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Antioxidant, Anti-Tyrosinase, and Anti-Collagenase Properties of *Elephantopus scaber* Leaf and Root Extracts

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ABSTRACT

Excessive exposure to ultraviolet (UV) radiation is the most common cause of accelerated skin aging. Due to Malaysia's geographical location near the equator, skin aging has become a growing health concern, leading to an increasing demand for skincare products. However, some over-the-counter skincare products contain hazardous chemicals such as parabens and formaldehyde. As a result, consumers are now switching to natural-based skincare products. *Elephantopus scaber* (*E. scaber*), also known as "Tutup Bumi", is a medicinal herb found in Malaysia. Although *E. scaber* is widely used for various medicinal purposes, its antiaging properties remain largely unexplored. The objective of this study was to determine the antioxidant and anti-skin aging potentials of *E. scaber* leaf and root extracts. Total phenolic content (TPC), total flavonoid content (TFC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were conducted to assess the antioxidant properties of *E. scaber* extracts. The extracts were also tested for tyrosinase and collagenase inhibition to determine their anti-skin aging properties. *E. scaber* root (ESR) extract showed higher TPC (63.34 ± 0.56 mg GAE/g) and TFC (156.46 ± 7.43 mg QE/g), but lower DPPH radical scavenging activity compared to *E. scaber* leaf (ESL) extract. The ESR also exhibited stronger tyrosinase and collagenase inhibitory activities. Therefore, *E. scaber*, particularly the root part, is a promising candidate for the development of novel natural-based antiaging skincare treatments.

INTRODUCTION

Skin is the largest organ and serves as a protective barrier for the body. In addition to protection, the skin also plays a crucial role in various physiological functions such as immunological defence,

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thermoregulation, sensation, and endocrine and exocrine activities [1]. The skin is composed of several different types of cells arranged in multiple layers contributing to its complexity and functionality. Skin aging is a significant health concern, compromising skin structure and functions such as wrinkle formation, increased fragility, uneven pigmentation and hyperpigmentation, aberrant wound healing, and others. The aging process is influenced by intrinsic factors, such as genetic predisposition, and extrinsic factors like ultraviolet (UV) radiation [2]. Intrinsic aging typically results in thin, dry skin with fine wrinkles, whereas extrinsic aging leads to more pronounced damage such as deep wrinkles, dry and sagging skin, and discoloration [3].

Chronic UV exposure contributes to skin aging via various pathways. Reactive oxygen species (ROS) are one of the key mediators of skin aging. UV radiation increases ROS production, which in turn activates various signalling molecules, leading to reduced extracellular matrix (ECM) production and increased ECM degradation. Notably, these signalling mediators markedly upregulate the expression and activity of matrix metalloproteinase enzymes such as MMP1 (collagenase), MMP12 (elastase), gelatinases, and other enzymes, exacerbating ECM degradation causing wrinkled, dry, and sagging skin [4]. UV exposure also elevates the level of α-melanocyte-stimulating hormone (α-MSH), and ROS enhance skin pigmentation by mobilizing α-MSH to melanocytes. α-MSH activates microphthalmia-associated transcription factor (MITF) in melanocytes to stimulate the production and activation of tyrosinase [5]. Activated tyrosinase catalyses the formation of melanin, the dark pigment in the skin, causing uneven and hyper skin pigmentation. A deep understanding of the molecular events of skin aging is paramount in skincare, and targeting skin aging mediators such as the ROS and MMPs is important in combating skin aging.

The World Health Organization estimates that sun exposure contributes to approximately 20 % of UV-related health issues, particularly in equatorial regions like Peninsular Malaysia, which consistently experiences high UV radiation levels [6]. Hence, the cosmetic industry in Malaysia has seen substantial growth to cater consumer demand for effective skincare solutions, especially in the organic and natural products sector [7]. This trend reflects a growing awareness of consumers in the potential adverse effects associated with hazardous synthetic substances in some skincare products in the market [7,8].

Elephantopus scaber (*E. scaber*), locally known as "Tutup Bumi", is a flowering weed. Its green leaves are elongated, with irregular or serrated edges and arranged in a rosette formation at the base [9]. *E. scaber* can be found worldwide, particularly in East Asia and Southeast Asia, Australia, Africa, Europe, India, and South America [9]. *E. scaber* contains high levels of sesquiterpene lactones such as deoxyelephantopin (DET), isodeoxyelephantopin (IDET) [10], as well as other unique sesquiterpene lactones such as scabertopin, elescaberin, and scabertopino [9]. Other phytochemicals present in *E. scaber* include phenolic compounds, flavonoids, triterpenoids, steroids such as stigmasterol and lupeol, and essential oils [9,11,12]. Most of these phytochemicals are present throughout the whole plant. However, some phytochemicals such as scabertopinol, molephantinin, indole-3-carbaldehyde, and numerous essential oils were reported to concentrate in the aerial parts, whereas some guaianolides, curcuphenol, patriscabratine, and stearic acid were extracted only from the roots [13,14]. *E. scaber* extracts have been shown to exhibit various bioactive properties such as anticancer, antidiabetic, antibacterial, antioxidant, anti-inflammatory, and hepatoprotective effects, as well as stimulation of hair growth, memory enhancement, and wound-healing acceleration [9,15]. However, the anti-skin aging properties of this plant are currently unclear.

We theorized that *E scaber* extracts could reduce ROS production and inhibit the activity of collagenase and tyrosinase enzymes. In this study, we sought to determine the antioxidant, tyrosinase and collagenase inhibitory properties of *E. scaber* extracts from the roots and leaves prepared using methanol as the solvent.

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EXPERIMENTAL

Extraction of Elephantopus scaber

The leaf and root samples of *E. scaber* were collected from a garden located in Kangar, Perlis. The samples were washed with tap water and patted dry using paper towels. The leaves and roots were separated and dried in the oven at 45°C for 72 hours. After drying, the plant samples were ground into fine powders using a mechanical grinder. The powders were weighed, labelled, and stored at 4°C. Extraction was performed as reported by Widyaningrum [16] with minor modifications. 20 grams of the leaf and root fine powders were extracted with 250 ml of 99.8 % methanol using the Soxhlet method. The temperature was set to 65°C and the extraction was performed for 8 hours until the colour of the extract faded. The solvent was removed using a rotary evaporator (Heidolph) at 65°C to obtain the crude extracts which were preserved at 4°C until further use. The extraction yield was calculated using the Equation 1:

$$Yield\ (\%) = \frac{Weight\ of\ dried\ extract}{Weight\ of\ dried\ plant\ sample} \times 100 \qquad (1)$$

Total phenolic content (TPC)

The TPC of *E. scaber* extracts was determined using a colorimetric assay based on our previous study [17]. Gallic acid standard solutions were prepared at 0.025, 0.050, 0.075, and 0.100 mg/ml. The extracts were prepared at 1 mg/ml in methanol. The crude extracts and standard solutions were mixed with the Folin-Ciocalteu solution (R&M Chemicals 6590-00). 7 % sodium carbonate was added to the mixture. The solutions were mixed and incubated at room temperature for 30 minutes. After incubation, the absorbance of the samples at 760 nm was obtained using a Genesys-20 spectrophotometer. Methanol was used as the blank. The TPC of the samples was computed based on the standard curve generated using the gallic acid standard solutions. The TPC of the crude extracts was calculated using Equation 2. The TPC was represented as mg gallic acid equivalent (GAE)/g dry extract.

$$TPC = \frac{cV}{m} \qquad (2)$$

c = phenolic concentration of the sample obtained from the standard curve
V = volume of the extract in ml
m = mass of the extract in gram

Total Flavonoid Content

The total flavonoid content (TFC) of *E. scaber* extracts was determined using a colorimetric assay [17]. Quercetin standard solutions were prepared at 0.025, 0.050, 0.100, 0.150, and 0.200 mg/ml. The extracts were prepared at 1 mg/ml in methanol. The extracts and standard solution were mixed with 5 % sodium nitrite and incubated for 5 minutes at room temperature. 10 % aluminium chloride was added, mixed, and incubated at room temperature for another 5 minutes. 1 mM sodium hydroxide was added to the mixture and topped with distilled water to 1 ml. After incubation, the absorbance of the samples at 510 nm was obtained using a Genesys-20 spectrophotometer. Methanol was used as the blank. The TFC of the samples was computed based on the standard curve generated using quercetin standard solutions. The TFC of the extracts was calculated using Equation 3. The TFC was represented as mg quercetin equivalent (QE)/g dry extract.

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$$TFC = \frac{cV}{m} \qquad (3)$$

c = flavonoid concentration of the sample obtained from the standard curve
V = volume of the extract in ml
m = mass of the extract in gram

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Assay

The free radical scavenging activity of the methanolic extract of the leaf and root extracts of *E. scaber* was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay [17]. Briefly, the samples and controls were diluted with methanol. 0.25 mM DPPH (Sigma-Aldrich D9132) was added to all samples. The mixtures were thoroughly mixed and incubated at room temperature for 30 minutes. After the incubation, the final absorbance at 517 nm was recorded. Ascorbic acid and methanol served as the positive control and negative control, respectively. Extracts diluted with the buffer served as the background controls. Background absorbance was corrected by subtracting the sample absorbance with the background control absorbance. DPPH scavenging activity was determined using the Equation 4:

$$DPPH\ scavenging\ activity\ (\%) = \frac{A0 - A1}{A0} \times 100\ \% \qquad (4)$$

A0: Absorbance of control reaction
A1: Absorbance of the test or standard sample.

Tyrosinase Inhibition Assay

Tyrosinase inhibition assay was performed according to Adli [18] with slight modifications. 50 mM phosphate buffer (pH 6.5) was added to the extracts, kojic acid, and methanol. 0.1 U/ml of tyrosinase (Sigma-Aldrich T3824) was added to the mixtures. Then, 0.5 mM of L-DOPA (Sigma-Aldrich D9628) was added to the mixtures, and an additional buffer was added to make a 1 ml reaction volume. The mixtures were incubated for 30 minutes in the dark at room temperature. Kojic acid and methanol were used as the positive and negative controls, respectively. Extracts diluted with the buffer served as the background controls. After incubation, the absorbance was measured at 492 nm. Background absorbance was corrected by subtracting the sample absorbance with the background absorbance. The percentage of enzyme inhibition activity was calculated using the Equation 5:

$$Tyrosinase\ inhibition\ activity\ (\%) = \frac{A - B}{A} \times 100\ \% \qquad (5)$$

A: Enzyme activity without extract (methanol negative control)
B: Enzyme activity in the presence of the extract or kojic acid

Collagenase Inhibition Assay

Collagenase inhibition assay was performed based on a previously reported method by Mat Yasin [17] with slight modifications. Collagenase enzyme (Sigma-Aldrich C0130) was mixed with the extracts, oleanolic acid, and methanol. The mixtures were incubated for 15 minutes. Next, tricine buffer and FALGPA (Sigma-Aldrich F5135) were added to the mixture. The mixtures were incubated for another 15 minutes at room temperature. Background absorbance control was prepared by diluting the extract with the buffer. After incubation, the absorbance was measured at 345 nm using a UV-Vis spectrophotometer (Shimadzu UV-1601). Oleanolic acid was used as the positive control, and methanol served as the negative control. The

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background absorbance was corrected, and the percentage of enzyme inhibition activity was calculated using the Equation 6:

$$Collagenase\ inhibition\ activity = \frac{1-B}{A} \times 100 \qquad (6)$$

A = enzyme activity without extract
B = enzyme activity in the presence of the extract or control.

Statistical Analysis

All assays were repeated at least three times. The average and standard error of the mean were calculated. T-test and ANOVA tests were used to determine statistical significance with the alpha value set at 0.05.

RESULTS AND DISCUSSION

Extraction yield

In this study, phytochemicals from the roots and leaves of *E. scaber* was extracted using Soxhlet method with methanol as the solvent. The results indicated that the *E. scaber* leaf extract (ESL) contains a higher level of methanol-soluble compounds (19.40 %) than the *E. scaber* root extract (ESR) (13.25 %) (Table 1). This finding is consistent with previous studies by Ahmad [19] and Efendi [20] where the methanolic extracts from the leaves showed higher yields than the roots.

Table 1. Extraction yield from the root and leaves of *E. scaber*

Extract	Yield (gram)	Percent yield (%)
ESL	3.88	19.40
ESR	2.65	13.25

In this study, methanol was chosen as the solvent because methanol has been consistently reported to produce higher yields than other solvents. Methanol was superior to n-hexane in extracting oil and steroids from the leaves and roots of *E. scaber* [19]. Similarly, the methanolic extract yield was greater than ethyl-acetate and n-hexane extract [20]. Another study compared the extraction of phytochemicals from *E. scaber* using six different solvents. Phytochemical screening revealed that methanol extracted higher amounts and more diverse phytochemicals than ethanol, water, ethyl acetate, and petroleum ether [21]. Together, this study and previous studies demonstrate that methanol is a suitable solvent and the leaves of *E. scaber* contain a higher concentration of potentially beneficial compounds than the roots.

Total phenolic and flavonoid content

Next, the total phenolic (TPC) and flavonoid content (TFC) of the extracts were determined. *E. scaber* root (ESR) extract displayed higher levels of TPC and TFC (Table 2). A previous study reported similar TPC values of 45 and 60 mg GAE/g and TFC values of 50 and 120 mg QE/g for *E. scaber* extracts using n-hexane and ethyl-acetate as the solvents, respectively [22]. However, another study showed a TPC value of 193.05 ± 1.17 mg GAE/g and a TFC value of 120.87 ± 0.61 mg CE/g for ethanolic *E. scaber* extract [23]. These different results could be due to the growth conditions of the plant, the extraction process, solvent, and the spectrophotometric analysis method.

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Table 2. TPC and TFC values of methanolic ESL and ESR

Assay	ESL	ESR
TPC (mg GAE/g extract)	35.62 ± 0.90	63.34 ± 0.56
TFC (mg QE/g extract)	125.81 ± 14.34	156.46 ± 7.43

Flavonoids are a subclass of polyphenols. Therefore, the TPC values were expected to be higher than the TFC values which contradicts the current findings. Nonetheless, these observations were common in the literature [24,25]. Orsavova [24] evaluated the TPC and TFC values of sweet rowanberry extracts using conventional spectrophotometry and high-performance liquid chromatography (HPLC) methods. They reported conflicting results where the spectrophotometric method showed lower TPC than the TFC values, whereas the HPLC method revealed significantly higher TPC values than the TFC, potentially due to the number of standards used in the study.

The discrepancies in the TPC and TFC values could be attributed to several factors. Folin-Ciocalteu (F-C) reagent, used to estimate the level of phenolic compounds in the extracts, is susceptible to interference by compounds such as ascorbic acid, tyrosine, formic acid, and acetic acid [26]. In this study, ascorbic acid could be one of the interfering compounds since it was reported to be present in *E. scaber* leaves [27]. Other phytochemicals in ESL and ESR could also interfere with F-C readings, causing the unexpected low TPC values. Another possible reason is the use of different standards for TPC and TFC assays. Although gallic acid is commonly used as the standard for TPC assay, TPC estimation using gallic acid alone as the standard may underestimate the actual TPC values. A combination of gallic acid with catechol, vanillic acid, guaiacol, and vanillin provides a more accurate TPC approximation as this combination represents different types of polyphenols with varying numbers of hydroxyl groups [26]. Although the spectrophotometric method lacks accuracy, this method is simple, rapid, and reproducible. In short, this study showed that both the leaves and roots of *E. scaber* contain phenolic and flavonoid compounds at levels comparable to a published study.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Assay

Next, the extracts were tested for antioxidant activity using DPPH radical scavenging assay. Both extracts showed dose-dependent responses, with the ESL exhibiting a stronger antioxidant activity (Figure 1). Ascorbic acid, served as the positive control, showed 91.58 ± 0.72 % radical scavenging activity. The ESL at 0.5 mg/ml showed a strong radical scavenging activity at 87.5 ± 0.54 %, which was close to that of ascorbic acid.

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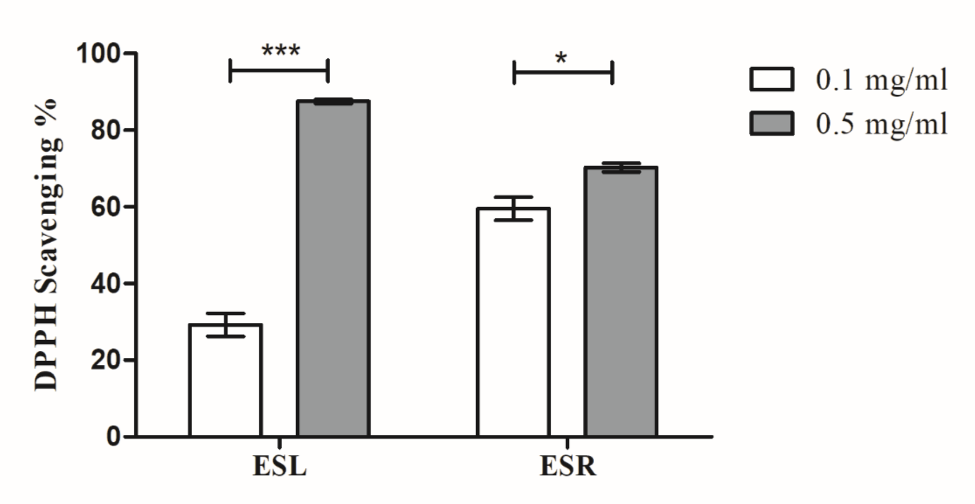

Figure 1. DPPH scavenging activity of *E. scaber* leaves (ESL) and roots (ESR) methanolic extracts. The bar graph depicts the average percentage with standard error of the mean as the error bars. Two-way ANOVA with Bonferroni post-test was used to determine statistical significance. * and *** denotes p-value of less than 0.05 and 0.001.

A previous study reported a 21 % radical scavenging activity of 0.1 mg/ml methanolic *E. scaber* leaf extract which is similar to the result of this study (29.2 % at 0.1 mg/ml) [21]. The study, however, did not investigate antioxidant activity at 0.5 mg/ml nor did they test the antioxidant activity of the root. Another study showed a slightly better DPPH scavenging activity of aqueous *E. scaber* leaf extract at 37.56 % but poor antioxidant activity of the aqueous root extract (4.85 %) [28]. The discrepancies in DPPH scavenging percentages could be due to the solvent and the extraction method. Nonetheless, the present findings are consistent with published studies.

As shown in Table 1, the roots extract (ESR) had higher TPC and TFC values. Since, phenolic and flavonoid compounds are well-known antioxidants, ESR was expected to show a stronger antioxidant activity than the ESL. However, 0.5 mg/ml leaves extract (ESL) showed the strongest antioxidant activity (Figure 1). This may be due to other potent antioxidant compounds in the leaves such as chlorophylls and carotenoid pigments which have been shown to mediate antioxidative effects via various mechanisms [29]. High concentration of essential oils such as n-tetradecane, n-pentadecane, and hexadecenoic acid in *E. scaber* leaves also provide excellent antioxidant properties [13,30]. Additionally, sterols such as stigmasterol and lupeol also contribute to the antioxidant effects of *E. scaber* leaves [11,31]. These findings suggest that the leaves contain strong antioxidant compounds than the roots.

The miscorrelation between TPC/TFC and antioxidant activity, as observed in the present study, has been previously reported. Although phenolic and flavonoid compounds typically exert potent antioxidant activities, Muflihah [25] reported low correlation values (r^2) of merely 0.699 and 0.541 between the TPC and TFC with DPPH activities, respectively. Another study even reported negative correlation values between TPC and TFC with DPPH scavenging activities [32]. Since DPPH assay is more sensitive to hydrophobic antioxidant compounds [32], the weaker antioxidant activity of ESR could be due low level of these compounds in the methanolic ESR extract. Using hydrophobic or less polar solvent may improve the antioxidant properties of ESR extract.

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Ascorbic acid is a potent antioxidant and is widely used in the skincare industry. However, ascorbic acid showed limited absorption and bioavailability and is prone to oxidation and degradation [33]. Therefore, current research is geared towards improving the stability and bioavailability of ascorbic acid, as well as discovering better alternatives. The high radical scavenging activity of the ESL in this study highlights the potential of *E. scaber* extract as a valuable alternative antioxidant in skincare products.

Tyrosinase inhibition

The extracts were then assayed for tyrosinase inhibition by measuring the production of dopachrome. Both extracts showed dose-dependent tyrosinase inhibition, though only at low to moderate levels (Figure 2). All the test samples demonstrated statistically lower inhibitory activities (20.6 - 44.7 %) compared to the positive control, kojic acid (91.7 ± 4.2 %). The ESR showed better tyrosinase inhibition than ESL but the differences among all test samples were not statistically significant.

To the best of our knowledge, the anti-tyrosinase activity of *E. scaber* extracts has not been reported. However, a previous study isolated and extracted an alkaloid from *E. scaber* that could significantly inhibit tyrosinase compared to the positive control arbutin [34]. In addition, stigmasterol has also been shown to inhibit melanin synthesis by reducing ROS levels and blocking tyrosinase activity in B16F10 melanoma cells stimulated with α-MSH hormone [35]. Lupeol is another phytochemical in *E. scaber* that could also inhibit mushroom tyrosinase [36]. Thus, our study together with previous studies demonstrate the potential of *E. scaber* extracts and phytochemicals in inhibiting tyrosinase. Further studies are required to determine the anti-tyrosinase activity of other phytochemicals in *E. scaber*. Additionally, research on enriching the composition of tyrosinase inhibitors in the extract is crucial to improve this activity.

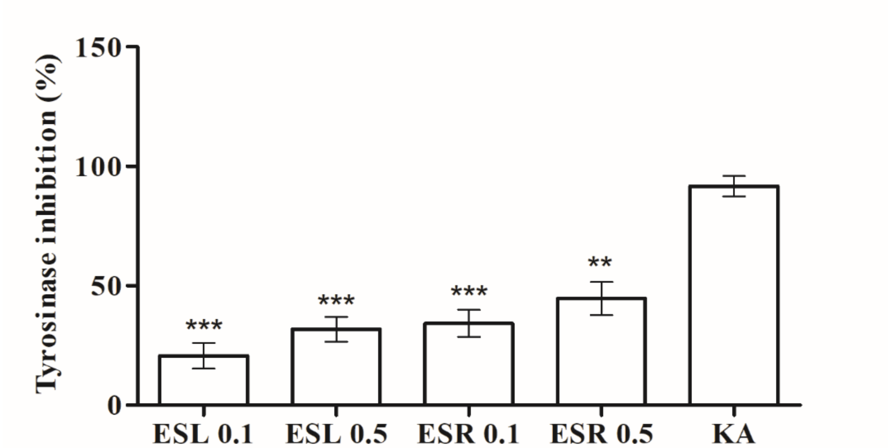

Figure 2. Tyrosinase inhibition activity of ESR and ESL extract. The bar graph depicts the average percentage with standard error of the mean as the error bars. One-way ANOVA with Tukey's Multiple Comparison test was used to determine statistical significance. ** and *** denotes p-value of less than 0.01 and 0.001, respectively, when compared to kojic acid (KA). ESL: *E. scaber* leaves extract; ESR: *E. scaber* roots extract. Extracts were tested at 0.1 mg/ml and 0.5 mg/ml.

In this study, kojic acid was used as the positive control. Kojic acid is a well-known tyrosinase inhibitor. Kojic acid was generally well-tolerated at a low dose (1 %) and treatment with 5000 mg/kg of *E.*

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scaber extract did not cause any adverse effects or histopathologic abnormalities in vivo [28]. However, kojic acid exhibited limited stability and efficacy, and clinical evaluation kojic acid safety revealed burning sensations in some participants [37]. Thus, although *E. scaber* extracts exhibit moderate tyrosinase inhibition, the extracts might still be beneficial in skincare applications due to their low toxicity. These extracts may be used to supplement kojic acid treatment to reduce the adverse effects while improving treatment efficacy.

Collagenase inhibition

Lastly, the extracts were evaluated for anti-collagenase activity. Both extracts showed moderate collagenase inhibition. Due to limited resources, only one concentration (0.1 mg/ml final concentration) of both extracts was assayed. ESR showed slightly better collagenase inhibition (50.7 ± 7.1 %) than ESL (39.6 ± 9.1 %) (Figure 3). However, the activity of both extracts was inferior to oleanolic acid positive control (81.5 ± 8.7 %), even though the differences were not statistically significant.

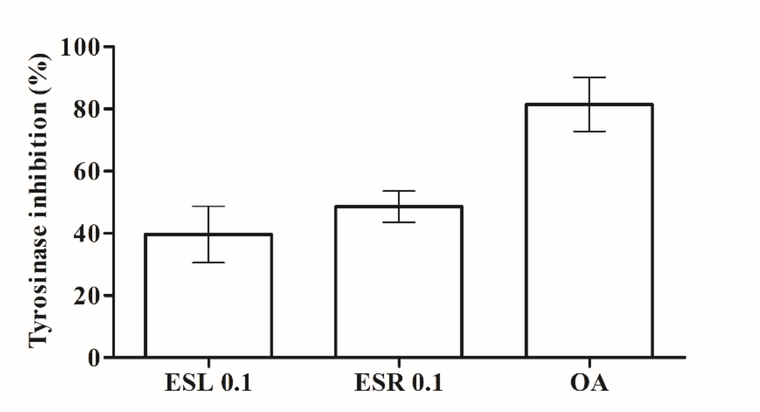

Figure 3. Collagenase inhibition activity of ESL and ESR extract. The bar graph depicts the average percentage with standard error of the mean as the error bars. The extracts were tested at 0.1 mg/ml final concentration. One-way ANOVA with Tukey's Multiple Comparison test was used to determine statistical significance. ESL: *E. scaber* leaves extract; ESR: *E. scaber* roots extract; OA: Oleanolic acid.

Thus far, the anti-collagenase activity of *E. scaber* extracts has not been documented. The present finding demonstrates a novel bioactivity of *E. scaber* extracts even though the activities are moderate. Phytochemicals in *E. scaber* may contribute to the anti-collagenase activity. For example, lupeol-based topical cream was reported to accelerate wound healing by reducing inflammation and stimulating tissue regeneration by increasing collagen synthesis and inhibiting MMPs in cells [31,38]. Further studies are required to demonstrate the contribution of other phytochemicals in anti-collagenase activity.

Oleanolic acid is a common collagenase inhibitor. We and others have previously demonstrated substantial collagenase inhibition by oleanolic acid [17,39,40]. However, the clinical efficacy of oleanolic acid in skincare has not been thoroughly investigated [41]. Moreover, oleanolic acid exhibits a low bioavailability due to its hydrophobic nature [41]. Therefore, the discovery of a novel collagenase inhibitor with superior absorption, distribution, metabolism, excretion, and toxicity profiles would tremendously benefit those with chronic skin aging.

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The potent antioxidant capacity and novel anti-skin aging properties of *E. scaber* extracts reported in this study may lead to the development of a novel skincare formulation to combat accelerated and chronic skin aging. However, the scope of this study is limited to biochemical assays only. Further in vitro study is required to determine the effects of the extracts on the expression and activity of collagenase and tyrosinase and other MMPs in skin fibroblasts and melanocytes. Investigations on the effects of these extracts on ROS-mediated skin aging, MITF-mediated pigmentation, and other signalling pathways are also pivotal to improve the efficacy of the extracts. Additionally, the cytotoxicity of the extracts needs to be comprehensively evaluated in vitro and in vivo to avoid any adverse effects.

CONCLUSION

Bioactive compounds extracted from the leaf and root of *E. scaber* showed substantial antioxidant activity, especially the ESL extract. The ESR extract, on the other hand, showed better anti-tyrosinase and anti-collagenase activities. The findings suggest that the *E. scaber* extract is a promising local plant in developing novel natural-based skincare products.

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AUTHOR'S CONTRIBUTION

FNAA and MASZ performed the experiments and wrote the drafts. SAK facilitated sample collection and reviewed the manuscript. SES performed data analysis and reviewed the manuscript. ASK conceptualized the central research idea, supervised the experiments, wrote, reviewed and approved the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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