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# Protective Effects of *Schinus terebinthifolius* Leaf Supercritical Fluid Extract Against UVC-Induced Oxidative Stress: A Comprehensive Gene Expression Study

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## Abstract

Ultraviolet (UV) exposure accelerates skin aging by inducing oxidative stress, extracellular matrix (ECM) degradation, and epidermal barrier dysfunction. This study investigated the protective effects of Brazilian pepper tree (SB), neem (SD), and Vietnamese coriander (PP) leaf extracts obtained by supercritical fluid extraction (SFE) using CO<sub>2</sub> with ethanol as a co-solvent against radiation-induced cellular damage. Among these, SB yielded the greatest amount of extract and exhibited the highest levels of phenolic and flavonoid constituents, including naringin, epicatechin gallate, and rosmarinic acid. These compounds, identified through HPLC profiling, were associated with strong inhibition of collagenase, elastase, and hyaluronidase, and exhibited potent antioxidant activity in the DPPH assay. Under UVC-induced oxidative stress in HaCaT keratinocytes, SB markedly enhanced the mRNA expression of key genes involved in ECM integrity (*COL1A1*, 3.04 ± 0.15-fold), epidermal barrier and hydration (*FLG*, 4.66 ± 0.17-fold; *HAS1*, 1.90 ± 0.14-fold), and cellular defense mechanisms (*SIRT1*, 3.83 ± 0.54-fold), demonstrating superior efficacy to reference antioxidants (EGCG and ascorbic acid) in upregulating key barrier genes like FLG. Overall, the findings highlight SB as the extract with the most comprehensive photoprotective properties and support the use of SFE-derived botanical extracts as promising agents for natural and photoprotective skincare applications.

**Keywords:** supercritical fluid extraction; *Schinus terebinthifolius*; anti-photoaging; UVC-induced oxidative stress; keratinocytes



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## 1. Introduction

Skin aging results from both internal factors, such as genetics and chronological aging, as well as external environmental influences. Extrinsic contributors encompass ultraviolet exposure, environmental pollutants, lifestyle habits, and cigarette smoke [1]. Among these factors, ultraviolet (UV) radiation is considered the primary external driver of photoaging, initiating both structural and functional damage in the skin [2]. Chronic UV exposure disrupts epidermal homeostasis, accelerates wrinkle formation, and contributes substantially to visible aging and increased skin cancer risk [3].

Solar UV radiation is classified into UVC (200–280 nm), UVB (280–320 nm), and UVA (320–400 nm). The UVA spectrum, which accounts for the majority of UV exposure, penetrates into the dermal layer, where it stimulates excessive ROS accumulation, disrupts redox homeostasis, and accelerates the degradation of collagen-rich tissues [2]. UVB constitutes only about 5% of terrestrial UV but carries higher energy. It primarily affects the epidermis, directly damaging DNA and initiating inflammatory cascades. These processes contribute to photoaging and skin carcinogenesis [3]. UVC possesses the highest photon energy and is the most efficient at generating ROS. However, it is naturally blocked by the ozone layer [2]. UV-induced oxidative stress results in excessive ROS production, leading to damage of critical cellular components, including DNA, proteins, and lipids [4]. This oxidative burden subsequently activates matrix metalloproteinases (MMPs), which catalyze the fragmentation of collagen and elastin fibers. This enzymatic breakdown disrupts the structural stability of the extracellular matrix (ECM) and results in a progressive reduction in cutaneous elasticity [5]. In addition, UV radiation impairs epidermal barrier function by reducing the expression of hydration-related genes responsible for skin moisture retention and barrier integrity, leading to dryness and visible signs of aging [6]. Together, these processes contribute to the development of wrinkles, decreased skin elasticity, and the appearance of noticeable signs of aging. UVC efficiently induces ROS generation, DNA damage, and oxidative damage of lipid-rich membranes [7]. Despite its limited physiological relevance in natural sunlight exposure, UVC is widely employed as a robust and highly reproducible experimental model to induce acute oxidative stress and direct DNA damage under controlled conditions [7]. Accordingly, UVC-irradiated HaCaT cells serve as a practical and mechanistically relevant model for investigating antioxidant defenses and assessing anti-photoaging efficacy of natural extracts at the molecular level. In this context, the cumulative evidence of UV-induced damage underscores the need to investigate natural antioxidants and bioactive phytochemicals as potential photoprotective agents. Among these, polyphenols and flavonoids, in particular, exhibit potent radical scavenging activity, inhibit collagen-degrading enzymes, and modulate the expression of genes involved in extracellular matrix (ECM) remodeling, skin hydration, and antioxidant defense [8]. Unlike synthetic compounds with a single mechanism, plant-derived extracts often act synergistically through multiple pathways for broader, more sustainable skin protection [9]. Therefore, the comprehensive identification and characterization of new plant sources rich in phenolic compounds represent a promising strategy for developing effective cosmeceutical and anti-aging formulations [10].

In addition, numerous studies have reported the potential of phytochemicals as anti-aging agents due to their antioxidant, anti-inflammatory, and enzyme-inhibitory activities [11]. Several medicinal plants have been traditionally used in Southeast Asia to treat skin inflammation, promote wound healing, and manage various dermatological conditions. Among them, *Schinus terebinthifolius* Raddi (Sadao Bahrain), *Azadirachta indica* A. Juss. (Sadao), and *Persicaria odorata* (Lour.) Soják (Phak pai) are of particular interest due to their rich phytochemical profiles and potential cosmeceutical applications. These plants are abundant in Thailand and are commonly consumed as fresh culinary herbs in northern

Thai cuisine, reflecting their long-standing traditional use and a history of safe dietary consumption, suggesting a favorable safety profile [12]. The selection of these three species was guided by a shared ethnobotanical and translational rationale. While all are edible medicinal herbs implying a favorable safety profile through long-term dietary exposure, they exhibit complementary phytochemical compositions. This diversity provides a comparative model to investigate how specific bioactive compounds contribute to antioxidant, anti-inflammatory, and skin aging mechanisms under a unified experimental framework.

*S. terebinthifolius* Raddi (locally known in Thailand as Sadao Bahrain; SB) is an American evergreen shrub that is now established across various tropical and subtropical regions, Thailand included [13]. The potent antioxidant and anti-inflammatory effects of the leaves and fruits are attributed to their high content of phenolic constituents, such as *p*-coumaric acid, gallic acid, and rutin [14]. Traditionally, leaves have been used for wound healing and as an antimicrobial rinse, and recent clinical and preclinical studies have further demonstrated their potential benefits in the treatment of inflammatory skin disorders [15–17].

*A. indica* A. Juss. (Sadao, or neem; SD) is a fast-growing deciduous tree reaching 15–20 m in height, characterized by serrated pinnate leaves and small, fragrant white flowers. It is native to the Indian subcontinent. It is now widely cultivated throughout Southeast Asia and tropical regions worldwide. The seeds, leaves, and bark contain high levels of limonoids, such as azadirachtin and nimbidin, which exhibit strong capacities to neutralize oxidative damage, suppress inflammatory responses, and inhibit microbial activity [18]. Neem-based preparations are widely applied in Ayurvedic and traditional Thai medicinal practices for managing eczema, acne, and other chronic skin disorders, with several clinical studies supporting their efficacy in managing inflammatory dermatoses [19].

*P. odorata* (Lour.) Soják (syn. *Polygonum odoratum* Lour.; PP), commonly known in Thailand as “Phak pai,” is a creeping perennial herb reaching 20–40 cm in height, characterized by lanceolate, dark green aromatic leaves with a distinctive peppery scent [20]. Phytochemical profiling has demonstrated high concentrations of polyphenols, with quercetin and catechin being particularly prominent for their antioxidant and anti-inflammatory properties [21,22]. Traditionally, this species is consumed as a culinary herb and applied topically to alleviate skin irritation. Experimental studies have further demonstrated its promising antimicrobial and wound-healing properties [23,24].

Given the rich polyphenol content and diverse bioactive properties of these plants, selecting an extraction method that preserves thermolabile compounds while ensuring high extract purity is essential. Supercritical fluid extraction (SFE) utilizes carbon dioxide under supercritical conditions, providing liquid-like solvating power and gas-like diffusivity. Compared with conventional solvent extraction, SFE operates at lower temperatures, leaves minimal solvent residues, and allows selective recovery of target phytochemicals. These characteristics make SFE particularly suitable for obtaining high-quality antioxidant extracts from medicinal plants while preserving the integrity and bioactivity of thermolabile compounds [25].

Despite the well-documented anti-inflammatory and antioxidant properties of SB, SD, and PP, their comparative phytochemical compositions and the molecular mechanisms underlying their photoprotective effects remain insufficiently elucidated. Most previous studies have focused on individual species, often examining single bioactive compounds or a limited number of biochemical assays, with minimal insight into how their extracts modulate key genes involved in ECM remodeling, skin-barrier function, and antioxidant defense under UV-induced oxidative stress. Importantly, no prior study has systematically compared these three species using SFE, a green and selective extraction technique suitable for producing high-purity extracts enriched in thermolabile polyphenols, which is essential for the rational development of natural photoprotective and anti-aging formulations.

Therefore, to the best of our knowledge, the present study provides the first systematic comparison of the anti-aging properties of SFE-derived extracts from these three plants. We evaluated their polyphenol content, antioxidant capacity, inhibition of skin-degrading enzymes, and gene regulation in UV-irradiated HaCaT keratinocytes, focusing on pathways related to extracellular matrix remodeling (*COL1A1*, *ELN*, *TIMP1*), skin-barrier function (*AQP3*, *FLG*, *HAS1*), oxidative defense (*SOD1*, *CAT*, *GPX*), and longevity (*SIRT1*). Collectively, our findings provide insight into their photoprotective roles and establish their potential as evidence-based candidates for natural anti-aging cosmeceutical development.

## 2. Results

### 2.1. Extract Yield, Quantification of Bioactive Compounds, and Antioxidant Activities of Plant Extracts

Table 1 summarizes the extraction efficiency and phytochemical constituents of the studied plants. Among the species evaluated, SB consistently demonstrated superior extraction efficiency and phytochemical density. It achieved the highest extraction yield ( $7.88 \pm 0.08\%$ ) and contained the greatest concentrations of total phenolics ( $186.26 \pm 1.69$  mg GAE/g extract) and flavonoids ( $133.43 \pm 0.82$  mg RE/g extract).

**Table 1.** Extract yield, Bioactive compound content; total phenolic content (TPC), total flavonoid content (TFC), and the antioxidant capacities of the plant extracts as measured by ABTS and DPPH methods.

| Code | Scientific Name                                       | Yield (%)         | TPC<br>(mg GAE/g Extract) | TFC<br>(mg RE/g Extract) | ABTS<br>(mg TE/g Extract) | DPPH<br>(mg TE/g Extract) |
|------|-------------------------------------------------------|-------------------|---------------------------|--------------------------|---------------------------|---------------------------|
| SB   | <i>Schinus terebinthifolius</i> Raddi (Sadao Bahrain) | $7.88 \pm 0.08^a$ | $186.26 \pm 1.69^a$       | $133.43 \pm 0.82^a$      | $62.61 \pm 1.55^a$        | $20.82 \pm 1.11^a$        |
| SD   | <i>Azadirachta indica</i> (Sadao)                     | $3.75 \pm 0.65^c$ | $76.88 \pm 1.83^b$        | $85.52 \pm 0.30^b$       | $60.89 \pm 0.63^a$        | $10.32 \pm 0.18^b$        |
| PP   | <i>Persicaria odorata</i> (Phak pai)                  | $4.86 \pm 0.26^b$ | $68.77 \pm 0.45^c$        | $131.78 \pm 0.85^a$      | $61.21 \pm 0.94^a$        | $11.54 \pm 0.14^b$        |

Data are presented as mean  $\pm$  SD; SB: *Schinus terebinthifolius* Raddi (Sadao Bahrain) extract; SD: *Azadirachta indica* (Sadao) extract; PP: *Persicaria odorata* (Phak pai) extract; GAE: gallic acid equivalent; RE: rutin equivalent; TE: Trolox equivalent. Different letters indicate significant differences (Tukey's HSD,  $p < 0.05$ ).

In contrast, SD exhibited the lowest extraction yield ( $3.75 \pm 0.65\%$ ) and comparatively lower levels of both phenolics ( $76.88 \pm 1.83$  mg GAE/g extract) and flavonoids ( $85.52 \pm 0.30$  mg RE/g extract). Notably, while PP displayed an intermediate extraction yield ( $4.86 \pm 0.26\%$ ), its flavonoid content ( $131.78 \pm 0.85$  mg RE/g extract) was remarkably high, comparable to that of SB.

Regarding antioxidant capacity, all extracts demonstrated significant radical-scavenging potential. While ABTS assays indicated comparable electron-donating abilities across the three species, distinct differences emerged in the DPPH assay. SB exhibited significantly stronger DPPH radical-scavenging activity ( $62.61 \pm 1.55$  mg TE/g extract), suggesting a superior capacity to neutralize stable free radicals, likely driven by its higher phenolic abundance.

### 2.2. Determination of Phenolic Constituents Using High-Performance Liquid Chromatography (HPLC) Analysis

HPLC analysis revealed distinct phenolic profiles among the three plant extracts, underscoring significant qualitative and quantitative differences (Table 2). The identification of these phenolic compounds was rigorously confirmed by comparing their retention times

with those of authentic reference standards under identical chromatographic conditions and was further validated through the standard addition (spiking) method.

**Table 2.** Phenolic compounds identified in extracts of *Schinus terebinthifolius* (SB), *Azadirachta indica* (SD), and *Persicaria odorata* (PP) determined by HPLC (mg/100 g extract).

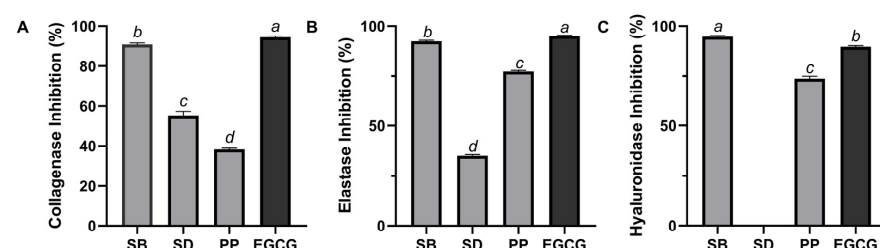
| No. | Compound                | SB<br>(mg/100 g Extract)     | SD<br>(mg/100 g Extract)   | PP<br>(mg/100 g Extract)   |
|-----|-------------------------|------------------------------|----------------------------|----------------------------|
| 1   | Gallic acid             | 193.31 ± 7.59 <sup>a</sup>   | 104.76 ± 4.16 <sup>b</sup> | 97.03 ± 5.59 <sup>b</sup>  |
| 2   | Catechin                | 16.57 ± 0.20 <sup>b</sup>    | 210.84 ± 0.76 <sup>a</sup> | ND                         |
| 3   | Epicatechin             | 301.86 ± 33.66 <sup>a</sup>  | 259.84 ± 2.43 <sup>a</sup> | 52.77 ± 1.06 <sup>b</sup>  |
| 4   | Caffeic acid            | 195.87 ± 0.50 <sup>a</sup>   | ND                         | 4.81 ± 0.26 <sup>b</sup>   |
| 5   | Epicatechin gallate     | 1207.22 ± 11.89 <sup>a</sup> | ND                         | 7.28 ± 1.53 <sup>b</sup>   |
| 6   | <i>p</i> -coumaric acid | ND                           | 34.78 ± 1.74 <sup>b</sup>  | 136.27 ± 7.53 <sup>a</sup> |
| 7   | Naringin                | 4628.30 ± 57.12 <sup>a</sup> | ND                         | 214.12 ± 0.57 <sup>b</sup> |
| 8   | Rosmarinic acid         | 479.04 ± 0.56 <sup>a</sup>   | ND                         | 21.93 ± 0.77 <sup>b</sup>  |
| 9   | <i>o</i> -coumaric acid | 458.76 ± 0.20 <sup>a</sup>   | ND                         | ND                         |

Data are presented as mean ± SD. ND: not detected (<LOD); SB: *Schinus terebinthifolius* Raddi (Sadao Bahrain) extract; SD: *Azadirachta indica* (Sadao) extract; PP: *Persicaria odorata* (Phak pai) extract. Different letters indicate statistically significant differences (Tukey's HSD,  $p < 0.05$ ). Limit of detection (LOD) of catechin = 0.030 mg/g, caffeic acid = 0.006 mg/g, epicatechin gallate = 0.423 mg/g, *p*-coumaric acid = 0.007 mg/g, naringin = 0.014 mg/g, rosmarinic acid = 0.088 mg/g, *o*-coumaric acid = 0.005 mg/g.

SB exhibited the most diverse phenolic composition, predominantly characterized by high concentrations of naringin, alongside substantial levels of epicatechin gallate and rosmarinic acid. In contrast, SD presented a more focused phenolic signature, with epicatechin serving as the primary constituent, supported by catechin and gallic acid, while lacking several compounds found in SB. PP displayed a unique profile distinguished by moderate concentrations of naringin and *p*-coumaric acid, complemented by lower levels of other phenolic acids. These variations in phytochemical composition provide a chemical basis for the differential antioxidant and biological activities observed among the extracts. Method validation parameters including precision, linearity, calibration curves, and retention times are presented in Supplementary Table S1.

### 2.3. Determination of Enzyme Inhibition

The inhibitory profiles of the plant extracts against collagenase, elastase, and hyaluronidase are illustrated in Figure 1. Among the evaluated species, SB exhibited superior broad-spectrum efficacy, suppressing the activity of all three enzymes with potency comparable or superior to the positive control, EGCG. Specifically, SB achieved inhibition rates of  $90.67 \pm 0.75\%$ ,  $92.31 \pm 0.48\%$ , and  $98.58 \pm 0.72\%$  against collagenase, elastase, and hyaluronidase, respectively.

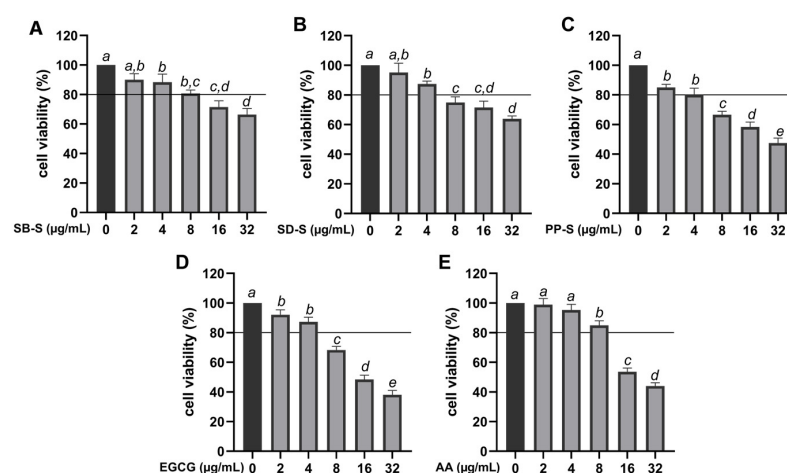


**Figure 1.** Inhibitory activities of *Schinus terebinthifolius* (SB), *Azadirachta indica* (SD), and *Persicaria odorata* (PP) (0.1 mg/mL) extracts and EGCG (250  $\mu$ M) against collagenase, elastase, and hyaluronidase. The inhibition activities for collagenase (A), elastase (B), and hyaluronidase (C) are presented. Data are presented as mean ± SD ( $n = 3$ ). Statistically significant differences between groups ( $p < 0.05$ ) are indicated by different superscript letters.

In contrast, SD and PP displayed distinct selectivity profiles. While SD showed negligible inhibitory potential against hyaluronidase, PP demonstrated moderate efficacy ( $73.67 \pm 1.30\%$ ) against this target. Notably, in the hyaluronidase assay, SB significantly outperformed EGCG ( $89.50 \pm 0.60\%$ ), identifying it as a robust multi-target candidate capable of mitigating extracellular matrix (ECM) degradation through the simultaneous inhibition of key structural remodeling enzymes.

#### 2.4. Cytotoxicity Assessment

The cytotoxicity of the extracts was assessed using a dose–response SRB assay HaCaT keratinocytes over a concentration range of 2–32  $\mu\text{g/mL}$ . As shown in Figure 2A–C, all three extracts exhibited concentration-dependent effects on cell viability. Importantly, cell viability remained above 80% at concentrations up to 4  $\mu\text{g/mL}$  for all ex-tracts, establishing a non-cytotoxic threshold within this range. Based on these dose–response profiles, 4  $\mu\text{g/mL}$  was defined as the maximum non-toxic concentration (MNTC) and was selected as a conservative working concentration for subsequent gene expression analyses to ensure comparability among the three plant species and to avoid confounding effects related to cytotoxicity during this initial comparative screening.



**Figure 2.** Dose–response assessment of HaCaT cell viability via SRB assay following treatment with plant extracts and standard antioxidants. (A–C) Cell viability following exposure to *Schinus terebinthifolius* (SB), *Azadirachta indica* (SD), and *Persicaria odorata* (PP) extracts at 2–32  $\mu\text{g/mL}$ . (D,E) Viability profiles of HaCaT cells exposed to reference antioxidants, epigallocatechin gallate (EGCG) and ascorbic acid (AA) at equivalent concentrations. Values represent mean  $\pm$  standard deviation from triplicate experiments ( $n = 3$ ). Cells incubated with the vehicle control (culture medium containing 0.1% DMSO) served as the baseline (100% viability). Distinct letters above bars indicate significant differences ( $p < 0.05$ ) calculated by one-way ANOVA with Tukey’s post hoc test.

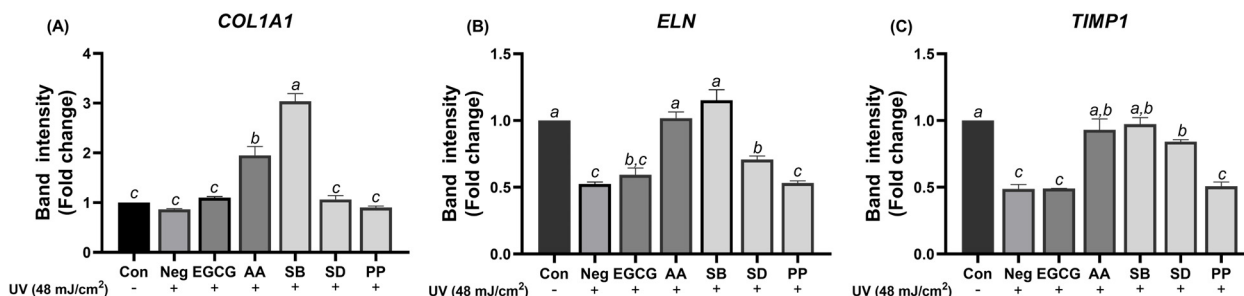
#### 2.5. Modulation of Gene Expression by Plant Extracts

HaCaT keratinocytes were exposed to UV irradiation at  $48 \text{ mJ/cm}^2$  to induce oxidative stress-mediated cellular damage, corresponding to approximately 50% cell viability. The effects of the plant extracts and reference antioxidants on gene expression related to extracellular matrix (ECM) remodeling, skin barrier function, and antioxidant defense were subsequently evaluated in UV-irradiated cells. Gene expression levels were expressed as fold changes relative to untreated controls and normalized to the GAPDH housekeeping gene.

##### 2.5.1. ECM-Related Genes (*COL1A1*, *ELN*, and *TIMP1*)

UV exposure significantly reduced the expression of ECM-related genes (*COL1A1*, *ELN*, and *TIMP1*), confirming UV-induced disruption of extracellular matrix homeostasis. Among all treatments, SB was the most effective, markedly increasing *COL1A1* expression

to  $3.04 \pm 0.15$ -fold, which was significantly higher than both reference antioxidants. Additionally, SB restored *TIMP1* to baseline levels  $0.97 \pm 0.05$ -fold and partially recovered *ELN* expression  $1.15 \pm 0.08$ -fold. In contrast, SD and PP exerted minimal effects on ECM-related genes. Ascorbic acid primarily improved *TIMP1* expression  $0.97 \pm 0.06$ -fold, whereas EGCG showed limited overall activity (Figure 3A–C).



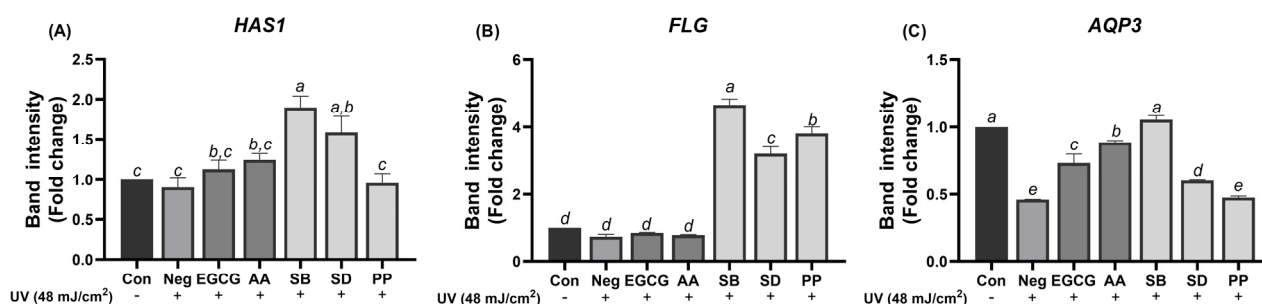
**Figure 3.** Modulatory effects of the plant extracts on the mRNA expression of extracellular matrix (ECM)-associated genes in UV-exposed HaCaT keratinocytes. Expression levels of the ECM-related genes (A) *COL1A1*, (B) *ELN*, and (C) *TIMP1*. Before being exposed to 48 mJ/cm<sup>2</sup> of UV irradiation, cells were incubated with plant extracts (4 µg/mL) or standard antioxidants (epigallocatechin gallate: EGCG, ascorbic acid: AA). Con = untreated control; Neg = negative control (UV irradiation only); SB, SD, PP = extracts from *Schinus terebinthifolius*, *Azadirachta indica*, and *Persicaria odorata*, respectively. Relative gene expression was calculated using GAPDH as internal control. Values represent the mean  $\pm$  SD. Different letters indicate significant differences ( $p < 0.05$ ) determined by one-way ANOVA with Tukey's post hoc test.

#### 2.5.2. Skin Barrier-Related Genes (*HAS1*, *FLG*, and *AQP3*)

UV irradiation significantly reduced the expression of genes associated with skin hydration and barrier integrity (*HAS1*, *FLG*, and *AQP3*).

Among all treatments, SB showed the strongest protective effect. It markedly increased *FLG* ( $4.66 \pm 0.17$ -fold) and *HAS1* expression ( $1.90 \pm 0.14$ -fold), while partially restoring *AQP3* levels ( $1.05 \pm 0.03$ -fold). This indicates a coordinated improvement across all three barrier markers.

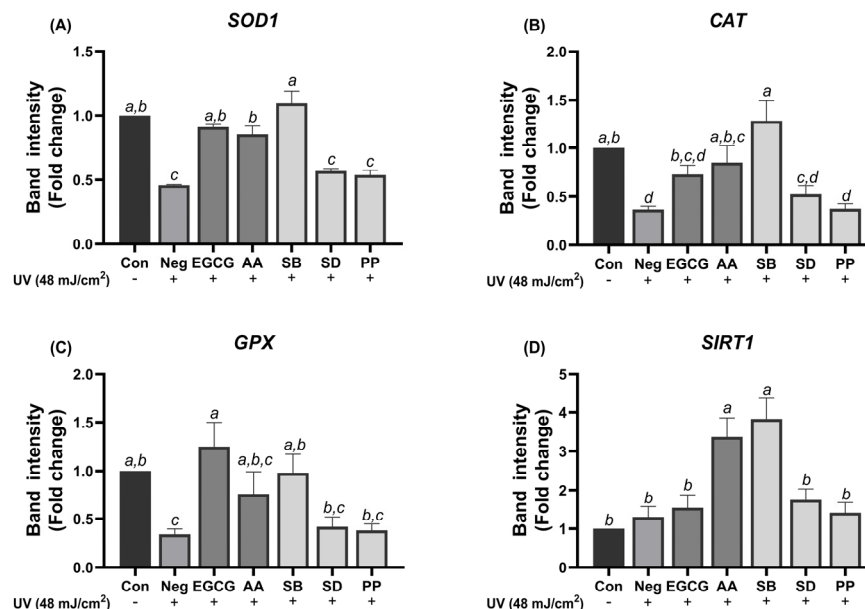
In contrast, SD and PP were selective; they primarily improved *FLG* expression but had limited effect on *HAS1* and *AQP3*. Ascorbic acid and EGCG showed only mild effects on these genes overall (Figure 4A–C).



**Figure 4.** Effects of the plant extracts on the mRNA expression of skin-barrier-related genes: (A) *HAS1*, (B) *FLG*, and (C) *AQP3*. HaCaT cells were pre-treated with plant extracts (4 µg/mL) or reference standards epigallocatechin gallate (EGCG) and ascorbic acid (AA) before exposure to UV irradiation (48 mJ/cm<sup>2</sup>). Con = untreated control; Neg = negative control (UV irradiation only); SB, SD, PP = extracts from *Schinus terebinthifolius*, *Azadirachta indica*, and *Persicaria odorata*, respectively. Relative gene expression was calculated using GAPDH as internal control. Values represent the mean  $\pm$  SD. Different letters indicate significant differences ( $p < 0.05$ ) determined by one-way ANOVA with Tukey's post hoc test.

### 2.5.3. Antioxidant Response Genes (*SOD1*, *CAT*, *GPX*, and *SIRT1*)

UV irradiation markedly suppressed the expression of antioxidant response genes, indicating compromised cellular redox homeostasis in HaCaT keratinocytes (Figure 5A–D). Among the plant extracts, SB demonstrated the most comprehensive activation of antioxidant defense pathways, markedly enhancing the expression of *SOD1*, *CAT*, and *SIRT1*.



**Figure 5.** Effects of plant extracts on mRNA expression of antioxidant related genes: (A) *SOD1*, (B) *CAT*, (C) *GPX*, (D) *SIRT1*. HaCaT cells were pre-treated with plant extracts (4 µg/mL) or epigallocatechin gallate (EGCG) and ascorbic acid (AA) before exposure to UV irradiation (48 mJ/cm²). Con = untreated control; Neg = negative control (UV irradiation only); SB, SD, PP = extracts from *Schinus terebinthifolius*, *Azadirachta indica*, and *Persicaria odorata*, respectively. Relative gene expression was calculated using *GAPDH* as internal control. Values represent the mean  $\pm$  SD. Different letters indicate significant differences ( $p < 0.05$ ) determined by one-way ANOVA with Tukey's post hoc test.

In particular, SB strongly upregulated *SIRT1*  $3.83 \pm 0.54$ -fold, suggesting a potential role in regulating stress resistance and cellular longevity-associated pathways. SB also effectively restored *SOD1*  $1.10 \pm 0.09$ -fold and *CAT*  $1.28 \pm 0.21$ -fold expression, supporting reinforcement of endogenous antioxidant defenses.

In contrast, EGCG exhibited a distinct and selective effect on *GPX*  $1.25 \pm 0.25$ -fold, inducing the strongest upregulation among all treatments, whereas SD and PP showed limited modulation of antioxidant-related genes overall. Ascorbic acid exerted moderate effects, primarily enhancing *SIRT1* expression (Figure 5A–D).

Overall, the SB extract demonstrated the most prominent protective effects against UV-induced damage by significantly enhancing the expression of genes related to ECM remodeling (*COL1A1*, *ELN*, *TIMP1*), skin hydration and barrier function (*HAS1*, *FLG*, *AQP3*), and antioxidant defense (*SIRT1*, *SOD1*, *CAT*). In comparison, ascorbic acid and EGCG exerted moderate effects, with EGCG showing a distinct and selective impact on *GPX* expression.

## 3. Discussion

The present study provides a comprehensive evaluation of the phytochemical composition, antioxidant potential, enzyme-inhibitory activities, and gene-regulatory effects of three medicinal plant extracts: *Schinus terebinthifolius* (SB), *Azadirachta indica* (SD), and *Persicaria odorata* (PP), obtained by supercritical fluid extraction (SFE) using ethanol as a

co-solvent. Among these extracts, SB exhibited the greatest extraction yield, total phenolic content, and total flavonoid content, reflecting its superior antioxidant activity. While SB showed the numerically highest ABTS antioxidant capacity, no significant difference was observed among the extracts. In contrast, SB demonstrated significantly stronger DPPH scavenging activity than the other extracts. These results indicate that SB accumulates more phenolics and contains compounds with potent radical-scavenging abilities, consistent with earlier reports of *S. terebinthifolius* phenolic richness and DPPH activity [26] and extend those observations by demonstrating comparable performance in SFE extracts. HPLC analysis confirmed that SB is rich in naringin, epicatechin gallate, and rosmarinic acid, well-known antioxidants with protective roles against oxidative stress, which possess several hydroxyl groups capable of donating hydrogen atoms to balance reactive oxygen species [27–29]. Previous studies have shown that combinations of such flavonoids often exert synergistic antioxidant effects, which might elucidate the superior radical-scavenging capacity observed in the SFE extract [30].

The inhibitory assays against collagenase, elastase, and hyaluronidase revealed that SB exerted broad-spectrum and potent enzyme inhibition. These enzymes are central mediators of skin aging, as they mediate the breakdown of collagen, elastin, and hyaluronic acid [31]. SB strongly inhibited collagenase and elastase. It also showed near-complete inhibition of hyaluronidase. This suggests SB can preserve structural proteins and maintain extracellular matrix integrity. Beyond the biochemical assays, analysis of gene expression provided key mechanistic insights. SB significantly upregulated ECM-related genes (*COL1A1*, *ELN*, *TIMP1*), indicating its ability to stimulate collagen and elastin synthesis while suppressing matrix degradation. Polyphenols such as epicatechin gallate and rosmarinic acid have been reported to promote ECM remodeling and inhibit UV-induced MMP activation, which supports our findings [32,33]. In addition, SB strongly upregulated the expression of hydration and barrier-related genes (*HAS1*, *FLG*, *AQP3*), suggesting restoration of skin moisturization and barrier function after UV-induced suppression. Specifically, the high induction of *FLG* expression reflects the potential of SB to improve epidermal differentiation and resilience. Supporting this, previous studies have demonstrated that natural flavonoids can enhance hyaluronic acid synthesis by upregulating *HAS1* and stimulating aquaporin-3 expression, thereby improving skin hydration and barrier recovery [34]. Notably, this transcriptional activation of *HAS1* was accompanied by the near-complete inhibition of hyaluronidase activity observed in our enzymatic assays, indicating that SB exerts a dual-protective mechanism in maintaining hyaluronic acid homeostasis by promoting its biosynthesis while preventing its degradation. Such a coordinated action is less commonly reported for single compounds and may underlie the superior moisturizing and anti-aging potential of SB, particularly for dry and barrier-compromised skin.

Moreover, SB markedly induced antioxidant defense genes, highlighting the engagement of a longevity-associated regulatory axis. Notably, the strongest activation was observed for *SIRT1*, a nuclear  $\text{NAD}^+$ -dependent class III histone deacetylase that plays a central role in cellular defense against oxidative stress by regulating apoptosis, cellular senescence, and proliferation, thereby contributing to metabolic homeostasis, cell survival, differentiation, and protection against UV-induced skin aging [35]. Flavonoids, such as rosmarinic acid, gallic acid, and naringin, have been shown to activate *SIRT1* signaling, a pathway associated with anti-aging effects [36–38]. These results align with previous in vitro studies demonstrating that plant-derived polyphenol-rich extracts can mitigate skin aging by modulating oxidative stress-responsive and longevity-associated genes, including *SIRT1*, while simultaneously enhancing collagen biosynthesis [39].

Importantly, among the quantified phytochemicals, naringin was the most abundant constituent in SB, and correlated with the strongest *SIRT1* activation observed in this study.

Supporting this link, previous *in silico* studies have suggested that flavonoids like naringin can interact directly with the *SIRT1* binding region [40,41]. While this suggests a potential contribution from naringin, the robust biological response is likely attributable to the synergistic effect of the extract's complex phytochemical profile rather than a single constituent. In addition to naringin, SB contains substantial levels of epicatechin gallate and rosmarinic acid, both well-documented for their ability to inhibit matrix metalloproteinases (MMPs) and mitigate UV-induced oxidative stress [42,43]. The extract also features significant concentrations of epicatechin, a flavonoid known to enhance cellular antioxidant status and decrease intracellular ROS levels [44]. Furthermore, the presence of epicatechin, alongside catechin and caffeic acid, broadens the bioactive profile of SB; these compounds have been reported to upregulate genes associated with extracellular matrix (ECM) stability and antioxidant defense [45,46]. Together with *o*-coumaric acid [47], these constituents likely act in a coordinated manner to drive the pronounced upregulation of *COL1A1* and *TIMP1* observed herein. This phenomenon exemplifies the 'entourage effect,' wherein the collective interaction of phytochemicals confers a more comprehensive photoprotective activity than that of any single purified compound.

SB also restored *SOD1* (superoxide dismutase 1) and *CAT* (catalase) expression, confirming its ability to reinforce enzymatic antioxidant defenses. Beyond its role in redox regulation, *SIRT1* is increasingly recognized as a master regulator of extracellular matrix homeostasis. *SIRT1* activation has been reported to suppress NF- $\kappa$ B-mediated inflammatory signaling and downstream *MMP* expression, thereby indirectly preserving collagen integrity. In this context, the remarkable upregulation of *COL1A1* and *TIMP1* observed in our study might be partially orchestrated by *SIRT1* activation, suggesting a functional crosstalk between antioxidant signaling and ECM remodeling pathways in SB-treated keratinocytes.

Although EGCG (epigallocatechin gallate) demonstrated the most pronounced effect on *GPX* (glutathione peroxidase) expression, consistent with previous reports on its role as a strong inducer of *GPX* activity [48], SB induced a broader antioxidant response, pointing to synergistic activation of multiple protective pathways. Ascorbic acid exerted moderate effects on *COL1A1*, *ELN*, and *TIMP1* [49], as well as on *SIRT1* [50]. Unlike these reference standards which showed selective efficacy, SB exhibited a more coordinated modulation across diverse signaling axes. Taken together, these findings indicate that SB exhibits not only superior antioxidant capacity but also exerts a multifaceted photoprotective effect, in which *SIRT1* activation, which is potentially associated with the high naringin content alongside other synergistic polyphenols, may act as a central regulatory hub linking antioxidant defense, ECM remodeling, and epidermal homeostasis. Notably, the concurrent stimulation of collagen biosynthesis (*COL1A1*) and reinforcement of anti-degradative control (*TIMP1*), together with strong inhibition of collagenase and elastase activities, suggests that SB may restore ECM homeostasis by coordinately enhancing anabolic processes while restraining catabolic degradation. Such balanced regulation is critical for maintaining dermal structural integrity during skin aging. Consistent with previous studies, naringenin (the aglycone of naringin), which was found at the highest levels in SB, has been shown to bind to the N-terminal domain (NTD) of the *SIRT1* enzyme. Computational docking studies by other researchers reveal that naringenin occupies three binding sites identical to those of resveratrol, forming hydrogen bonds with residue E230 and targeting key residues Q222 and N226 [41]. Similarly, the role of rosmarinic acid, the phenolic compound in SB in this regulatory network is supported by prior docking simulations, which indicate that rosmarinic acid exhibits high binding affinity for the catalytic domains of MMP-9. By forming stable hydrogen bonds with residues such as Tyr248 and His364, RA effectively inhibits enzymatic degradation [51]. Furthermore, other phenolic compound identified in SB extract such as epicatechin has been documented to anchor within the MMP-1 active

site, coordinating with the catalytic zinc ion [52]. Consequently, these established molecular interactions reported in the literature provide a robust structural rationale for our observed preservation of *COL1A1* and *ELN* expression. This further reinforces the concept of the ‘entourage effect,’ where the combined action of these documented bioactives confers a more comprehensive photoprotective activity than any single compound.

The pronounced cellular responses elicited by SB may also be attributed, at least in part, to the extraction strategy employed. Supercritical fluid extraction (SFE), particularly when combined with a polar co-solvent, is known to preferentially enrich non-polar to moderately polar phytochemicals, including lipophilic flavonoids and phenolic derivatives, which often exhibit superior membrane permeability. The enrichment of compounds such as naringin and epicatechin gallate in the SFE-derived extract may therefore facilitate enhanced cellular uptake, providing a plausible explanation for the robust gene-regulatory effects observed in HaCaT cells. Overall, these findings support a multifaceted mode of action for SB, in which coordinated modulation of antioxidant defense, ECM remodeling, and hydration/barrier pathways converge to restore skin homeostasis. This integrated response reflects the synergistic potential of the phytochemical complex inherent in the extract, allowing it to modulate multiple biological targets simultaneously.

Regarding the other extracts, while *A. indica* (SD) showed moderate antioxidant potential, it failed to exhibit comparable enzyme-inhibitory or gene-regulatory effects to SB. In contrast, *P. odorata* (PP) exhibited selective anti-aging benefits, which can be attributed to its diverse phytochemical composition. The phytochemical profile of this extract is characterized by a variety of phenolic and flavonoid derivatives, such as caffeic, gallic, rosmarinic, and *p*-coumaric acids, alongside naringin and epicatechin gallate. These constituents are widely recognized for their robust antioxidant, anti-inflammatory, and dermo-protective capabilities. Furthermore, the literature indicates that *P. odorata* possesses additional bioactive molecules, including (+)-catechin, methyl gallate, and volatile compounds like n-dodecanal and  $\alpha$ -humulene, as well as eupatoriochromene and anthraquinone. Such diverse chemical components contribute to its broad spectrum of pharmacological activities, ranging from antimicrobial and anticancer to potent radical scavenging effects [20].

Several limitations should be acknowledged in the present study. First, this investigation was performed exclusively *in vitro* using human keratinocyte (HaCaT) cells, which may not fully represent the complex biological processes of living human skin particularly the intricate crosstalk between different cell types. Second, our findings were limited to the mRNA level; therefore, protein expression and functional activity were not confirmed in this stage of research. Finally, the stability and skin permeation of the SB extract in topical formulations were not evaluated, which remains a necessary step before clinical or commercial application.

To address these limitations, we are planning a follow-up study to validate our key findings specifically the upregulation of *SIRT1*, *COL1A1*, and *FLG* in primary human dermal fibroblasts (HDFs) and human epidermal keratinocytes (HEKa). This subsequent work will focus on protein-level confirmation (via Western blot or ELISA) and functional assays to fully establish the mechanistic basis of the observed effects. Furthermore, developing the SB extract into a nanoemulsion system could be a viable strategy to enhance its delivery. Previous studies have successfully utilized ultrasound-assisted nanoemulsification to improve the physical stability and bioactivity of natural anti-aging oils [53], suggesting a promising approach that could be adapted for our SFE extracts. Future clinical studies will also be necessary to verify the protective effects of SB on human skin and to determine optimal concentrations for topical use.

## 4. Materials and Methods

### 4.1. Chemicals and Reagents

For the antioxidant evaluations, reagents such as rutin, Trolox, DPPH, and ABTS were obtained from Sigma-Aldrich (St. Louis, MO, USA). The same manufacturer supplied the substrates for enzymatic activity assays, specifically N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAPVN) and N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA). Additionally, sulforhodamine B (SRB), used to assess cytotoxicity, was also procured from Sigma-Aldrich. Folin–Ciocalteu’s phenol reagent and K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (potassium persulfate) were provided by Loba Chemie (Mumbai, India). The reference antioxidant EGCG (epigallocatechin gallate) was purchased from Biosynth (Louisville, KY, USA). RCI Labscan Ltd. (Bangkok, Thailand) supplied all analytical-grade solvents. The reference standards of gallic acid, catechin, epicatechin, caffeic acid, epicatechin gallate, *p*-coumaric acid, naringin, rosmarinic acid, and *o*-coumaric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA; purity ≥ 98%; HPLC grade). For cell culture, HaCaT cells were maintained in Dulbecco’s modified eagle medium (DMEM) enriched with fetal bovine serum (FBS) and a 1% antibiotic cocktail (100 U/mL penicillin and 100 mg/mL streptomycin), all obtained from Gibco Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Regarding gene expression studies, total RNA was isolated using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA), while the MyTaq™ One-Step RT-PCR Kit (Bioline Meridian Bioscience, Memphis, TN, USA) was utilized for the subsequent molecular analysis.

### 4.2. Extract Preparation

Fresh leaves of three plants, *Schinus terebinthifolius* (SB), *Azadirachta indica* (SD), and *Persicaria odorata* (PP), were purchased from Warorot market in Chiang Mai, Thailand, in January 2023. The Pharmaceutical and Natural Products Research and Development Unit at Chiang Mai University’s Faculty of Pharmacy verified and stored voucher specimens (SB: PNPRDU66015, SD: PNPRDU66016, and PP: PNPRDU66017).

Leaves were rinsed with distilled water, shade-dried for three days, and oven-dried at 50 °C for 3 days until a constant weight was obtained (moisture < 10%). Subsequently, the dried materials were ground in a stainless-steel grinder and sieved through a 40-mesh screen to achieve a uniform fine powder. To protect from light and moisture, the powder was stored in airtight containers until extraction.

SFE was conducted using a Spe-ed™ SFE-2 system (Applied Separations, Allentown, PA, USA). For each plant, 10 g of powdered leaves were mixed with 2.5 g of an inert dispersing matrix and 20 mL of 95% (*v/v*) ethanol as a co-solvent, then loaded into the extraction vessel and secured with glass wool (2.0 g) at both ends to ensure uniform fluid distribution. The extraction process was initiated with a static extraction period of 20 min at an operating pressure of 300 bar and an oven temperature of 40 °C. This was followed by a 15 min dynamic extraction phase with a constant CO<sub>2</sub> flow rate of 3.0 L/min. To prevent extract accumulation and clogging at the restrictor, the micrometering valve temperature was maintained at 110 °C. The separator temperature was controlled at 25 ± 2 °C throughout the collection process. After extraction, the SFE extracts were collected, and the ethanol was subsequently removed under reduced pressure using a rotary evaporator at 40 °C. Finally, the extracts of SB, SD, and PP were transferred to amber glass vials and stored at 4 °C until further analyses.

### 4.3. Quantity of Bioactive Compound

#### 4.3.1. Determination of Total Phenolic Content (TPC)

The Folin–Ciocalteu method was employed to evaluate the phenolic content of the extracts [54]. Briefly, each sample (1.0 mg/mL) or standard (20 µL) was reacted with 100 µL

of 10% Folin–Ciocalteu reagent within a 96-well plate. Subsequently, 80  $\mu\text{L}$  of 7.5% (*w/v*) sodium carbonate was added to the mixture. The reaction was allowed to proceed for 2 h at room temperature shielded from light. Measurement of absorbance was performed at 765 nm (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). TPC values were calculated based on a gallic acid standard curve and expressed in mg GAE/g extract.

#### 4.3.2. Determination of Total Flavonoid Content (TFC)

Flavonoid concentration was measured using a modified aluminum chloride assay based on the protocol by Herald et al. [55]. Briefly, distilled water (100  $\mu\text{L}$ ) was initially added to the wells, followed by sodium nitrite (10  $\mu\text{L}$ , 50 g/L) and the extract (25  $\mu\text{L}$ , 1.0 mg/mL). The mixture was incubated for 5 min before the addition of 15  $\mu\text{L}$  of  $\text{AlCl}_3$  (100 g/L). After standing for 6 min, the solution was neutralized with 50  $\mu\text{L}$  of 1 mol/L NaOH and diluted with an additional 50  $\mu\text{L}$  of water. Following a brief 30 s shaking period, absorbance at 510 nm was determined using a SPECTROstar Nano reader (BMG Labtech, Ortenberg, Germany). Results were calculated as rutin equivalents (mg RE/g extract) based on a prepared calibration curve.

#### 4.3.3. Identification and Quantification of Polyphenols by HPLC

Ten milligrams of sample were dissolved in 95% methanol to prepare a stock solution at a concentration of 10 mg/mL. For polyphenol analysis, the samples were diluted to 1 mg/mL in working solution and filtered through a 0.45  $\mu\text{m}$  nylon filter into a vial. The Polyphenol compound profiles were analyzed using a Shimadzu LC-20A HPLC system (Shimadzu Corporation, Kyoto, Japan) with a UV-Vis diode-array detector. The procedure followed the method of Sangta et al. with minor modifications [56]. Extracts were separated on an ultra-aqueous C18 column (250  $\times$  4.6 mm, 5  $\mu\text{m}$ ) (Restek, Bellefonte, PA, USA). The mobile phase used solvent A (5:95 formic acid: distilled water) and solvent B (85:5:10 acetonitrile: formic acid: distilled water). The gradient program was: 80% A for 4 min; linear decrease to 25% A over the next 4 min (4–8 min); maintained at 25% A for 2 min (8–10 min); increased back to 80% A over 7 min (10–17 min); and held at 80% A for the final 3 min (17–20 min). Total runtime was 20 min per sample. Compounds were monitored at 280 nm; flow rate, 1 mL/min; injection volume, 10  $\mu\text{L}$ . The identity of each phenolic compound was confirmed by comparing its retention time with those of the corresponding standards under identical chromatographic conditions. To further validate the identification and ensure the absence of co-eluting interferences, standard addition (spiking) was performed, where the target peaks showed a proportional increase in area without any shift in retention time or the appearance of extraneous peaks.

#### 4.4. Determination of Antioxidant Activity

##### 4.4.1. ABTS Radical Scavenging Activity

The scavenging capacity against  $\text{ABTS}^{\bullet+}$  radicals was assessed following the method [57]. Initially, the  $\text{ABTS}^{\bullet+}$  radical solution was produced by reacting 7 mM ABTS with 88  $\mu\text{L}$  of 140 mM potassium persulfate, followed by overnight storage (12–16 h) in darkness. A working solution was then prepared by diluting the stock 1:49 (*v/v*) with distilled water. Each sample (20  $\mu\text{L}$ , 1.0 mg/mL) was combined with 180  $\mu\text{L}$  of the diluted radicals and incubated for 6 min at room temperature. Measurement was performed at 734 nm (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). The antioxidant potential was reported in mg TE/g extract, using Trolox for the construction of the calibration curve.

##### 4.4.2. DPPH Radical Scavenging Activity

DPPH radical scavenging capacity was measured using a modified procedure [58]. Sample solutions (1.0 mg/mL) of varying concentrations were mixed with a 0.06 mM DPPH

solution in equal parts to a final volume of 200  $\mu$ L, then incubated in the dark for 30 min. Absorbance was measured at 517 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany). Results were calculated as milligrams of trolox equivalents (TE) per gram of extract (mg TE/g extract).

#### 4.5. Determination of Enzyme Inhibition

##### 4.5.1. Collagenase Inhibition Assay

Collagenase inhibitory activity was measured using a modified spectrophotometry assay [59]. To assess this, plant extracts (0.1 mg/mL) were first incubated with collagenase from *Clostridium histolyticum* in 50 mM Tricine buffer (pH 7.5, 400 mM NaCl, 10 mM  $\text{CaCl}_2$ ) for 15 min. The reaction was then initiated by adding 1 mM FALGPA substrate dissolved in Tricine buffer. Water was used as a negative control, and EGCG served as the positive control at a concentration of 0.115 mg/mL (equivalent to 250  $\mu$ M). Absorbance at 335 nm was monitored for 20 min following the addition of substrate using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany), and results were expressed as the percentage of enzyme inhibition.

##### 4.5.2. Elastase Inhibition Assay

In accordance with [59], elastase inhibition was determined via a colorimetric method. We utilized AAPVN at a final concentration of 1.6 mM (in 0.2 mM Tris-HCl, pH 8.0) and porcine pancreatic elastase (3.33 mg/mL stock). Each extract (0.1 mg/mL) was combined with the enzyme for a 15-min preliminary incubation phase, followed by the introduction of the substrate to trigger the enzymatic process. Epigallocatechin gallate (EGCG) at 250  $\mu$ M (0.115 mg/mL) served as the positive control, whereas deionized water was used for the negative baseline. Absorbance monitoring was conducted at 410 nm over a 20 min interval (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany), and results were expressed as percent inhibition relative to the control.

##### 4.5.3. Hyaluronidase Inhibition Assay

In accordance with the protocol by [60], hyaluronidase inhibitory properties were quantified. We utilized 0.115 mg/mL EGCG (250  $\mu$ M) as the reference standard. Briefly, 25  $\mu$ L of the sample extracts (0.1 mg/mL) were incubated with 100  $\mu$ L of hyaluronidase (4 U/mL) for 10 min at 37 °C. Subsequently, 100  $\mu$ L of hyaluronic acid substrate (0.03% in 300 mM phosphate buffer, pH 5.4) was added to initiate the reaction, which proceeded for 45 min at the same temperature. To stop the enzymatic activity and precipitate the undigested substrate, 1 mL of acid albumin reagent (0.1% BSA and 24 mM sodium acetate, pH 3.8) was incorporated. Following a 10 min stabilization at ambient temperature, the absorbance was recorded at 600 nm (UV-2600i, Shimadzu, Japan). Results were presented as percent enzyme inhibition relative to the enzyme-free blank.

#### 4.6. Assessment of Cell Viability

##### 4.6.1. Cell Culture

HaCaT cells were kindly provided by Prof. Dr. Chuda Chittasupho (Faculty of Pharmacy, Chiang Mai University). The cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin 100 U/mL, streptomycin 100 mg/mL) at 37 °C in a humidified 5%  $\text{CO}_2$  atmosphere. The medium was replaced every 2–3 days until the cells reached approximately 80% confluence. Cell morphology and viability were examined microscopically to ensure no detached or abnormal cells were present before treatment. An untreated control group was included as the experimental baseline.

#### 4.6.2. Cytotoxic Effect of Plant Extracts

The viability of HaCaT cells following extract treatment was assessed via the SRB assay, in accordance with the procedures outlined by [14] with slight variations. Human keratinocytes were initially seeded ( $2 \times 10^4$  cells/well) in 96-well plates and incubated overnight (24 h) to facilitate attachment. The dried SFE extracts were first solubilized in 10% (v/v) dimethyl sulfoxide (DMSO) and subsequently diluted with culture medium to achieve final concentrations ranging from 2 to 32 µg/mL. To ensure the absence of solvent-induced toxicity, the final DMSO concentration in each well was strictly maintained at 0.1% (v/v). After the initial incubation, the culture medium was replaced with either the prepared extract solutions or a vehicle control (medium supplemented with 0.1% DMSO), followed by an additional 24 h incubation. To fix the cells, 50 µL of 50% (w/v) trichloroacetic acid (TCA) was introduced for a 1-h incubation at 4 °C. Once the plates were washed and dried, the cellular proteins were stained with 0.04% SRB solution for 30 min. Unbound dye removal was achieved through four rinses with 1% acetic acid, whereas the bound SRB was dissolved in 10 mM Tris base. The optical density was recorded at 515 nm (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). The percentage of cell viability was subsequently calculated relative to the vehicle control. Following the recommendations of ISO 10993-5, extracts were classified as non-cytotoxic if they sustained a survival rate of more than 80% relative to the control.

#### 4.6.3. Effect of UV on Cell Viability of HaCaT Cells

To determine the UV dose resulting in approximately 50% reduction in HaCaT cell viability for use in further analyses, the protocol was adapted from Izawa et al. with minor modifications [7]. HaCaT cells were seeded into 96-well plates at a density of  $2 \times 10^4$  cells/well and allowed to adhere for 24 h. After the adherence period, the culture medium was replaced. Various UV doses (0, 24, 48, 72, 144 mJ/cm<sup>2</sup>) were then applied using the germicidal lamp installed in a Class II Type A2 biological safety cabinet (model SC2-4A1, Esco Lifesciences, Singapore). The lamp emitted radiation with a peak wavelength of 254 nm, which is typical for low-pressure mercury germicidal lamps. Culture plates were positioned approximately 55 cm below the center of the lamp. The irradiance (mJ/cm<sup>2</sup>) at the cell surface was measured and calibrated using a UV radiometer calibrated for 254 nm prior to each experiment. During UV exposure, the cabinet sash remained closed, and airflow was turned off to maintain a sterile environment. After irradiation, the medium was replaced with fresh medium, and the plates were incubated for 24 h. Serum-free medium without UV served as a control. Cell viability was measured by the SRB assay.

#### 4.7. RNA Extraction and Semi-Quantitative RT-PCR

Following a 2-h pre-incubation with either extracts or standard compounds, HaCaT cells were subjected to UV radiation (48 mJ/cm<sup>2</sup>). Total RNA was then isolated utilizing the E.Z.N.A.<sup>®</sup> Total RNA Kit I according to the provided instructions, with samples maintained at −80 °C for subsequent use. The transcriptional levels were assessed using the MyTaq<sup>™</sup> One-Step RT-PCR Kit and specific primers (Table 3) as per the manufacturer's guidelines. The resulting amplicons were resolved by 1% agarose gel electrophoresis and detected with the Gel Doc<sup>™</sup> EZ System (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analysis was performed using Image Lab software (version 6.1.0, Bio-Rad), where *GAPDH* served as the internal reference for normalizing the relative expression levels, presented as fold changes compared to the control group.

**Table 3.** The primer sequence for semi-quantitative RT-PCR.

| Primers | Forward Primer         | Reverse Primer          | Product Size (bp) | Ref. |
|---------|------------------------|-------------------------|-------------------|------|
| COL1A1  | GAGGGCCAAGACGAAGACATC  | CAGATCACGTCATCGCACAAC   | 140               | [61] |
| ELN     | CAAGGCTGGTTACCCAACAG   | CACCTGGGACAACCTGGAATC   | 350               | [61] |
| TIMP1   | GGAGAGTGTCTGCGGATACTTC | GCAGGTAGTGATGTGCAAGAGTC | 100               | [62] |
| HAS1    | CCACCCAGTACAGCGTCAAC   | CATGGTGCTTCTGTCTGCTCT   | 314               | [63] |
| AQP3    | TGCAATCTGGCACTTCGC     | GCCAGCACACACACGATAA     | 156               | [64] |
| FLG     | AGGGAAGATCCAAGAGCCCA   | ACTCTGGATCCCCTACGCTT    | 478               | [64] |
| SOD1    | AGGGCATCATCAATTCGAG    | ACATTGCCCAAGTCTCCAAC    | 217               | [65] |
| CAT     | CATCGCCACATGAATGGATA   | CCAACTGGGATGAGAGGGTA    | 317               | [65] |
| GPX     | TTCCCGTGCAACCAGTTTG    | GGACGTACTTGAGGGAATTCAGA | 214               | [65] |
| SIRT1   | GCAGATTAGTAGGCGGCTTG   | TCTGGCATGTCCCACTATCA    | 152               | [66] |
| GAPDH   | GGAAGGTGAAGGTCCGAGTC   | CTCAGCCTTGACGGTGCCATG   | 185               | [58] |

#### 4.8. Statistical Analyses

The results were presented as mean  $\pm$  standard deviation (SD). Statistical analyses were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) with a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A  $p$ -value  $< 0.05$  was considered statistically significant.

## 5. Conclusions

This study demonstrated that *Schinus terebinthifolius* (SB) extract provided the strongest protection against UV-induced skin damage among the tested Southeast Asian medicinal plants. The high polyphenolic content of SB suggests that naringin and epicatechin gallate work synergistically with other bioactive compounds to enhance its overall antioxidant and enzyme inhibitory potential. SB also exhibited the most comprehensive effects in enhancing the expression of genes related to skin structure, hydration, and defense. These findings suggest that SB may provide a valuable basis for photoprotective applications in the development of natural cosmeceuticals. While the current in vitro results are significant, further in vivo and clinical studies are warranted to fully validate these findings and evaluate the long-term efficacy of the extract.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052092/s1>.

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