP-1 ELISA. (D) HEKa cells were irradiated with UV-B and then treated with the indicated concentrations of reference compounds for 48 h. MMP-1 levels were analyzed using an ELISA kit. All the data are presented as the means $\pm$ SDs of triplicate experiments. $^{##}p < 0.01$ compared to the normal control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the UV-B-irradiated control group.

upregulated by MAPK [17, 55], the inhibitory effects of CTE on MMP-1 may be mediated by the inhibition of MAPK-dependent signal transduction. Finally, our results showed that CTE effectively inhibited the MAPKs signaling to diminish the expression of MMP-1 and MMP-9 in UVB-radiation damage skin. Further studies needed to be determined whether martynoside and isomartynoside could restore UVB-induced skin damage via anti-MMP expression through anti-inflammatory, antioxidant, and antiapoptotic effects.

Author Contributions

S. Chae and K.-W. Nam conceptualized and designed the study. K.M. Kim performed the in vitro study. A.-R. Im, S. Choi, and S. Lee performed the animal study. K.-S. Shim, A.Y. Lee, J.W. Hyun, and T. Kim collected the data and performed the data analysis. K.M. Kim and S. Chae wrote the manuscript. All authors approved the submitted version of the article.

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The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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