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Assessment of the Antioxidant Properties of Horned Turban (*Turbo cornutus*) Viscera, Marine By-Products with Potential Application in Nutraceuticals

Areumi Park [1], Nalae Kang [1], Eun-A Kim [1], Yeon-Ji Lee [1] and Soo-Jin Heo [1,2,*]

[1] Jeju Bio Research Center, Korea Institute of Ocean Science and Technology (KIOST), Jeju 63349, Republic of Korea; areumi1001@kiost.ac.kr (A.P.); nalae1207@kiost.ac.kr (N.K.); euna0718@kiost.ac.kr (E.-A.K.); leeyj0409@kiost.ac.kr (Y.-J.L.)

[2] Department of Marine Biotechnology, University of Science and Technology (UST), Daejeon 34113, Republic of Korea

* Correspondence: sjheo@kiost.ac.kr; Tel.: +82-64-798-6101

Abstract: Horned turban (*Turbo cornutus*) is an edible gastropod that occurs along the intertidal zone and basalt coastline, and is an important marine resource in Jeju, Korea. However, *T. cornutus* viscera are mostly discarded following processing. In this study, the antioxidant activity of viscera and muscle extracts was compared. In addition, the protective effect of *T. cornutus* viscera ethanol extract (TVEE) against H_2O_2-induced oxidative stress in human dermal fibroblasts (HDFs) was investigated. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and hydrogen peroxide scavenging activity of the viscera (IC_{50} = 0.64 ± 0.64 and 0.76 ± 0.01 mg/mL, respectively) showed higher activity than that in muscle. TVEE increased the reactive oxygen species (ROS) scavenging effect and cell viability in H_2O_2-induced HDFs without cytotoxicity. Furthermore, the fluorescence intensity of HDFs was high in those from H_2O_2-induced intracellular ROS production, but TVEE treatment decreased ROS production. H_2O_2 activated the mitogen-activated protein kinase (MAPK) signaling and matrix metalloproteinase 2 (MMP-2) in HDFs. However, MMP2 expression was confirmed to be reduced via MAPK (ERK, JNK, and p38) signaling phosphorylation. In conclusion, various antioxidant effects were confirmed in *T. cornutus* viscera, which instead of being discarded as marine by-products, can be applied as nutraceuticals in various industries.

Keywords: *Turbo cornutus*; viscera; by-products; human dermal fibroblasts; antioxidant; oxidative stress

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1. Introduction

The seafood processing industry produces a variety of by-products, including heads, tails, shells, scales, viscera, and bones, depending on the type of raw material used and the preferred product [1,2]. These materials are typically discarded as solid waste, offal, or by-products. However, the interest in the various uses of waste, such as functional ingredients, functional foods, and pharmaceuticals, continues to grow despite the low value of marine by-products [2,3].

Turbo cornutus (Phylum Mollusca, Class Gastropoda, Order Trochida; common name horned turban), is a species of sea snail that is distributed in the coastal waters of Taiwan, China, Japan, and Korea [4]. Although the habitat, maturation, spawning, and histology of *T. cornutus* have been extensively studied [5], the biological mechanisms associated with the species' functional activity have not [6]. *Turbo cornutus* muscle parts are used in several ways as local food in Jeju, but the viscera are mostly unused owing to low consumer preference and awareness [7]. However, *T. cornutus* viscera contain many essential amino acids and can be used as a functional food ingredient because of their potential antioxidant properties [7].

The skin plays a key role in protecting the body from the external environment and in maintaining homeostasis [8,9]. Environmental factors such as visible and ultraviolet light, ozone, and particulate matter can increase the production of reactive oxygen species (ROS) in the skin [10,11]. ROS damage proteins, lipids, and DNA, the major components of cells, and cause oxidative stress, which further damages cellular structures, causes a variety of diseases, and is an essential mediator of aging [12,13].

The mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 are activated by induced oxidative stress [14]. The MAPK pathways are also activated by external stimulation and are related to inflammation, immune response, stress response, differentiation, and apoptosis [15,16]. MAPKs are present in all eukaryotic cells and transduce signals to increase matrix metalloproteinase (MMP) expression. In addition, MAPKs are assumed to play a crucial role in maintaining the life of cells [17]. The expression of MMPs is associated with various diseases, including cancer, dermal fibrosis, and skin aging. Among the roles of MMPs, the activation of gelatinases (e.g., by MMP-2) plays a key role in ultraviolet-induced skin aging. Furthermore, MMP-2 is expressed in human dermal fibroblasts (HDFs) [18].

In the present study, the antioxidant activity of *T. cornutus* viscera and muscles were compared. Moreover, the antioxidant effects of *T. cornutus* viscera ethanol extract (TVEE) against H_2O_2-induced oxidative stress were assessed in HDFs to evaluate whether this could be a potential use of the viscera of *T. cornutus* obtained as a marine by-product.

2. Materials and Methods

2.1. Preparation of TVEE

Turbo cornutus individuals were purchased from a fishing village in Udo, Jeju (33°30′22″ N, 126°57′14″ E). After removing the shell, the viscera and muscles were separated, washed, dried, and grounded. The viscera and muscles of each individual were ultrasonically extracted for 30 min using 70% ethanol to increase the surface area of the raw material and maintained in a shaking incubator for 24 h. Then, the solution was centrifuged at 3200 rpm for 30 min, and the supernatant was filtered. The supernatant was evaporated, freeze-dried, and used in the experiment as TVEE.

2.2. General Components Analysis of TVEE

Carbohydrate content was measured using the phenol sulfuric acid method. Glucose (0.1 mg/mL) was used as a standard and measured using a calibration curve. A solution with 25 µL of 80% phenol and 2.5 µL of sulfuric acid in 0.1 mg/mL TVEE was prepared and after 30 min, absorbance was measured at 480 nm.

Protein content was measured using a BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Albumin was used as a standard material and measured using a calibration curve. Next, 200 µL of reagent (50:1 mixture of BCA protein kit regent A and regent B) was added to 10 µL of 1 mg/mL TVEE, left to react at 37 °C for 30 min, and absorbance was measured at 526 nm.

Total polyphenol content was measured by the Folin–Ciocalteu method with reference to the Folin–Denis method [19]. Gallic acid was used as a standard and measured using a calibration curve. After diluting the TVEE ranging from 1 mg/mL to 0.1 mg/mL, 500 µL of the TVEE, 500 µL of 95% ethanol, and 2.5 mL of distilled water were mixed. Then, 250 µL of 50% Folin–Ciocalteu reagent was added and left to react for 5 min, followed by addition of 500 µL of 5% Na_2CO_3, and allowed to react for 60 min of reaction. Then, absorbance was measured at 725 nm.

2.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical and Hydrogen Peroxide Scavenging Activity of TVEE

DPPH radical scavenging activity was measured using Blois' method [20]. TVEE (20 µL) diluted in each extract at 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 mg/mL for each concentration

and 180 μL of 0.15 mM DPPH solution were dispensed into a 96-well plate, left to react for 30 min at room temperature, and absorbance was measured at 515 nm.

Hydrogen peroxide scavenging activity was measured by using Muller's method [21]. Viscera and muscle (20 μL each) were diluted in each extract at 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 mg/mL for each concentration, and 20 μL of 10 mM hydrogen peroxide and 0.1 M sodium phosphate buffer were added and left to react in an incubator at 37 °C for 5 min. Then, 30 μL of 1.25 mM ABTS and peroxidase (1 unit/mL) was added, left to react in an incubator at 37 °C for 10 min, and absorbance was measured at 405 nm.

The radical scavenging activity was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = [\{\text{Blank} - (\text{Sample} - \text{Control})\}/\text{Blank}] \times 100.$$

2.4. Cell Culture

HDFs (#C0045C, Thermo Fisher Scientific) were cultured in DMEM + F12 medium containing 10% fetal bovine serum and 1% penicillin, and cultured in a 100 mm cell culture dish at 37 °C and under 5% CO_2.

2.5. Cytotoxicity

To measure the cytotoxicity of TVEE, cultured HDFs in culture were seeded on a 96-well plate at 0.8×10^5 cells/mL and cultured in a CO_2 incubator for 16 h. Thereafter, the cells in each well pre-treated with 50, 100, and 200 μg/mL TVEE and cultured for more than 24 h. After treatment with 10 μL of MTT reagent for 3 h, the culture medium was removed, dissolved in 200 μL of DMSO per well, and absorbance was measured at 540 nm.

2.6. Cell Viability

Cultured HDFs were seeded into a 96-well plate at 0.8×10^5 cells/mL and cultured in a CO_2 incubator for 16 h. Then, to determine the effect of TVEE on H_2O_2-induced cytotoxicity, HDFs were pre-treated with 50, 100, or 200 μg/mL for 1 h and then with H_2O_2 at a concentration of 0.85 mM for additional 24 h. After treatment with 10 μL of MTT reagent for 3 h, the culture medium was removed, dissolved in 200 μL of DMSO per well, and then cell viability was assessed by measuring absorbance at 540 nm [22].

2.7. ROS Scavenging Efficacy

The intracellular active oxygen scavenging effect was measured using the principle that 2′,7′-dichlorofluorescein diacetate (DCFH-DA) non-fluorescent material reacts with free radicals generated in cells and oxidizes to fluorescent 2′,7′-dichlorofluorescein (DCF). HDFs were cultured for 16 h in a 96-well plate at 0.8×10^5 cells/mL. Then, HDFs were pre-treated with 50, 100, or 200 μg/mL TVEE for 1 h, followed by treatment with 0.85 mM H_2O_2. After incubation for 30 min, DCFH-DA was added. The degree of intracellular free radical production was measured based on DCF fluorescence intensity in the range of 485 nm λ excitation and 528 nm λ emission [23].

In addition, the effect of TVEE on intracellular ROS production in H_2O_2-induced HDFs was confirmed using a fluorescence microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan). HDFs (5×10^5 cells/mL) were cultured for 16 h in a 60 mm cell culture dish with a coverslip. Then, the cells were incubated with 50, 100, and 200 μg/mL TVEE for 1 h, and treated with 0.85 mM H_2O_2. After incubation for 30 min, the treated cells were stained with DCFH-DA, washed with PBS, and observed under a fluorescence microscope.

2.8. Western Blot Analysis

HDFs were cultured in a 60 mm cell culture dish to observe changes in the expression of related genes according to the oxidative stress protection of TVEE. Cells were incubated with 50, 100, and 200 μg/mL TVEE for 1 h and then treated with 0.85 mM H_2O_2 for 15 min. After removing the culture medium and washing with PBS, cellular proteins were extracted using RIPA buffer (Rockland Immunochemical, Limerick, PA, USA), and protein

concentration was measured with a BCA™ protein assay kit (Thermo Fisher Scientific). The quantified TVEEs were heated at 70 °C for 5 min, and electrophoresis was performed using a 14% SDS-PAGE gel [24]. After electrophoresis, proteins separated from the gel were transferred to an iblot 2 system (Invitrogen, Waltham, MA, USA). Blocking was performed for 2 h with a TBS-T solution in which 3% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 2% skim milk (Sigma-Aldrich) were dissolved. The primary antibody was diluted at a ratio of 1:1000 (Santa Cruz Biotechnology, Dallas, TX, USA or Cell Signaling Technology, Danvers, MA, USA) in TBS-T solution in which 3% BSA was dissolved, and incubated overnight at 4 °C. The horseradish peroxidase (HRP)-conjugated secondary antibody was diluted with anti-mouse IgG and anti-rabbit IgG at a ratio of 1:3000. Densitometric analysis was revealed using ImageJ to quantify protein expression.

2.9. Statistical Analyses

All data are expressed as mean ± standard deviation (S.D.). Student's *t*-test and one-way analysis of variance (ANOVA) were performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). The test results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Comparison of Extraction Yield, DPPH Radical and Hydrogen Peroxide Scavenging Activity between T. cornutus Viscera and Muscles

The extraction yield, DPPH radical and hydrogen peroxide scavenging activity of *T. cornutus* viscera and muscles were compared (Table 1). A comparison of the dry weight of the *T. cornutus* viscera and muscles, the viscera were found to be 2.60 ± 0.83 g per individual (Muscle dry weight: 3.55 ± 0.36 g). Through this dry weight, it was confirmed that the viscera of the *T. cornutus* were not fully utilized compared to their muscles. The final extraction yields of *T. cornutus* viscera and muscles (22.33 ± 0.91% and 21.07 ± 2.80%, respectively) showed no significant difference. IC_{50} values were calculated for the comparison, and the viscera and muscles were found to inhibit DPPH radical and hydrogen peroxide scavenging activity by 50%. The IC_{50} values of viscera and muscle extracts for DPPH radical scavenging activity were 0.64 ± 0.64 mg/mL and >2 mg/mL, respectively; the IC_{50} values of viscera and muscle extracts for hydrogen peroxide scavenging activity were 0.76 ± 0.01 mg/mL and 0.86 ± 0.03 mg/mL, respectively. Based on these results, viscera with high antioxidant activity were selected and used in this study.

Table 1. Comparison of dry weight, extraction yield and DPPH radical and hydrogen peroxide scavenging activity of viscera and muscles of *T. cornutus*.

Sample	Dry Weight (g)	Extraction Yield (%)	IC_{50} Value (mg/mL)	
			DPPH	Hydrogen Peroxide
Viscera	2.60 ± 0.83	22.33 ± 0.91	0.64 ± 0.64	0.76 ± 0.01 [a]
Muscle	3.55 ± 0.36	21.07 ± 2.80	>2	0.86 ± 0.03 [b]

Dry weight represented the average of per individual of *T. cornutus* (Number = 3737). Different letters ([a], [b]) show a significant difference with $p < 0.05$.

3.2. General Components Contents of TVEE

The results of the general component analysis of TVEE are shown in Table 2. The carbohydrate and protein contents were 10.25 ± 0.02% and 23.04 ± 0.02%, respectively. The polyphenol content of TVEE was 4.95 ± 0.00%.

Table 2. General components contents of TVEE.

General Components (%)		
Carbohydrate	Protein	Polyphenol
10.25 ± 0.02	23.04 ± 0.02	4.95 ± 0.00

3.3. Effect of DPPH Radical and Hydrogen Peroxide Scavenging Activities in TVEE

2,2-diphenyl-1-picrylydrazyl (DPPH) radical and hydrogen peroxide scavenging activities can be used to rapidly assess the radical scavenging activity of functional ingredient in an extract through electron transfer reactions [25]. Free radical scavenging activity is related to cell aging [26]. DPPH radical and hydrogen peroxide scavenging activities increased in a concentration-dependent manner. The DPPH radical scavenging activity of TVEE was 7.4, 13.6, 21.2, 41.6, 62.2, 84.3 and 89.4% at 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 mg/mL, respectively, and the hydrogen peroxide scavenging activity was 10.8, 15.1, 24.7, 44.9, 66.4, 91.3 and 97.4% at 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 mg/mL, respectively (Figure 1). These results indicate that *T. cornutus* viscera have high radical scavenging activity. Previous studies using hydrolyzed viscera and muscle of *T. cornutus* showed that the viscera extract had higher radical scavenging activity than the muscle extract at 0.25–2 mg/mL [7]. Moreover, a previous study comparing DPPH radical scavenging activity of abalone muscles and viscera extracts confirmed the higher effects in the viscera than in the muscle [27].

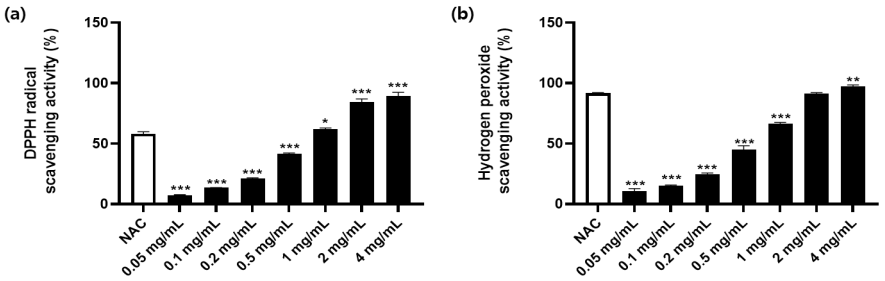

Figure 1. DPPH radical and hydrogen peroxide scavenging activity of TVEE. (**a**) DPPH radical scavenging activity. (**b**) Hydrogen peroxide scavenging activity. Values are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate values compared with N-acetylcysteine (NAC, 0.05 mg/mL).

3.4. Effects of TVEE on H_2O_2-Induced ROS Production and Cell Viability in HDFs

To investigate the oxidative stress-protective effect of TVEE in HDFs, ROS scavenging efficacy and cell viability were examined (Figure 2). TVEE did not show cytotoxicity at 50, 100, or 200 µg/mL; therefore, the experiment was conducted at these concentrations (Figure 2a). ROS present in HDFs were measured using DCFH-DA after treatment with 0.85 mM H_2O_2 (Figure 2b). When 0.85 mM H_2O_2 treatment was used to induce oxidative stress in HDFs, ROS production increased approximately four times compared with that in the control. However, TVEE treatment at concentrations of 50, 100, and 200 µg/mL improved ROS scavenging efficacy in a concentration-dependent manner. These results were similar to previous results of ROS scavenging efficacy in abalone viscera hydrolysates and *T. cornutus* viscera protamex extracts [7,28], and corroborate the ROS scavenging effect in viscera of various gastropods. To confirm the cell viability of TVEE-treated cells, HDFs were treated with 0.85 mM H_2O_2 (Figure 2c). The cell viability of TVEE untreated HDFs decreased to 52% compared with that in the control, but the viability of TVEE-treated cells was 65.4, 69.6, and 65.4% at concentrations of 50, 100, and 200 µg/mL, respectively.

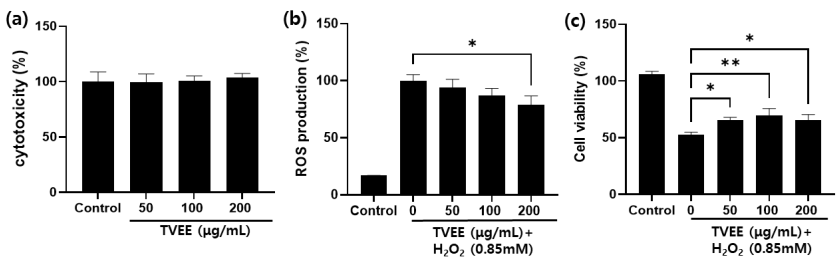

Figure 2. Protective effect of TVEE on H_2O_2-induced HDFs. (**a**) Cytotoxicity (**b**) ROS production (**c**) cell viability. Values are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$ indicate values compared with only the H_2O_2-induced group.

3.5. Effects of TVEE on the Production of Intracellular ROS

To confirm the protective effect of TVEE against H_2O_2, intracellular ROS production was assessed using DCFH-DA (Figure 3). The fluorescence intensity of H_2O_2-treated HDFs indicated higher intracellular ROS production than that in the control group. Moreover, ROS production was significantly reduced when TVEE was treated with H_2O_2 at concentrations of 50, 100, and 200 μg/mL. A previous study found that the hydrolysate from *T. cornutus* viscera decreased ROS production in H_2O_2-treated HaCaT cells [29]. These results suggest that TVEE regulates the production of intracellular ROS and alleviates H_2O_2-induced oxidative stress in HDFs.

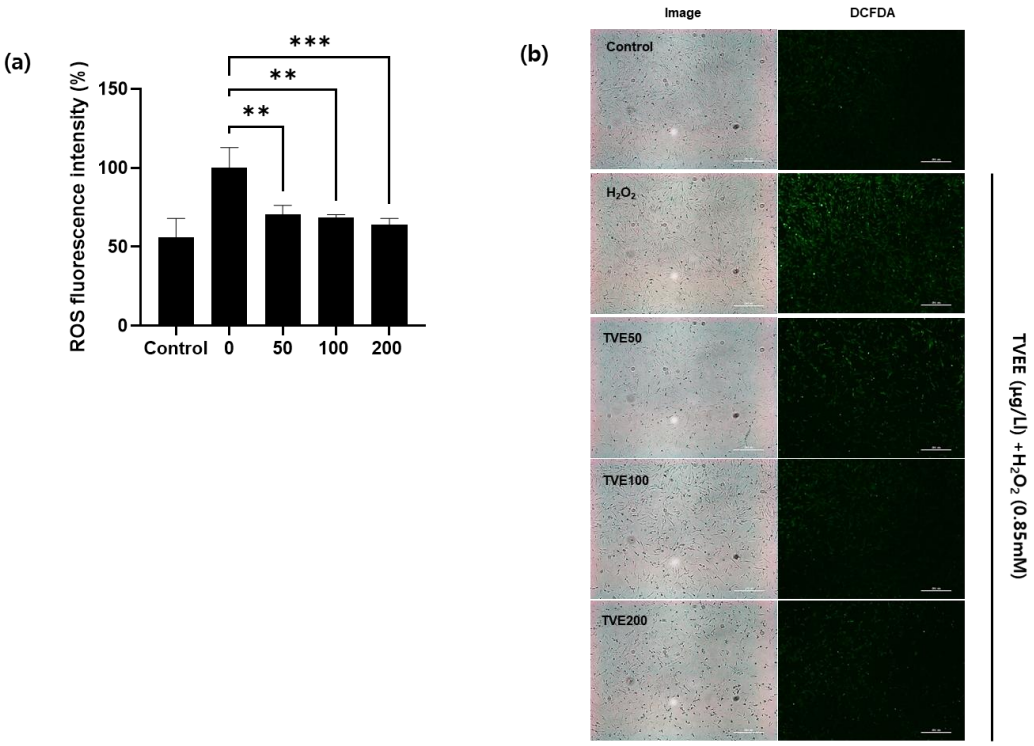

Figure 3. Intracellular ROS production in H_2O_2-induced HDFs. (**a**) ROS fluorescence intensity was detected by DCFH-DA using a fluorescence microscopy. Values are expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ indicate values compared with only the H_2O_2-induced group. (**b**) Image of the intracellular ROS production by fluorescence microscopy (scale bar = 500 μm).

3.6. TVEE Regulates MMP-2 via MAPK Signaling Pathways in HDFs

Excessive ROS production by H_2O_2 in HDFs can activate MAPK signaling pathways [15]. To determine the signal pathway of the protective effect of TVEE, we evaluated whether the protective effect of TVEE in HDFs exposed to H_2O_2 occurs via the regulation of the MAPK pathway. The phosphorylation of MAPK (ERK, JNK, and p38) increased compared with that of the control group when treated with 0.85 mm H_2O_2 (Figure 4). However, phosphorylation was significantly reduced after TVEE treatment at concentrations of 50, 100, and 200 μg/mL. In particular, ERK and JNK were the most effective in inhibiting phosphorylation in a concentration-dependent manner. Therefore, TVEE exhibited the protective effects against H_2O_2-induced cell injury in HDFs via the regulation of the MAPK pathways. The effect of TVEE on MMP-2 protein expression was confirmed (Figure 4). When treated with TVEE at concentrations of 50, 100, and 200 μg/mL, the protein expression level of MMP-2 was significantly decreased in a concentration-dependent manner. Previous studies have shown that MMP production was inhibited via MAPK inhibition [30]. These results confirm that TVEE reduces MMP-2 expression by affecting the regulation of the MAPK signaling pathway in HDFs.

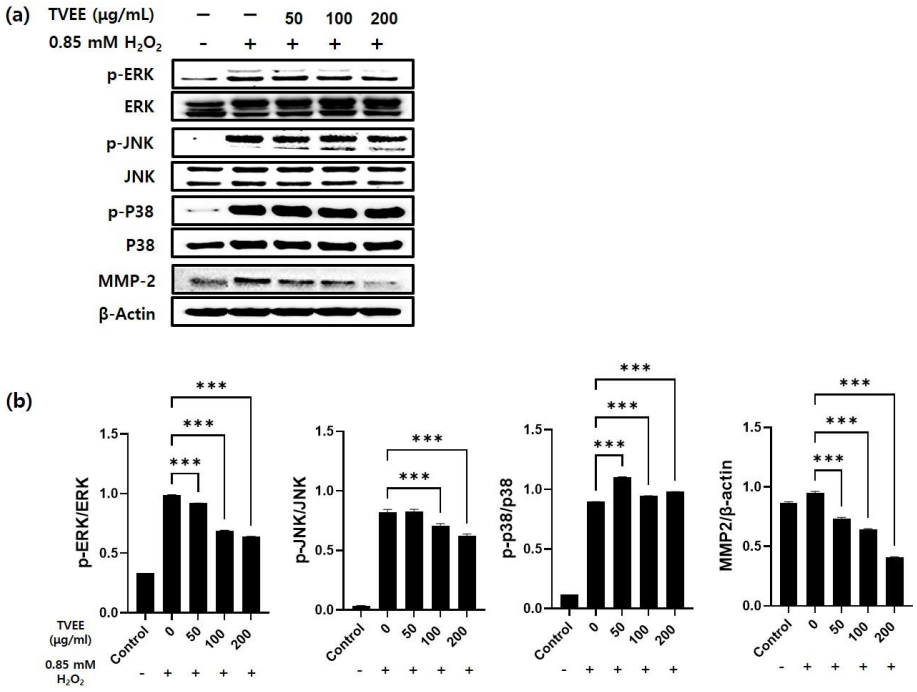

Figure 4. Effects of TVEE on H_2O_2-induced MAPK (ERK, JNK and P38) and MMP-2 in HDFs. (**a**) The protein levels of MAPK and MMP-2 were analyzed by Western blotting. (**b**) The density of each protein band was quantified by using the Image J program. Values are expressed as mean ± SD. *** $p < 0.001$ indicate values compared with only the H_2O_2-induced group.

4. Conclusions

Seafood processing results in various by-products that may cause environmental and human health problems. However, some by-products of such as *T. cornutus* viscera can be used as functional ingredients. Recently, various studies on *T. cornutus* viscera, a representative marine by-product found in Jeju, have been published; these focused on the anti-inflammatory activity of its extracts [6], myeloperoxidase inhibition effect of bioactive peptides [7], and antioxidant and anti-hyaluronidase activities of enzymatic hydrolysates [29]. Here, we demonstrated that TVEE has a protective effect against H_2O_2-induced oxidative stress in the HDFs as it increases antioxidant activity and reduces ROS generation in these cells. In addition, we confirmed that TVEE is related to MMP-2 expression via the regulation of phosphorylation of the MAPK signaling pathway. Thus, this study validated the antioxidant properties of *T. cornutus* viscera, suggesting their potential to be applied as nutraceuticals in various industries.

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