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Original Article

Anti-aging bioactivities of egg white hydrolysates

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Abstract

Egg whites can be readily hydrolyzed to produce small fragments which may be a good source of bioactive peptides. The aim of this study was to investigate anti-aging bioactivities of peptides produced by alkaline hydrolysis. The results demonstrated that neutralized egg white hydrolysates (EWH) at 5 mg/mL showed high antioxidant activity on free radical-scavenging activity at 67.1±1.7% and inhibited lipid peroxidation at 81.1±2.9%. Moreover, it possessed high reducing power equivalent to 0.2502 mg of vitamin C. EWH exhibited better antioxidant activity than ovalbumin. In dermal cell culture, EWH increased proliferation and cell migration of keratinocyte HaCaT cells and fibroblast NHDF cells and inhibited nitric oxide production of murine macrophage cell line RAW 264.7 which indicated its anti-inflammatory action at as low as 0.031 mg/mL. *In vivo* studies on the prevention of premature skin aging are worth further investigation

Keywords: egg white hydrolysates, antioxidant peptides, anti-aging properties, bioactive proteins

1. Introduction

Skin aging is characterized by a reduction of epidermal thickness, a flattening of the basal membrane, loss of elasticity, irregular keratinization, and a decrease in skin lipids. Signs of skin aging are usually noticed as fine lines and wrinkles. After the age of 40, there is a 1-2% annual decrease in collagen and elastin (Travis, Darren & Zimei, 2014). The external factors that cause premature aging of skin include oxidative stress, which is triggered by the release of free radicals in the skin mainly by UV rays from sunlight, environmental pollutants, and smoking. Free radicals are highly reactive molecules containing unpaired electrons that damage skin structures (Ratnam *et al.,* 2006). A number of reports describe the antioxidative properties of peptides purified from protein hydrolysates, such as those from α- and β-lactalbumin and lecithin-free egg yolk. Egg white proteins also possess antioxidative activities against free radicals (DaValos, Miguel, Bartolome, & LaPez-FandiO, 2004; Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Park, Suzuki, & Lennarz, 2001; Tanzadehpanah, Asoodeh, & Chamani, 2012).

Bioactive proteins and peptides have been used in anti-aging cosmeceutical products. These peptides are thought to act by stimulating fibroblast production of collagen or decreasing collagenase breakdown of existing collagen, reducing inflammation, improving cell migration and enhancing wound healing. There are three main categories of cosmeceutical peptides. First, signal peptides which help to increase fibroblast production of collagen or decrease collagenase enzymes, e.g., valine-glycine-valine-alanine-proline-glycine peptide and lysine-threonine-threonine-lysine-serine peptide. Second, neurotransmitter-affecting peptides which function to reduce muscle contraction and decrease wrinkle size and skin roughness, e.g., acetyl hexpeptide-3 (Argireline®) and peptapeptide-3 (Vialox®). Third, carrier peptides which function to stabilize and deliver important trace elements necessary for the wound healing process, e.g., copper tripeptide complex (Lupo & Cole, 2007; Secchi, 2008).

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Egg white proteins are commonly used in the food industry due to their gelling, foaming, and emulsifying properties and high nutritional quality. Several studies have demonstrated that the bioactivities and functional properties of egg white protein hydrolysates (EWH) are different depending on the methods of hydrolysis (Van der Plancken, Van Loey, & Hendrickx, 2006). For example, Xu *et al.* (2007) found that enzymatically derived ovalbumin, which is found mainly in egg white proteins, possessed antioxidant activity through the inhibition of superoxide anion, hydroxyl radicals, and lipid peroxidation *in vitro* as well as by elevating the activities of SOD, GSH-Px, and CAT which indicated tissue protection in mice. Chen *et al.* (2012) demonstrated that antioxidant activity and angiotensin-I converting enzyme inhibitory activity of EWH prepared with trypsin increased as the degree of hydrolysis increased. However, few studies on alkali treatment with egg white proteins have been reported. Mine (1997) found that the combination of dry heat and mild alkali treatment of egg white proteins improved their functional properties. Van der Plancken *et al.* (2005) demonstrated that a combination of pressure and alkali treatment of egg white solutions appeared to decrease protein solubility and total sulfhydryl (SH) content, whereas at high pressure and temperature, some of the SH groups remained unoxidized. However, their bioactivities were not reported. Therefore, the aim of this study was to investigate anti-aging bioactivities of alkaline hydrolysates of egg white treated under high pressure and temperature conditions.

2. Materials and Methods

2.1 Materials

Hen eggs were obtained from Polwittaya Farm (Khon Kaen, Thailand). Potassium hydroxide was purchased from RCI Labscan (Bangkok, Thailand). Hydrochloric acid (fuming 37%) was purchased from VWR International (PA, USA). Trolox, glutathione (GSH), and ovalbumin (OVA) were purchased from Sigma-Aldrich (MO, USA). Vitamin E acetate was purchased from Namsiang Co., Ltd. (Bangkok, Thailand). Vitamin C was purchased from S. Tong Chemicals Co., Ltd. (Nonthaburi, Thailand). Other chemicals used in the experiment tests were analytical grade. The following chemicals were purchased from Sigma-Aldrich (MO, USA): 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azo-bis-amidinopropane (ABAP), ferrous chloride, trichloroacetic acid, ammonium thiocyanate, potassium ferricyanide, ferric chloride, tris hydrochloride, β-mercaptoethanol, sodium dodecyl sulfate, Dulbecco's Modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS), antibiotic-antimycotic, L-glutamine and PrestoBlue®. TEMED was purchased from Thermo Fisher Scientific (MA, USA)

2.2 Egg white hydrolysates

2.2.1 Methods of egg white hydrolysate (EWH) preparation

Egg whites (EW) were separated from hen eggs and mixed with 0.4 N KOH at a ratio of 1:3. The mixture was hydrolyzed in a water bath at 55 °C for 2 h with continuous stirring and autoclaved at 121 °C and 15 psi for 2 h. Then, the solution was filtered through 5 layers of gauze and neutralized (pH=7) using hydrochloric acid (fuming 37%). The hydrolysate was lyophilized and stored at –40°C.

2.2.2 Percent yield

Egg white hydrolysate (EWH) solutions were weighed in pre-weighed jars. Then, the samples were lyophilized and weighed as a dry powder. The percent yield of dry EWH was calculated by Equation 1.

$$\% \text{ yield} = \frac{\text{Weight of lyophilized EWH}}{\text{Weight of fresh EW}} \text{x } 100 \quad (1)$$

2.3 Characterization of EWH

2.3.1 Determination of total protein

A stock solution of bovine serum albumin (BSA) as a standard protein at a concentration of 1000 µg/mL was prepared and diluted with deionized water (DI) in a range of 10-500 µg/mL. Samples of 20 µL were pipetted into a 96-well plate. Then, Bradford dry reagent (180 µL) was added to each well and mixed well. The mixtures were kept at room temperature (25 °C) for 5 min. The experiment was performed in triplicate. The absorbance of the test samples was measured using a UV-vis spectrophotometer at 595 nm. The absorbance was plotted versus the concentrations of BSA solutions to prepare a standard curve of BSA. The EWH samples were prepared in the same manner as the standards and measured the absorptions at 595 nm. The total protein of EWH was then calculated (Bradford, 1976).

2.3.2 Determination of molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the components of the EWH. Amersham Rainbow Markers (3.5-38 kDa) RPN755E (GE Healthcare Life Science, Sweden) were used to compare the hydrolysates. Briefly, 15% (v/w) acrylamide separating gel and 4% (v/w) acrylamide stacking gel were prepared. The hydrolysate samples at a protein concentration of 30 µg/µL were pipetted (10 µL) into 1.5 mL test tubes and mixed with 10 µL of 2X solubilizing dye with β-mercaptoethanol. Then, the mixture was heated at 100 °C for 5 min. The samples were pipetted onto the gel sheet and run at 140 V for about 40 min to separate the protein bands. After the electrophoresis process finished, the gel sheet was stained with Coomassie brilliant blue G-250 staining solution (Wang, Su, Jia, & Jin, 2013).

2.3.3 Amino acids analysis

The EWH was prepared by EZ:faast™ amino acid analysis of protein hydrolysates by the liquid chromato-graphy-mass spectrometry (LC-MS) method. Sample volumes of 1 µL were injected into the LC mounted with EZ:faast™ AAA-MS column 250×3.0 mm and eluted at 35 °C at a flow rate of 0.5 mL/min. The mobile phase A was water containing

10 mM ammonium formate, and B was methanol containing 10 mM ammonium formate. The gradient consisted of 68% B for 13 min, with a linear increase to 83% B in 13 min, and re-equilibration at 68% B until the end of the run (23 min). The mass spectrum was run in the positive ion mode scanning range from 100-600 *m/z*. The APCI ionization chamber temperature was 450 °C.

2.4 Bioactivities of EWH

2.4.1 Antioxidant activities tests

1) DPPH radical scavenging assay

Radical scavenging activity of EWH was measured in terms of DPPH radical-scavenging activity. Each 100 µL of sample of either hydrolysates or standards, i.e. Trolox, vitamin E acetate, glutathione, and vitamin C, were pipetted into the first column of the 96-well plate and diluted 2-fold with DI water or 80% (v/v) ethanol. DPPH (0.004 M) was prepared by dissolving in 80% (v/v) ethanol and then 50 µL was added to each well except the blank wells (samples without 0.004 M DPPH). The samples were mixed and then kept at room temperature in the dark for 25 min. The color reduction of the DPPH substrate was measured by a UV-vis spectrophotometer at the wavelength of 517 nm and calculated as DPPH radical-scavenging activity (%) using Equation 2 (Veerapan, & Khunkitti, 2011):

$$\text{DPPH radical} - \text{scavenging activity}\,(\%) = \frac{\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \quad (2)$$

$\text{Abs}_{\text{control}}$ = Absorbance of solvent with 0.004 M DPPH
$\text{Abs}_{\text{sample}}$ = Absorbance of sample with 0.004 M DPPH
$\text{Abs}_{\text{blank}}$ = Absorbance of sample without 0.004 M DPPH

2) Linoleic acid peroxidation (LPO) inhibition

A stock solution of linoleic acid (50 µl/mL in 80% (v/v) ethanol) was prepared. Then 50 µL of the stock solution was mixed with 50 µL of the standard solutions, i.e. Trolox, vitamin E acetate, glutathione, and vitamin C, and EWH in the 1.5 mL test tube. A 10 µL of 2,2'-azo-bis-amidinopropane (ABAP) was added into each tube, except the blank tubes (sample without 0.07 M ABAP). Then, 150 µL of 20% (v/v) acetic acid was pipetted into each tube. All test tubes were vortexed and incubated at 70 °C for 1 h. After that, 20 µL of the reaction mixtures were mixed with 160 µL of 75% (v/v) ethanol in a 96-well plate. Then 10 µL of 15% (w/v) ammonium thiocyanate and 0.05 M ferrous chloride were added sequentially. The absorbances were measured by a UV-vis spectrophotometer at 500 nm and values of % lipid peroxidation inhibition were calculated using Equation 3 (Ajibola, Fashakin, Fagbemi, & Aluko, 2011):

$$\%\text{Lipid peroxidation inhibition} = \frac{\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \times 100 \quad (3)$$

$\text{Abs}_{\text{control}}$ = Absorbance of solvent (water or ethanol) with 0.07 M ABAP
$\text{Abs}_{\text{sample}}$ = Absorbance of standards and EWH with 0.07 M ABAP
$\text{Abs}_{\text{blank}}$ = Absorbance of standards and EWH without 0.07 M ABAP

3) Reducing power assay

All hydrolysates and the reference standards, i.e. Trolox, vitamin E acetate, glutathione, and vitamin C, were dissolved in DI water or 80% (v/v) ethanol as solvent at different concentrations. Then 250 µL of each preparation was mixed with 250 µL of 0.2 M sodium phosphate buffer pH 6.6 and 250 µL of 1% (w/v) potassium ferricyanide. The samples were mixed and incubated at 50 °C for 20 min. Then 250 µL of 10% (w/v) trichloroacetic acid was added. The solutions were mixed together and incubated at 25 °C for 10 min. All preparations were centrifuged at 800 rpm for 10 min. The supernatants (30 µL) were diluted with distilled water (160 µL) in a 96-well plate and then 10 µL of 0.1% (w/v) ferric chloride was added and allowed to stand for 10 min at room temperature. The absorbance was measured using a UV-vis spectrophotometer at 700 nm and calculated as vitamin C equivalent (mg) using a vitamin C standard curve (Oyaizu, 1986).

2.5 Effects of EHW on dermal cell culture

2.5.1 Cell cultures

1) Normal human dermal fibroblast (NHDF) cells and human immortalized keratinocyte cell line (HaCaT)

Normal human dermal fibroblast (NHDF) cells and the human immortalized keratinocyte cell line (HaCaT) were used in this study. NHDF cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, USA), supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotic-antimycotic solution, and 1% (v/v) L-glutamine, whereas the HaCaT cells were cultured in DMEM, supplemented with 10% (v/v) FBS, and a 1% (v/v) antibiotic-antimycotic solution. All cells types were seeded in a 75 cm^2 T-flask and cultivated at 37 °C in 5% CO_2/95% relative humidity (RH). Cells were subcultured with 0.25 % (w/v) trypsin-EDTA when they reached about 80%-90% confluence (Sayes *et al.*, 2006).

2) Murine macrophage cell line RAW 264.7 (RAW 264.7)

Murine macrophage cell line RAW 264.7 was cultured in Roswell Park Memorial Institute medium (RPMI 1640) (Sigma-Aldrich, USA), supplemented with 10% (v/v) FBS and a 1% (v/v) antibiotic-antimycotic solution. The cells were seeded in 75 cm^2 T-flask and cultivated at 37 °C in 5% CO_2/95% RH. The cells were subcultured using a cell scraper when they reached around 80%-90% confluence (Sayes *et al.*, 2006).

2.5.2 Anti-inflammation activity using nitric oxide inhibition assay

The nitric oxide (NO) inhibition assay was determined in RAW 264.7. The cells were seeded into a sterile 96-well plate at 5×10^4 cells/well and cultured at 37 °C in 5% CO_2/95% RH for 24 h. Then, the medium was removed and the cells were washed twice with phosphate buffer solution pH 7.4. An aliquot of 200 µL of the samples and standard (diclofenac sodium) in RPMI 1640 medium containing 100 ng/mL of lipopolysaccharides (LPS) were pipetted into the cell culture plate. Controls received only fresh RPMI 1640. The plate was then incubated at 37 °C in 5% CO_2/95% RH for 24 h. To determine NO reduction, 100 µL of the sample was mixed with 100 µL of Griess reagent containing 1% (w/w) sulfanilamide and 0.1% (w/w) N-1-[naphthyl] ethylenediamine dihydrochloride in 2.5% (w/w) H_3PO_4. The absorbance of the solution was determined after 10 min by a Varioskan Flash microplate reader (Thermo Fisher, Finland) at 550 nm. The results were reported as % NO reduction which was calculated by Equation 4 (Hernández-Ledesma, Hsieh, & de Lumen, 2009).

$$\%\ \text{nitric oxide reduction} = \frac{\mathbf{Abs_{control}}\text{-}(Abs_{sample}\text{-}Abs_{blank})}{Abs_{control}} \times \mathbf{100} \quad (4)$$

$Abs_{control}$ = Absorbance of cell treated with medium with LPS
Abs_{sample} = Absorbance of cell treated with EWH and medium with LPS.
Abs_{blank} = Absorbance of cells treated with EWH and medium without LPS.

2.5.3 Scratch-wound assay

The NHDF cells and HaCaT cells were seeded into a 6-well plate at a density of 1.25×10^5 cells/well and 3.5×10^5 cells/well, respectively. The plates were incubated at 37 °C in 5% CO_2/95% RH for 24 h. After incubation, the adherent cell layer was scratched with a sterile yellow pipette tip (200 µL) to form three vertical "wound" lines and then the medium was removed. Cellular debris was removed by washing with phosphate buffer solution pH 7.4. The cells were treated with 2 mL of the medium containing 0.031, 0.125, 0.5, and 2 mg/mL of EWH and standard OVA with a concentration of 0.031 mg/mL which was prepared using the same method as the cell viability test. The medium was used as the control. The cells were incubated at 37 °C in 5% CO_2/95% RH for 0, 12, and 24 h and then imaging of the scratched areas was carried out at two different points using an inverted fluorescence microscope (Carl Zeiss Microscopy, Germany) at 0 h (just after scratching the cells) and at 12 h and 24 h after incubation with EWH, OVA, and the control. Data were analyzed with the Image Pro Plus 7.0 program (Media Cybernetics, USA) in order to determine the width of the scratch and thus to calculate the rate of migration of cells using Equation 5 (Z. Wang, Wang, Farhangfar, Zimmer, & Zhang, 2012):

$$\%\ \text{Migration rate} = \frac{(Area_0\text{-}Area_n)}{Area_0} \times 100 \quad (5)$$

$Area_0$ = wound areas at time 0 h
$Area_n$ = wound areas at 12, and 24 h

2.5.4 Cell viability assay

The NHDF cells and HaCaT cells were seeded in a 96-well sterile plate with flattened bottom at 1×10^5 cells/well and 2×10^5 cells/well, respectively. Both cell preparations were cultivated overnight at 37 °C in 5% CO_2/95% RH. The cells were then washed with phosphate buffer solution pH 7.4 (100 µL). The EWH and standard OVA were prepared in a range of 0.031-10 mg/mL with DMEM supplementation with 10% (v/v) FBS. Samples (200 µL) of either EWH or standards were added and the medium was used as the control.

The RAW 264.7 cells were seeded in a 96-well sterile plate with flattened bottom at 7.5×10^4 cells/well and cultivated overnight at 37 °C in 5% CO_2/95% RH. The cells were then washed with phosphate buffer solution pH 7.4. Samples (200 µL) were added and the medium was used as the control.

The cell viability was measured at 0, 6, 12, and 24 h. At the end of each incubation time, all solutions were removed and the cell viability was measured using a Prestoblue® mixture (50 µL) (Sigma-Aldrich, USA) prepared as 1:9 parts of Prestoblue®:cell medium. The plate was incubated at 37 °C in 5% CO_2/95% RH for 90 min. After reaction, the emission of the mixture was determined using the Verioskan Flash microplate reader (Thermo Fisher, Finland) at wavelengths 560/590 nm. The results were reported as % cell viability which was calculated using Equation 6 (Fischer, Li, Ahlemeyer, Krieglstein, & Kissel, 2003):

$$\%\ \text{cell viability} = \frac{Abs_{sample}}{Abs_{control}} \times 100 \quad (6)$$

$Abs_{control}$ = Absorbance of cells treated with medium at 0 h
Abs_{sample} = Absorbance of cells treated with EWH at the time intervals

2.6 Statistical analysis

All experiments were performed in triplicate. The results are expressed as mean±SD. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS version 17.0, SPSS Inc., Chicago, IL, USA). Significant differences among the test samples were analyzed by ANOVA. Tukey post hoc test was used for multiple comparison analysis. A value of $P<0.05$ was considered statistically significant.

3. Results

The percent yield and total protein of EWH were 22.40±0.24 with a BSA equivalent of 0.4695±0.7304 g. The EW without hydrolysis had molecular weights that clustered in the 13-14 kDa and about 15-38 kDa range (Figure 1). EWH displayed molecular weights in the range below about 3.5 kDa. The lower band intensities could be a result of significant migration of very low molecular weight species to the lower electrode buffer.

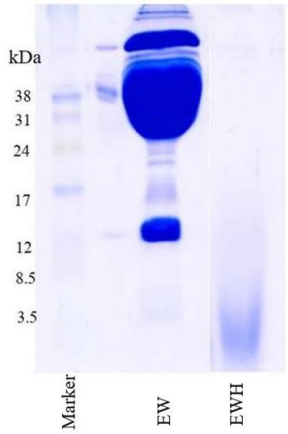


Figure 1. SDS-PAGE bands of Amersham Rainbow Markers, EW and EWH.

The amino acid profiles of EWH are shown in Table 1. Neutralized EWH contained many types of amino acids with the highest amounts for aspartic acid, which has a negatively charged carboxyl side chain at pH 7, lysine, leucine, and alanine, which contains protonated amine groups at pH 7.

Table 1. Amino acid profiles of neutralized EWH.

Types of amino acid	Neutralized EWH
	g/100g of sample
Alanine	9.38
Arginine	1.78
Aspartic acid	19.24
Cystine	0.00
Glutamic acid	8.93
Glycine	6.10
Histidine	2.2
Hydroxylysine	0.00
Hydroxyproline	0.00
Isoleucine	6.75
Leucine	9.86
Lysine	16.49
Methionine	4.42
Phenylalanine	5.78
Proline	8.20
Serine	2.23
Threonine	0.84
Tryptophan	1.55
Tyrosine	3.06
Valine	8.44
Asparagine	0.00
Cysteine	0.00
Glutamine	3.87

Antioxidant activities of EWH were determined using three methods: free radical scavenging capacity assay (DPPH), LPO inhibition assay, and reducing power assay. In the DPPH assay, EWH at 5 mg/mL possessed DPPH radical scavenging activity and the % DPPH radical-scavenging activity was 67.1%. Moreover, it exhibited significantly higher values than OVA, which is the main protein in egg white, and vitamin E acetate ($P<0.0001$) (Table 2).

In the LPO inhibition assay, EWH showed activities in lipid peroxidation inhibition at 81.1% (Table 2). However, it seemed that the EWH possessed significantly more inhibition activity than OVA, vitamin E acetate or glutathione ($P<0.05$).

The reducing power activities of EWH were substantial. At a concentration of 50 mg/mL of hydrolysate, EWH exhibited high reducing power with abilities equivalent to vitamin C 0.2502 mg. Furthermore, the reducing power of EWH was significantly higher than 50 mg/mL of OVA, 0.24 mg/mL of vitamin E acetate, 0.15 mg/mL of GSH and 0.125 mg/mL of trolox ($P<0.05$) (Table 2).

Figure 2 shows the effect of EWH on NO production. The lowest concentration of EWH (0.031 mg/mL) exhibited the highest % NO reduction at 20.87±5.02% compared with the control medium and it was not significantly different from 0.1 mg/mL diclofenac sodium. It showed that the activity decreased as the concentration increased.

This study showed that EWH enhanced migration of the keratinocytes (Table 4). At 12 h of incubation, EWH at 0.031 mg/mL and 0.125 mg/mL exhibited a significantly higher percentage of keratinocyte migration at 37.83% and 36.66%, respectively, compared to the control medium (26.47%) ($P<0.05$). Furthermore, it significantly increased cell migration at 52.32% and 49.74%, respectively, compared to the control (38.08%) ($P<0.05$) at 24 h of incubation (Table 3 and Table 4). However, it should be noted that the percent fibroblast migration of EWH at the same concentration was significantly faster than the control at 12 h but a slower rate was found at 24 h with no significant difference (Table 3).

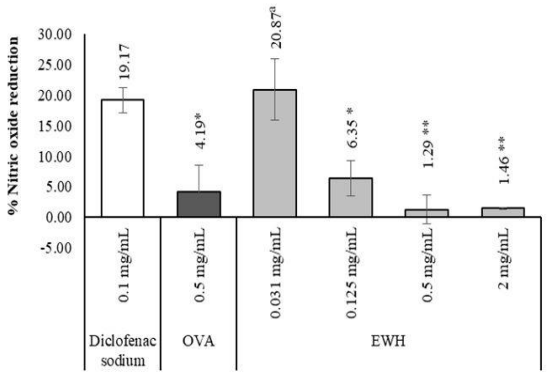


Figure 2. Nitric oxide inhibition abilities of standard diclofenac sodium, OVA and EWH in the RAW 264.7.
$^{**}P<0.01$, $^{*}P<0.05$ compared with diclofenac sodium (standard) $^{a}P<0.05$ compared with OVA.

PrestoBlue® Cell Viability Reagent was used to measure cell proliferation. It was a ready-to-use cell permeable resazurin-based solution. When added to cells, the PrestoBlue® reagent is reduced by enzymes in the mitochondria of viable cells and turns into the red-colored resorufin whose fluorescence emission at 560/590 nm was determined. Cell proliferation was evaluated in the NHDF cells, HaCaT cells, and RAW 264.7.

In RAW 264.7 at 12 h and 24 h, although the viability of RAW 246.7 exposed to EWH at all concentrations was not significantly different from the control and 0.031

Table 2. Antioxidant activities of EWH, OVA, and standards.

Samples	% DPPH radical-scavenging activity (at 5 mg/mL)	% Linoleic acid peroxidation inhibition (at 5 mg/mL)	Reducing power* equivalent to Vitamin C (mg)
Vitamin C	85.9 ± 0.8 [a]	100.6 ± 0.9 [a]	-
Trolox	95.9 ± 1.1 [b]	102.5 ± 3.1 [a]	0.0884 ± 0.0052[a]
Glutathione (GSH)	75.4 ± 1.1 [c]	65.8 ± 4.2 [b]	0.0927 ± 0.0019[a]
Vitamin E acetate	42.2 ± 0.7 [d]	58.7 ± 3.3 [b]	0.0126 ± 0.0034[b]
Ovalbumin (OVA)	22.8 ± 2.2 [e]	66.4 ± 1.5 [b]	0.0225 ± 0.0007[b]
EWH	67.1 ± 1.7 [f]	81.1 ± 2.9 [c]	0.2502 ± 0.0174[c]

Values with different letters (a-f) in each column indicate significant differences (P<0.05)
*Concentration of each substance in reducing power assay: trolox 0.125 mg/mL; GSH 0.15 mg/mL; vitamin E acetate 0.24 mg/mL; OVA 50 mg/mL and EWH 50 mg/mL.

Table 3. Percent migration abilities of EWH, OVA and control medium in fibroblast and keratinocyte cell.

Time	Concentration (mg/ml)	% Fibroblast migration	% Keratinocyte migration
12 h	EWH 0.031 mg/mL	27.39 ± 0.82[*a]	37.83 ± 1.26[**a]
	EWH 0.125 mg/mL	25.04 ± 2.61	36.66 ± 2.88[*a]
	EWH 0.5 mg/mL	23.70 ± 1.56	27.55 ± 4.04
	EWH 2 mg/mL	24.55 ± 1.22	25.97 ± 2.12
	OVA 0.031 mg/mL	23.22 ± 1.60	23.45 ± 3.53
	Control	23.79 ± 1.40	26.47 ± 3.93
24 h	EWH 0.031 mg/mL	52.11 ± 0.63	52.32 ± 0.57[**a]
	EWH 0.125 mg/mL	49.11 ± 3.00	49.74 ± 1.15[*a]
	EWH 0.5 mg/mL	48.62 ± 0.99	39.18 ± 4.54
	EWH 2 mg/mL	50.00 ± 2.09	38.00 ± 1.92
	OVA 0.031 mg/mL	50.96 ± 1.67	34.92 ± 3.65
	Control	49.11 ± 3.81	38.08 ± 6.11

[**]P<0.01, [*]P<0.05 compared with control medium
[a]P<0.05 compared with OVA.

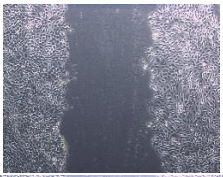
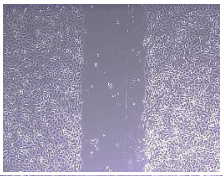
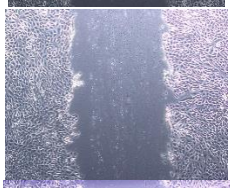
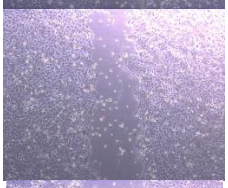
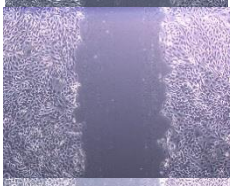
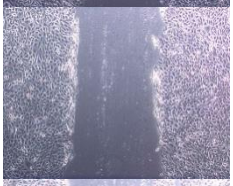
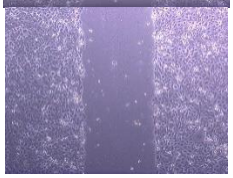
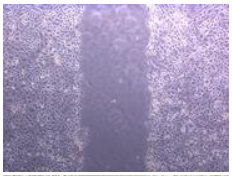
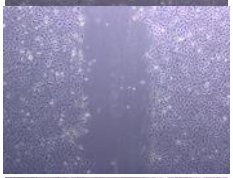
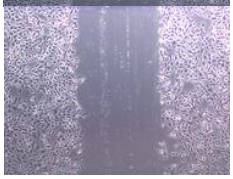
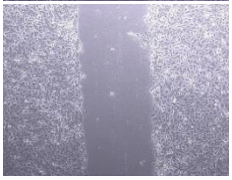
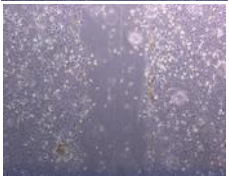
mg/mL OVA, it was found that the viability of RAW 264.7 treated with 0.031 mg/mL EWH was significantly less than the cells treated with 0.125-0.5 mg/mL EWH. The viability of RAW 264.7 tended to increase as the concentrations of EWH increased (Figure 3). Moreover, this study also demonstrated that EWH stimulates fibroblast proliferation. At 12 h of incubation, the EWH at 0.031 mg/mL significantly increased cell viability up to 111.23% which was greater than the control (105.10%) (P<0.05) (Figure 4). Moreover, EWH at 0.031 mg/mL promoted keratinocyte proliferation (Figure 5). At 6 h of incubation, it had significantly higher cell viability (120.46%) than the control (104.59%). At 24 h of incubation, EWH at 0.031 mg/mL and 0.125 mg/mL had significantly increased cell viability at 191.84% and 166.64%, respectively which were greater than the control (166.27%) (P<0.05). However, keratinocyte proliferation of EWH was not significantly different than the OVA.

4. Discussion

In this study, it was found that the antioxidant activities of EWH were greater than OVA. EWH possessed moderate free radical scavenging, high lipid peroxidation inhibition, and reducing power activities in comparison with trolox, vitamin C and glutathione, respectively. The antioxidant activity of EWH might be due to low molecular weight polypeptides (<3.5 kDa) and amino acid components. Several studies demonstrated that the antioxidant abilities of peptides depended on their molecular weight, types of amino acid, and amino acid sequence in the peptide chains. Small peptides can act as antioxidant agents better than long chain peptides (Cho *et al.*, 2014).

Although the detailed nature of the peptide and amino acid composition of the hydrolysates has not been studied, the amino acid profiles of hydrolysates show high amounts of electrically charged side chains of amino acids, such as aspartic acid, glutamic acid, and lysine, and hydrophobic amino acid contents, such as leucine, valine, and alanine. Some amino acids in hydrolysates, including tyrosine, histidine, glutamic acid, and leucine, can donate hydrogen or electrons to the DPPH free radical (Abeyrathne, Lee, & Ahn, 2013). Accordingly, the hydrolysate components act as radical scavenging compounds. Moreover, this hydrolysate inhibited lipid peroxidation activities. Most of the amino acid residues in EWH contain of hydrophobic amino acids such as alanine, leucine, tyrosine, valine, and phenylalanine. Hydrophobic amino acids are probably important for protecting hydrophobic substances such as lipids (Zhuang, Zhao, & Li, 2009). In particular, hydrophobic peptides in hydrolysates may donate protons to lipid radicals. In contrast, OVA contains large protein molecules which make them harder to insert its chains into the lipid cell membrane. As a result, it can not inhibit lipid radicals (Chen & Chi, 2012; Memarpoor-Yazdi, Asoodeh, & Chamani, 2012). Furthermore, EWH, which contained high amounts of acidic and basic amino acids, such as aspartic acid, lysine, glutamic acid, and arginine

Table 4. Cell migration of EWH, OVA, and control medium in keratinocytes at different time points.

Samples	0 h	12 h	24 h
EWH 0.031 mg/mL			
EWH 0.125 mg/mL			
EWH 0.5 mg/mL			
EWH 2 mg/mL			
OVA 0.031 mg/mL			
Control			

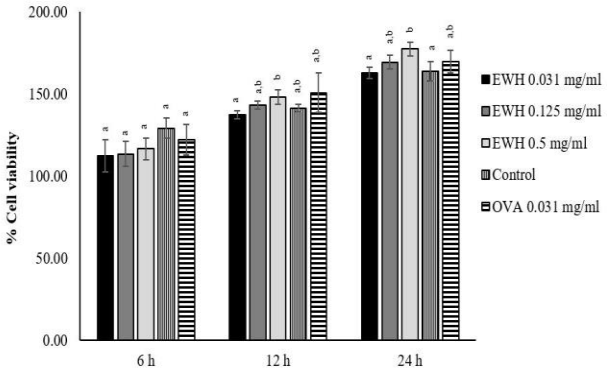


Figure 3. Cell proliferation activities of EWH, OVA, and control medium in RAW 264.7.
The different letters (a-b) indicate significant differences ($P<0.05$).

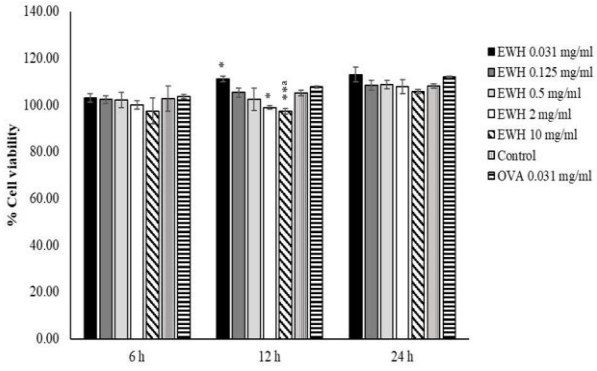


Figure 4. Cell proliferation activities of EWH, OVA, and control medium in NHDF cells,
$^{**}P<0.01$, $^{*}P<0.05$ compared with control medium, $^{a}P<0.05$ compared with OVA.

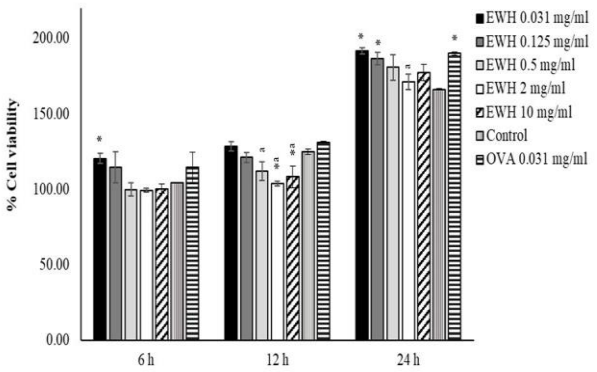


Figure 5. Cell proliferation activities of EWH, OVA, and control medium in HaCaT cells.
$^{**}P<0.01$, $^{*}P<0.05$ compared with control medium
$^{a}P<0.05$ compared with OVA.

in peptides chains, possessed good reducing power. They may act as metal chelators through binding with the side chains and N-terminal and C-terminal groups (Abeyrathne *et al.*, 2013).

RAW 264.7, a murine macrophage cell line, was activated by LPS to produce a large quantity of NO which is a major inflammatory mediator and can induce cell and tissue damage around a wound area. In this study, a range of EWH concentrations (0.031-0.5 mg/mL) had no cytotoxic effects on RAW 264.7. The proliferation of RAW 264.7 exposed to EWH in a range of 0.031-0.5 mg/mL increased in a dose-dependent manner. EWH at a low concentration (0.031 mg/mL) appeared to have anti-inflammatory action to some extent. However, NO inhibition decreased as EWH concentrations increased. Napoli *et al.* (2013) reported that the effect of NO production on the cellular process depends on its concentration and on the presence of other free radicals. The results showed that NO inhibition at low concentration appeared to exert a direct effect on cell proliferation and survival, whereas at higher EWH some parts of EWH might inhibit NO production and the remaining EWH might nurture RAW 264.7 proliferation and produce NO during the proliferation process resulting in lower NO inhibition. However, the anti-inflammatory action of EWH could also occur by inhibition of other inflammatory pathways, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-1β, interferon gamma (IFN-γ), IL-17, and cytokines, in the inflammatory pathway. Several studies have shown that egg white possesses anti-inflammation properties. For example, Choi *et al.* (2013) found that egg white-chalcanthite could inhibit NO and prostaglandin E2 in LPS-stimulated BV2 microglia. It also attenuated the expression of NO synthase, cyclooxygenase-2, and pro-inflammatory cytokines such as, IL-1β and TNF-α. Zhao *et al.* (2017) found that simulated gastrointestinal digestion from preserved egg white can inhibit the secretion of interleukin (IL)-8 and reduce TNF-α in a concentration-dependent manner.

It should be noted that keratinocyte and fibroblast migration in the proliferation assay decreased as the EWH concentration increased. This was probably due to the formation of soluble salts from the neutralization process in the EWH solutions at high concentrations that interfered with the electrolyte balance of cells that led to hypertonicity resulting in cell death (Robbins, 1970). In addition, EWH at a low concentration motivated fibroblast and keratinocyte proliferation and migration. This finding suggested that EWH at a low concentration might have a wound healing effect. However, fibroblast migration was found to be significant at 12 h but had slower migration at 24 h. It might be possible that EWH contains essential amino acids which are important for cell activities and amino acid sequences in the various peptide chains similar to cytokines and growth factors, thus providing cell nutrients (Ye *et al.*, 2016). According to a review on the wound healing process (Gonzalez *et al.*, 2016), these findings suggested that EWH at a low concentration might involve controlling the inflammation phase of wound healing. Then, fibroblasts located on the skin edge begin to proliferating and probably synthesize and secrete keratinocyte growth factors which stimulate neighboring keratinocytes to migrate into the wound area, proliferate, and differentiate in the epidermis in the proliferative phase.

5. Conclusions

EWH prepared by alkaline hydrolysis under high pressure and temperature appeared to have multifunctional anti-aging cosmetic activities, such as free radical scavenging, inhibited lipid peroxidation, and reducing power activities. Moreover, EWH at an optimal concentration may help to improve wound healing process. Therefore, EWH may be a good candidate as an anti-aging ingredient. However, *in vivo* studies and clinical trials are necessary to establish the anti-aging properties and allow for development of cosmeceutical products.

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