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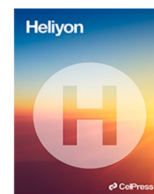

Research article

Egg white hydrolysate peptides act as antimicrobial and anti-inflammatory agents for acne

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ABSTRACT

A simple method to generate antibacterial peptides by alkaline hydrolysis of hen egg whites is reported. The method reproducibly generates short peptides with molecular weight of less than 14.4 kDa that exhibit low to no cytotoxicity on RAW 264.7 macrophage cells, but do inhibit the bacterial growth of *Cutibacterium acnes* (*C. acnes*), *Staphylococcus aureus* (*S. aureus*) and antibiotic-resistant *S. aureus* (MRSA), while also reducing nitric oxide production from heat-killed *C. acnes*-treated RAW 264.7 cells. Peptidomics revealed at least thirty peptides within the complex mixture, of which eight were evaluated individually. Three peptides (PK8, EE9 and RP8) were potent anti-inflammation and antibacterial agents, but notably the complex egg white hydrolysate (EWH) was more effective than the individual peptides. Electron microscopy suggests the antibacterial mechanism of both the hydrolysate and the selected peptides is through disruption of the cell membrane of *C. acnes*. These findings suggest that EWH and EWH-derived peptides are promising candidates for infection and inflammation treatment, particularly in managing acne and combating antibiotic-resistant bacteria like MRSA.

1. Introduction

Acne vulgaris is a chronic skin disorder that affects ~10 % of the human population [1]. It is caused by *Cutibacterium acnes*, an

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opportunistic bacterium that colonizes the hair follicle, hair shaft and sebaceous gland (pilosebaceous unit). Acne can have a significant effect on a person's physical appearance and psychological well-being [2–5]. As a result, there is a constant demand for treatments. Typically, topical antibiotics such as erythromycin and clindamycin are used as the first line of defense, while oral antibiotics such as tetracyclines and macrolides are used for more severe cases [6]. However, the prolonged use and misuse of these drugs can promote drug resistance and alter the local microbiomes. For example, *Staphylococcus aureus* is a common skin surface bacterium. While its role in the promotion of acne is controversial [7], the antibiotics used can cause the development of drug resistant variants. Treatments of acne over 12 weeks using erythromycin resulted in over a ~20 % increase in erythromycin-resistant *Staphylococcus aureus* carriage rates [8]. This is growing global problem [9] with methicillin-resistant *Staphylococcus aureus* (MRSA) for example being a heavy burden on healthcare systems [10].

To this end, food-derived antimicrobial peptides (AMPs) provide an interesting solution. AMPs have remarkable activity against bacteria, fungi, and viruses, and also promote wound healing and modulate immune responses [11–13]. More importantly, AMPs are less likely to induce drug resistance in bacteria than other antibiotic treatments due to their generic membrane disrupting mechanism of action [14]. AMPs can be sourced both naturally and through biomimetic synthesis. For example, AMPs can be generated from enzymatic lysis of natural proteins or through fractionation of natural proteins by molecular weight [15–17]. Currently, the identification of new sources and methods to reproducibly generate AMPs is a key focus in this field.

Egg white, especially hen egg white, is a rich source of AMPs and has been used in various food, beauty and skincare practices for centuries. Previous studies have shown that egg white and its derived peptides have potential pharmacological properties, including antibacterial, anti-cancer, anti-inflammatory, ACE inhibition, and immunomodulatory effects, making them valuable for disease prevention and treatment [18]. Additionally, peptides derived from egg white trypsin and chymotrypsin hydrolysates exhibited tyrosinase inhibition [19]. There are also reports indicating alternative applications for egg whites, such as using ointment-based egg whites for the effective treatment of second-degree burn wounds [20].

In our previous study [21,22], we hydrolyzed hen egg white proteins using an alkaline hydrolysis method (EWH). The resulting hydrolysate protein demonstrated antioxidant and anti-inflammatory activities. However, the antimicrobial and anti-inflammatory (*C. acnes* induced inflammation) potential of this EWH has not been investigated. Therefore, in this study, we evaluate the antimicrobial activity of EWH and its derived peptides from alkaline hydrolysis against *C. acnes*, *S. aureus* and MRSA, the major causes of acne and secondary infections, respectively. The anti-inflammatory activity was also evaluated using *C. acnes*-induced RAW 264.7 cells as an inflammatory model. By exploring the origin, structure, and mechanism of action of these peptides, we aim to shed light on their use as an innovative treatment for managing acne.

2. Materials and methods

2.1. Microbial strains and reagents

The bacterial strains used in this study were methicillin-resistant *Staphylococcus aureus* (MRSA) (DMST 20646), *Staphylococcus aureus* (ATCC 25923) and *Cutibacterium acnes* (DMST 14916). Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (MO, USA). Schaedler broth was purchased from Condalab (Madrid, Spain). The nutrient broth was purchased from Himedia (Mumbai, India). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco (CA, USA).

2.2. Egg white sample preparation

Egg white hydrolysates (EWH) were prepared by alkaline hydrolysis as previously described [22]. In brief, the hen egg white (EW) was separated from the yolk and mixed with a 0.4 M KOH solution (1:3 v/v). The solution was incubated at 55 °C for 2 h with the pH monitored every 30 min to ensure it remained close to the initial value (pH 12–13). The solution was then autoclaved at 121 °C for 2 h. Next, the solution was filtered twice through five layers of gauze and the filtrate was collected. The pH of the filtrate was neutralized by fumigating concentrated hydrochloric acid. Finally, the filtrate was centrifuged at 8000×*g* for 15 min to yield a supernatant which was subsequently spray-dried and stored at −40 °C until use.

2.3. Confirmation of protein hydrolysis

2.3.1. Degree of hydrolysis

To determine the success of the egg white hydrolysate preparation, the degree of hydrolysis was measured by analyzing the α-amino acid content based on modification of a previously reported method [23]. In brief, 125 μL of peptide hydrolysate or completely digested egg white (control; using 6 M HCl at 100 °C for 24 h) was mixed with 2 mL of 0.2 M phosphate buffer, pH 8.2. Next, 1 mL of 0.1 % 2,4,6-trinitrobenzene sulfonic acid solution was added, mixed well by vortex, and incubated in a temperature-controlled bath at 50 °C for 10 min in the dark. The reaction was stopped by the addition of 2 mL of 0.1 M sodium sulfite and incubated at room temperature for 15 min. The absorbance of the solution was measured at a wavelength of 420 nm to analyze the α-amino acid content by comparing it with a calibration curve created by using a leucine solution (0.1–0.6 mM). The degree of hydrolysis was calculated from the following equation:

$$\text{Degree of hydrolysis (\%DH)} = \left(\frac{L_t - L_0}{L_{max} - L_0}\right) \times 100$$

where: L_t is the amount of α-amino acid in the solution after hydrolysis.

L_0 is the amount of α-amino acid in the egg white solution before hydrolysis.

L_{max} is the amount of α-amino acid in completely digested egg white (control)

2.3.2. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the hydrolysis of egg white proteins into peptides. SDS-PAGE was composed of a 4 % (w/v) stacking gel and a 15 % (w/v) separating gel. Before electrophoretic separation, the protein and peptide concentrations of EW and EWH were determined using the Bradford method [24]. 5 μg of sample were mixed with 2 × solubilizing dye containing β-mercaptoethanol and boiled in boiling water for 5 min before loading into the gel. The gel was run using an electric potential of 150 V for 1 h. Protein bands in the electrophoretic gel were visualized by staining with a Coomassie brilliant blue R-250 staining solution.

2.4. RAW 264.7 cytotoxicity assay

Murine macrophage cells (RAW 264.7) were used to assess the impact of synthetic peptides on cell cytotoxicity using the previously described MTT assay [25]. These RAW 264.7 were cultured in DMEM, supplemented with 10 % heat-inactivated FBS and 1 % antibiotic-antimycotic. The cell cultures were maintained at 37 °C with 5 % CO_2 and 95 % relative humidity. RAW 264.7 cells were seeded into 96-well plates at a density of 2×10^4 cells/well and cultured at 37 °C overnight. Subsequently, a final concentration of each synthetic peptide at 1000 μg/mL was added to the cell culture (total volume 100 μL). After 24 h incubation in a 5 % CO_2 incubator, the culture medium was removed and an MTT solution was added to the cells (final concentration of 0.5 mg/mL in fresh culture medium). Following 30 min incubation, the culture medium was aspirated and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density at 570 nm was measured using a microplate reader (ALLSHENG, Zhejiang, China). Cell viability was calculated using the following formula:

$$\%\ \text{cell viability} = \left(\frac{OD_{570}\ \text{of sample}}{OD_{570}\ \text{of untreated control}}\right) \times 100$$

2.5. Peptidomics analysis of egg white hydrolysate

2.5.1. Peptidomics analysis

Lowry's assay [26] was used to determine the protein concentration in EWH using bovine serum albumin (BSA) as a standard. EWH was purified by C18 ZipTip (Merck Millipore, Darmstadt, Germany) and analyzed by LC-MS/MS. The peptide samples were prepared for injection into an Ultimate 3000 Nano/Capillary LC System (Thermo Scientific, UK) coupled to a Hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics) which was equipped with a Nano-captive spray ion source. 1 μL of peptide (100 ng) was enriched on a μ-Precolumn 300 μm i.d. × 5 mm C18 Pepmap 100, 5 μm, 100 A (Thermo Scientific, UK), separated on a 75 μm I.D. × 15 cm and packed with Acclaim PepMap RSLC C18, 2 μm, 100 Å, nanoViper (Thermo Scientific, UK). The C18 column was enclosed in a thermostatically controlled column oven set to 60 °C. Solvents A and B containing 0.1 % formic acid in water and 0.1 % formic acid in 80 % acetonitrile respectively were supplied in the analytical column. A gradient of 5–55 % solvent B was used to elute the peptides at a constant flow rate of 0.30 μL/min for 30 min. Electrospray ionization was carried out at 1.6 kV using the CaptiveSpray. Nitrogen was used as a drying gas (flow rate of 50 L/h). Collision-induced-dissociation (CID) product ion mass spectra were obtained using nitrogen gas as the collision gas. Mass spectra (MS) and MS/MS spectra were obtained in the positive-ion mode at 2 Hz over the range of *m/z* 150–2200. The collision energy was adjusted to 10 eV as a function of the *m/z* value. The LC-MS analysis of each sample was carried out in triplicate.

MaxQuant 2.0.3.0 was used to quantify the proteins in individual samples using the Andromeda search engine to correlate MS/MS spectra to the Uniprot Araceae family database [27]. Label-free quantitation with MaxQuant's standard settings was performed: mass tolerance of 0.6 Da for main search, unspecific digestion and the oxidation of methionine and acetylation of the protein N-terminus as variable modifications. Only peptides with a minimum of 7 amino acids, as well as at least one unique peptide, were required for protein identification. Only proteins with at least two peptides and at least one unique peptide were identified and used for further data analysis. The maximal number of modifications per peptide was set to 5.

2.5.2. Peptide selection and synthesis

Based on peptidomics analyses, eight peptides were chemically synthesized. The peptide selection criteria were semi-random. Firstly, only peptides with a hydrophobicity of less than 65 % were selected to facilitate re-dissolution after synthesis. Secondly, the analyzed peptides were split into the following groups based on net molecular charge, and peptides were selected such that each group is represented in the final synthetic peptides: strong negative charge (−4), weak to moderate negative charge (−1 to −2), neutral (0), weak to moderate positive charge (+1 to +2) and strong positive charge (+4).

The selected peptides are shown in Table 2 and were synthesized using standard Fmoc solid phase synthesis. All peptides were purified to 95 % purity by reverse-phase high-performance liquid chromatography (HPLC) and verified for identity with electrospray

ionization mass spectrometry at GL Biochem Ltd. (Shanghai, China).

2.5.3. *Circular dichroism of peptides*

The selected peptides were diluted to a concentration of 0.4 μg/μL in 1 × phosphate buffer saline solution (PBS) or 80 % aqueous trifluoroethanol (TFE) in a total volume of 100 μL. Circular dichroism (CD) spectra were recorded at a scanning speed of 20 nm/min from 180 to 260 nm using a Jasco spectrometer (Easton, MD, USA). Data were plotted as mean residue ellipticities.

2.6. *Antimicrobial assay*

2.6.1. *Antimicrobial activity tests*

C. acnes was grown in Schaedler broth medium at 37 °C for 72 h in the absence of oxygen. The single colony of *S. aureus* and MRSA was inoculated into Nutrient broth (NB) and incubated at 37 °C for 18 h. After that, the bacteria cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.1 and 0.001 for *C. acnes* and both *S. aureus* strains, respectively. 50 μL of each bacteria suspension were placed in a 96-well plate and mixed with 50 μL of samples including intact EW (93.75–3000 μg/mL), EWH (93.75–3000 μg/mL), peptides (1000 μg/mL) and antibiotics ampicillin (AMP; 500 μg/mL) or clindamycin (CLN; 500 μg/mL). Plates were incubated at 37 °C for 18–22 h. After the incubation period, the optical density at 600 nm in each well was measured to calculate the inhibition of bacterial growth. Cultured bacteria that were not incubated with any substances were used as a negative control (i.e. 0 % growth inhibition). The percentage inhibition was used to calculate the MIC_{50} and MIC_{90} using GraphPad Prism 8 (San Diego, CA, USA). The MIC_{50} represents the minimum inhibitory concentration (MIC) at which 50 % of the bacteria growth was inhibited, while the MIC_{90} represents the MIC at which 90 % of the bacteria growth was inhibited.

To investigate thermal stability, each synthetic peptide was incubated at 90 °C for 15 min and then at 121 °C for 30 min. After cooling to room temperature, their antimicrobial activity against *C. acnes*, *S. aureus* and MRSA were measured as described above.

2.6.2. *Examination of cell* ***membrane*** *destruction of C. acnes by scanning electron microscopy*

The morphology change of *C. acnes* was observed based on the scanning electron microscopy (SEM) method as described previously [17]. Bacterial suspensions were mixed with selected peptides (1000 μg/mL) and were then incubated at 37 °C for 18–22 h. 50 μL of treated bacterial suspensions were pipetted onto a polycarbonate membrane and left at room temperature for 10 min. 1 mL of 2.5 % (v/v) glutaraldehyde was added to the membrane to immobilize the bacterial cells. The cells were incubated at room temperature for 1 h. Dehydration was then performed by submerging the membrane in aqueous ethanol solutions with concentrations of 30, 50, 70, 90 and 100 % at room temperature for 15 min. The polycarbonate membrane was dried in a desiccator for 12–24 h and subsequently attached to the stub with carbon tape. Samples were coated with gold palladium and imaged using a SEM (Quattro-S E-SEM, Thermo Fisher Scientific, USA).

2.7. *Anti-inflammatory assay*

2.7.1. *Heat-killed C. acnes preparation*

C. acnes was grown in Schaedler broth medium at 37 °C for 72 h under anaerobic conditions. Following centrifugation at 1800×*g* for 5 min, the cells were harvested and washed three times with phosphate buffer saline (PBS). The cell pellets were resuspended in PBS after the final wash and heat-killed at 90 °C for 10 min. The heat-killed *C. acnes* was then aliquoted and stored at 4 °C before use.

2.7.2. *Examination C. acnes induced the inflammation in RAW 264.7 macrophage cells*

RAW 264.7 macrophage cells were plated at a density of 2×10^4 cells/well in a 96-well plate (total volume 100 μL/well) and were exposed to heat-killed *C. acnes* (wet weight 100 μg/mL), either alone or in combination with various test peptides. Following 24 h of incubation at 37 °C with 5 % CO_2 and 95 % relative humidity, 100 μL of cell culture medium from each treatment was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. Subsequently, the absorbance at 540 nm was measured, and NO production was calculated as a percentage relative to the no peptide control. Additionally, the MTT assay was used to assess the cell viability of RAW 264.7 cells following each treatment, with the viability of cells treated with heat-killed *C. acnes* set as 100 % viability.

Table 1
The MIC_{50} and MIC_{90} values of EW and EWH against *C. acnes*, *S. aureus* and MRSA.

Sample	*C. acnes*		*S. aureus*		MRSA	
	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
EW	1358 ± 33.23	2928 ± 78.83	ND	ND	ND	ND
EWH	218 ± 5.69	480 ± 61.06	656 ± 27.54	1574 ± 70.72	425 ± 5.66	950 ± 20.22

ND = not detected within the concentration range of the samples used in the test.

Table 2
Peptidomics analysis of egg white hydrolysate.

Sequence	Mass (Da)	Proteins	Score	Protein name	Organism	Hydrophobic ratio	Net charge	Note
LPDEVSDLE[a]	1015.47	P01013	23.02	Ovalbumin-related protein X	*Gallus gallus*	33 %	−4.00	Selected as LE9
EVSGLEQLE[a]	1002.49	P01012	53.17	Ovalbumin	*Gallus gallus*	33 %	−3.00	Selected as EE9
FDKLPGFGD	994.48	P01012	147.20	Ovalbumin	*Gallus gallus*	33 %	−1.00	
ALAMVYLGAKD	1150.61	P01012	180.80	Ovalbumin	*Gallus gallus*	64 %	0.00	
LAMVYLGAKD	1079.57	P01012	149.94	Ovalbumin	*Gallus gallus*	60 %	0.00	
AMVYLGAKD	966.48	P01012	138.54	Ovalbumin	*Gallus gallus*	56 %	0.00	
KILELPFA	929.56	P01012	114.78	Ovalbumin	*Gallus gallus*	63 %	0.00	
ALAMVYLGAK	1035.58	P01012	173.88	Ovalbumin	*Gallus gallus*	70 %	+1.00	
AVAMITFA	822.43	P19121	17.74	Albumin	*Gallus gallus*	88 %	0.00	
PKAPFSEVSK	1088.59	P19121	19.78	Albumin	*Gallus gallus*	30 %	+1.00	
EDGKVMVL	889.46	P01005	143.00	Ovomucoid	*Gallus gallus*	50 %	−1.00	
ARDDNKVE	945.45	P02789	31.94	Ovotransferrin	*Gallus gallus*	25 %	−1.00	
IIDVKMLS[a]	917.53	P20740	73.04	Ovostatin	*Gallus gallus*	63 %	0.00	Selected as IS8
QTPLPQVP	878.49	P20740	20.70	Ovostatin	*Gallus gallus*	25 %	0.00	
EDVHVDTE	942.39	F1NSM7	29.34	Ovocleidin-116	*Gallus gallus*	25 %	−3.75	
EDVHVDTEG	999.41	F1NSM7	27.53	Ovocleidin-116	*Gallus gallus*	22 %	−3.75	
ARTQPEVASAP	1125.58	F1NSM7	22.74	Ovocleidin-116	*Gallus gallus*	36 %	0.00	
PAPSTGGRIVA[a]	1024.57	F1NSM7	22.74	Ovocleidin-116	*Gallus gallus*	36 %	+1.00	Selected as PA11
FLVRANVF	964.55	P20740	13.99	Ovostatin	*Gallus gallus*	75 %	+1.00	
PEKKAKKK[a]	955.62	Q9PRR7	16.96	Ovofactor-1	*Gallus gallus*	13 %	+4.00	Selected as PK8
DIDKIQLE	972.51	P02845	57.56	Vitellogenin-2	*Gallus gallus*	38 %	−2.00	
GEHEAKIV	881.46	P02845	23.97	Vitellogenin-2	*Gallus gallus*	38 %	−0.75	
TLVGSQKF	878.49	P02845	19.36	Vitellogenin-2	*Gallus gallus*	38 %	+1.00	
ALHHDAGWE	1034.46	Q8JGM4	46.66	Sulfhydryl oxidase 1	*Gallus gallus*	44 %	−1.50	
VALDLLQY[a]	933.52	Q8JGM4	59.21	Sulfhydryl oxidase 1	*Gallus gallus*	63 %	−1.00	Selected as VY8
VSEQRIEL	972.52	P30371	59.21	Transforming growth factor beta-2 proprotein	*Gallus gallus*	38 %	−1.00	
RKLLEGEE[a]	972.52	Q49MC0	32.47	Vimentin	*Gallus gallus*	25 %	−1.00	Selected as RE8
VAGVNYFLD	996.49	P01034	21.30	Cystatin-C	*Gallus gallus*	56 %	−1.00	
AVVPVPVS	766.46	A0A146J2U8	18.96	Protein TENP	*Gallus gallus*	63 %	0.00	
RVTNLGRP[a]	911.53	P26007	23.97	Integrin alpha-6	*Gallus gallus*	25 %	+2.00	Selected as RP8

[a] Selected for chemical synthesis.

2.8. Statistical analysis

Statistical comparisons of all experiments were performed by repeating the experiments three times. Experimental results are expressed as the mean ± standard deviation (SD). A one-way ANOVA was used to compare differences in means with a Duncan post-

hoc multiple comparison. Differences in means were determined by a P value < 0.05. All statistical tests were performed using the Statistical Package for Social Sciences (SPSS version 17.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. KOH hydrolyzes egg white proteins to a mixture of peptides

Research increasingly focuses on developing food ingredients with biological activities beyond basic nutrition. These bioactive compounds potentially offer health benefits such as disease prevention, improved well-being and a reduced reliance on traditional drugs. Food proteins, in particular, exhibit various biological activities alongside their nutritional role [28]. Notably, egg white proteins such as ovalbumin, ovotransferrin and lysozyme exhibit significant antioxidant properties [28]. Recent studies highlight egg proteins, particularly those in egg white, as a promising source of novel bioactive peptides obtained through enzymatic hydrolysis [28,29]. In this study, hen egg white proteins were subjected to KOH hydrolysis to produce hydrolyzed short peptides with potentially enhanced or novel biological activities compared to their original protein forms. The degree of protein hydrolysis was determined to assess the extent of peptide formation, which is a crucial metric for ensuring reproducible antimicrobial and anti-inflammatory peptide production. To determine it, the α-amino acid content after hydrolysis was determined using 2,4,6-trinitrobenzene sulfonic acid and the hydrolyzed samples were run on SDS-PAGE to evaluate the average molecular weight of the resulting peptides. As a positive control, 100 % hydrolysis of egg whites was achieved by treating the egg whites with 6 M hydrochloric acid at 100 °C for 24 h. In comparison, upon treatment of egg whites with 0.4 M KOH, the degree of hydrolysis was 37.64 ± 3.81 % (N = 3). This value suggests that the EWH is likely to contain active peptides as a higher degree of hydrolysis often results in enhanced biological activity [30,31]. Furthermore, SDS-PAGE (Fig. 1 and Fig. S1) showed the hydrolyzed peptides (EWH) have a low molecular weight (below 14.4 kDa) and that they are unlikely to be contaminated with non-hydrolyzed intact egg white proteins (*cf*. unhydrolyzed EW negative control). In combination, these results suggest KOH hydrolysis produces uniform low molecular weight peptides from egg whites.

3.2. Antibacterial activities of EW and EWH

Next, the ability of the hydrolysate to inhibit bacterial growth was evaluated by comparing their performance with known antibacterial drugs ampicillin and clindamycin. Ampicillin disrupts peptidoglycan cross-linking resulting in the weakening of the cell wall and also inactivates autolytic enzyme inhibitors aiding in cell wall degradation [32]. For *C. acnes*, *S. aureus* and MRSA, 500 μg/mL ampicillin inhibited growth by 92.92, 97.85 and 100.00 % respectively (Fig. 2A–C). Clindamycin is used to treat acne and specifically targets *C. acnes* colonization and proliferation through binding the 50S subunit of bacterial ribosomes thereby inhibiting protein synthesis [33]. For *C. acnes*, *S. aureus* and MRSA, 500 μg/mL clindamycin inhibited growth by 100.00, 96.35 and 51.86 % respectively (Fig. 2A–C).

EW and EWH both exhibited dose-dependent antibacterial activity (93.75–3000 μg/mL; Fig. 2A–C; Table 1). Notably EWH displayed greater potency than EW at almost all concentrations. In the specific case of *C. acnes*, the MIC_{50} for EW was 1358 μg/mL, whereas for EWH it was 218 μg/mL, almost an order of magnitude lower. More strikingly, for *S. aureus* and MRSA, MIC_{50} and MIC_{90} values could only be determined for EWH (656 μg/mL and 1574 μg/mL against *S. aureus*, 425 μg/mL and 950 μg/mL against MRSA).

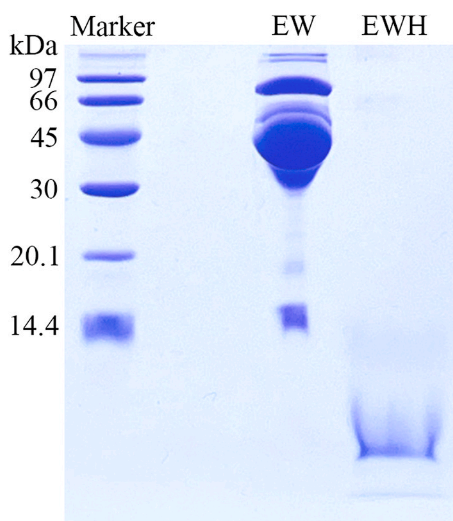

Fig. 1. Confirmation of egg white protein hydrolysis using 15 % SDS-PAGE. The marker lane comprises low molecular weight protein ranging from 14.4 to 97 kDa (GE Healthcare, UK). EW is the intact egg white (5 μg) and EWH is the egg white hydrolysate (5 μg) after treatment with a KOH solution. The uncropped version of Fig. 1 was shown as a supplementary file, named Fig. S1.

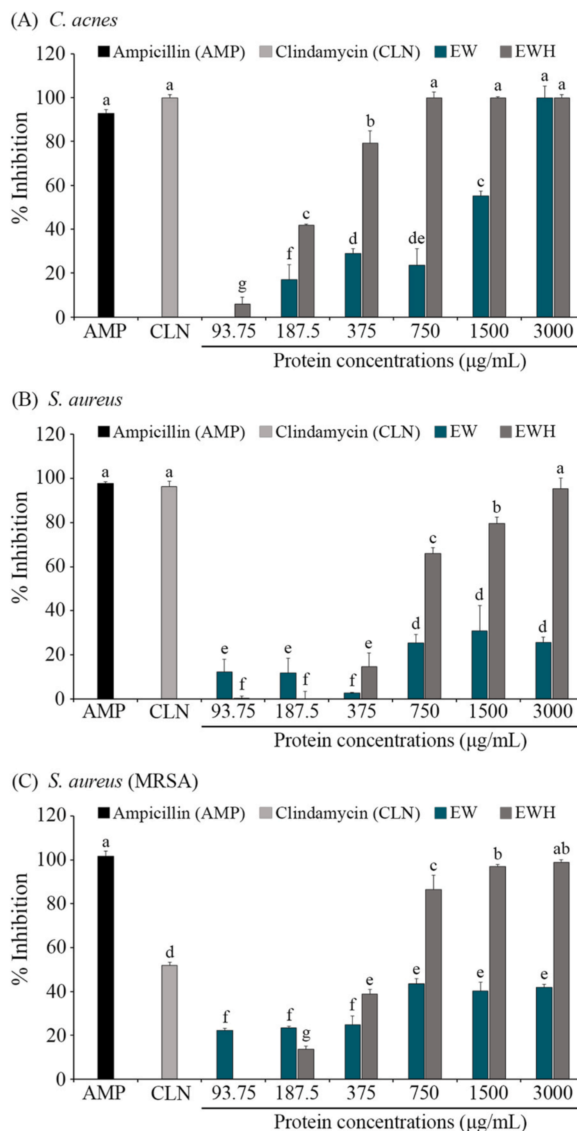

Fig. 2. Egg white hydrolysate can completely inhibit bacterial growth. The graphs show antibacterial activities of ampicillin, clindamycin, intact egg white (EW) and egg white hydrolysate (EWH) against (A) *C. acnes*, (B) *S. aureus* and (C) MRSA. The distinct letters (a–g) presented on the bar graphs indicate significant differences in the mean values ($P < 0.05$).

This demonstrates alkaline hydrolysis of the egg whites is important and corroborates previous studies that showed pepsin hydrolyzed egg white is effective against *S. aureus* [34–36]. EWH exhibited enhanced effectiveness in inhibiting the growth of MRSA compared to *S. aureus* itself. Finally, it is interesting to note that at lower concentrations EWH is more potent against *C. acnes* than *S. aureus* suggesting it could be used to specifically target acne causing *C. acnes*.

3.3. Peptidomics analysis of EWH

Given these promising results, we sought to identify the specific peptides in the EWH that are responsible for the majority of the antibacterial activity. Peptidomics analysis of EWH identified at least 30 enriched peptides with molecular weights below 14.4 kDa (8–11 amino acid residues; ~766.46–~1150.61 Da; net charge from −4 to +4). These peptides were derived from the 15 egg white proteins listed in Table 2.

Classically AMPs are amphiphilic with a net cationic charge (+2 to +9) [14], a hydrophobic region [14] and an α-helical structure [37]. This enables them to interact non-specifically with the anionic phospholipids of the cell membrane and subsequently penetrate the membrane disrupting normal cellular function. However, there are notable exceptions such as anionic Fibrinopeptide A, Fibrinopeptide B and Thymosin-β4 [38] and those that adopt β-sheets [39]. A small subset of natural AMPs have extended/random-coil

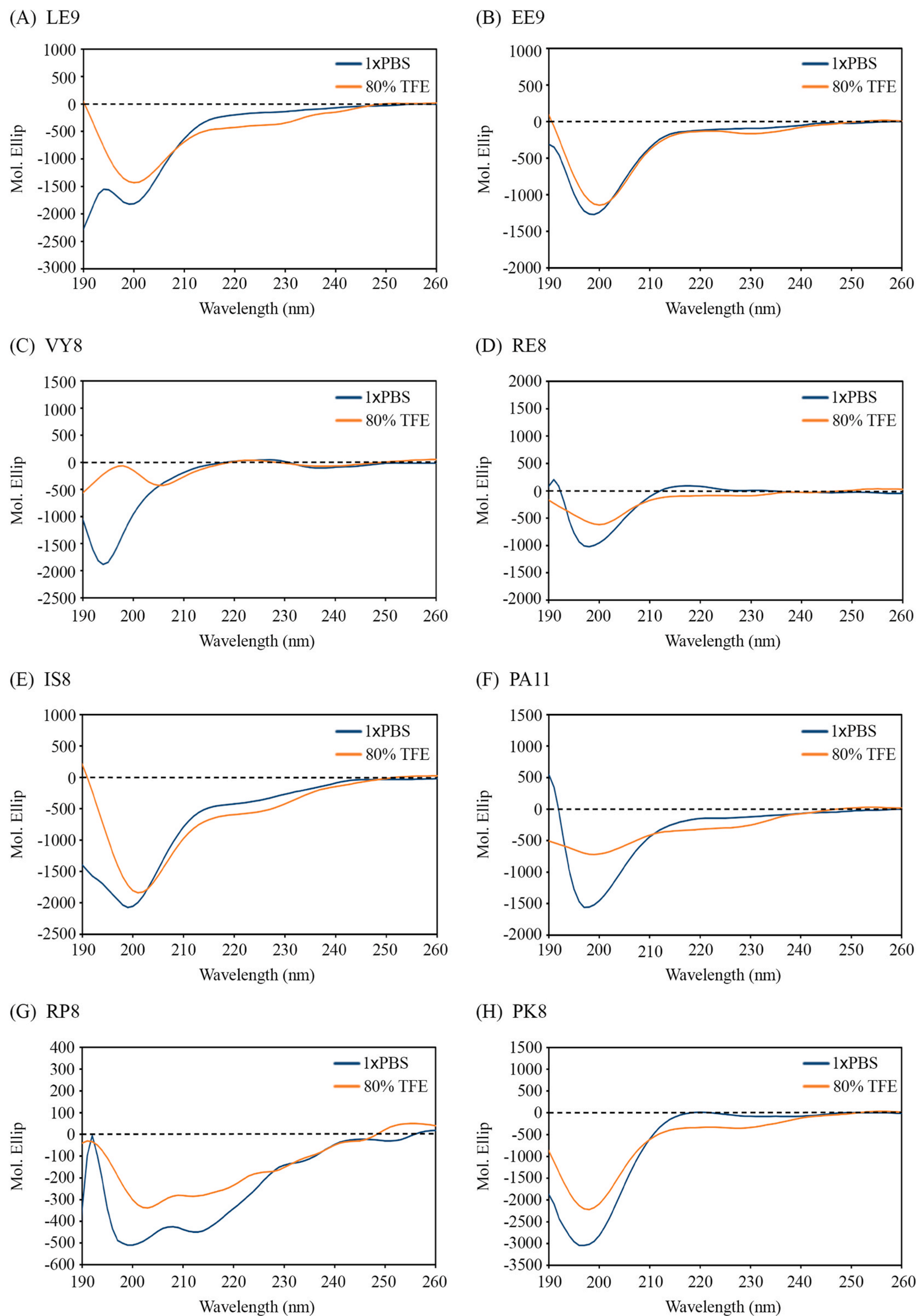

Fig. 3. The circular dichroism (CD) spectra of 8 synthetic peptides including LE9 (A), EE9 (B), VY8 (C), RE8 (D), IS8 (E), PA11(F), RP8 (G) and PK8 (H). The peptides were dissolved in 1 × phosphate buffered saline (PBS) and 80 % trifluoroethanol (TFE). The mean residue ellipticity was plotted against wavelength.

secondary structure and generally lack a well-defined conformation. These peptides often have a high content of arginine, proline, tryptophan and/or histidine residues [40]. As a consequence, when narrowing down the 30 peptides to test, we took a pseudo-random approach. Firstly, we rejected any peptides with hydrophobicity greater than 65 %. This was to ensure only peptides with good solubility were selected. Secondly, we split the 30 peptides into groups based on their net charge and selected representative examples for each group (Table 2): strong negative charge (−3 to −4; peptides EE9 and LE9), weak to moderate negative charge (−1 to −2; peptides VY8 and RE8), neutral charge (0; peptide IS8), weak to moderate positive charge (+1 to +2; peptides PA11 and RP8) and strong positive charge (+4; peptide PK8). From each of these groups, we selected a representative peptide.

In total, 8 peptides were selected and their secondary structures were investigated by CD spectroscopy in either PBS (mimicking an aqueous environment) or TFE (mimicking the hydrophobic environment of the microbial membrane) solutions. In PBS, most peptides adopted a random coil or extended conformation, as evidenced by a strong negative band at ~195–200 nm and a positive band near zero at ~217–220 nm (Fig. 3A–F, H) [41]. RP8, however, displayed a distinct profile with a positive peak at ~192 nm and a typical negative peak at ~197–203 nm, along with a negative band at ~214–217 nm, indicating primarily an α-helical structure (Fig. 3G) [41]. In TFE, LE9, EE9, RE8, IS8 and PK8 retained their random coil or extended conformations (Fig. 3A, B, D, E, H). VY8 adopted a β-sheet structure, as indicated by its CD spectrum with a minimum peak at ~205–219 and a positive peak at ~196–199 nm (Fig. 3C) [42]. Conversely, PA11 and RP8 exhibited characteristic α-helical spectra with double minima around ~200–208 and ~214–227 nm (Fig. 3F and G). This demonstrates that the local environment is critical to peptide structure and potentially function on interaction with the cell membrane.

3.4. Cytotoxicity effect of selected synthetic peptides

To screen these peptides, a single concentration (1000 μg/mL) was used for all screening experiments. Cytotoxicity was assessed using the MTT assay and RAW 264.7 cells. After 24 h of treatment, all of the peptides showed minimal cytotoxicity (cell viability ranging from 84.93 to 105.55 %; Fig. 4). RE8 caused the greatest reduction in cell viability (~15 %). No significant conclusions were drawn from this observation other than all peptides showing minimal cytotoxicity, which is promising given the high concentrations of peptides used in this screen.

3.5. Antibacterial activities of selected synthetic peptides

Next, the antibacterial properties of these peptides were evaluated. At a concentration of 750 μg/mL, EWH caused >65 % inhibition of bacterial (*C. acnes*, *S. aureus* and MRSA) cell growth (Fig. 2). At a concentration of 1000 μg/mL, all eight selected peptides inhibited bacterial growth by ~20–60 % (Fig. 5A). While less potent than EWH, these peptides did inhibit bacterial growth and clear trends could be observed.

Firstly, positively charged peptides performed better than neutral or negatively charged peptides (PA11, +1 charge, 37.81–50.42 % inhibition; RP8, +2 charge, 35.90–48.17 % inhibition; PK8, +4 charge, 26.51–60.08 % inhibition). Indeed, neutrally charged IS8 was the least potent peptide (Fig. 5A). These findings corroborate the general design principles for AMPs reported in literature and discussed in the peptide selection section of this work [14].

Secondly, there was a notable exception in EE9 (−3 charge, 38.06–59.29 % inhibition) (Fig. 5A). Given that interaction with a negatively charged membrane is critical to AMP activity, it is surprising that EE9 is active. It has been postulated that negatively charged residues can interact with microbial membranes by utilizing metal ions to establish cationic salt bridges with negatively charged components of microbial membranes [14,43].

Thirdly, the most potent peptides PA11, PK8 and EE9 inhibited *C. acnes* growth by >50 % and some did display partial selectivity for one strain over another (~2-fold selectivity). PK8 (+4 charge) was the most selective for *C. acnes* relative to *S. aureus* and MRSA (60.08, 26.51 and 29.43 inhibition respectively). Conversely, only VY8 (−1 charge) and LE9 (−4 charge) were more selective for *S. aureus* compared to *C. acnes* (VY8: 45.66 and 33.72 % inhibition respectively; LE9: 36.41 and 28.90 % inhibition respectively). VY8

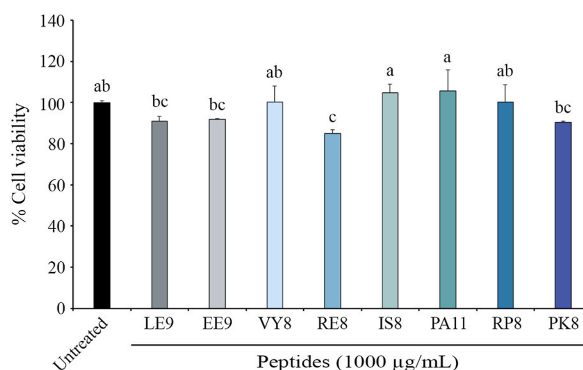

Fig. 4. The cytotoxicity of the 8 synthetic peptides that were enriched in EWH against RAW 264.7 cells using the MTT assay. Each value is expressed as the mean ± SD. The distinct letters (a–c) presented on the bar graphs indicate significant differences in the mean values ($P < 0.05$).

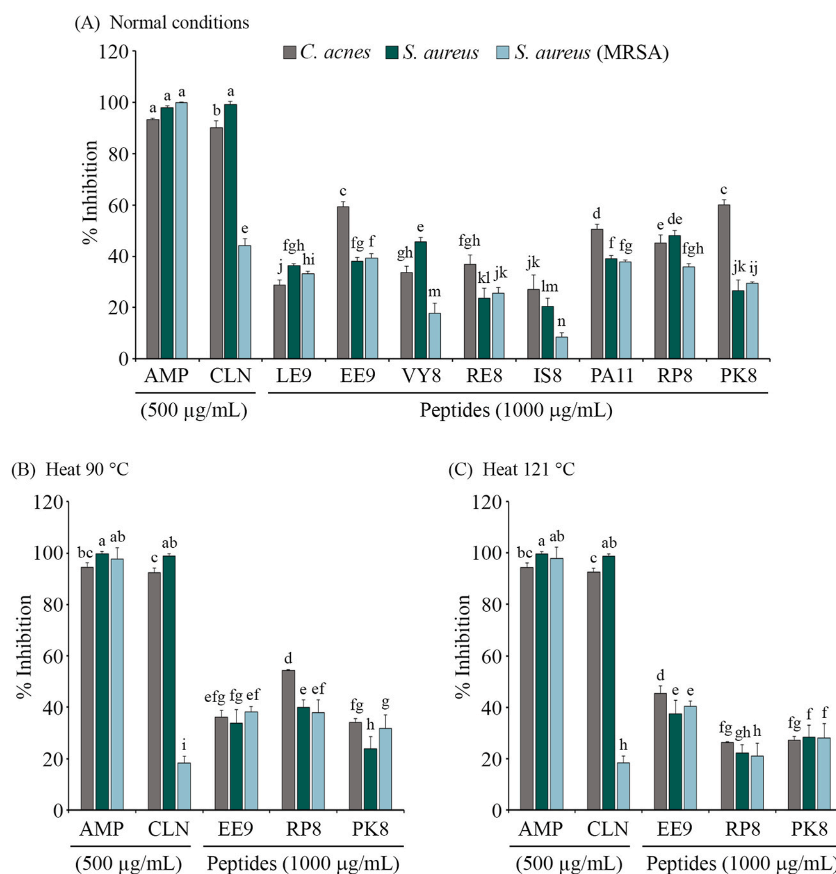

Fig. 5. Antibacterial activities against *C. acnes*, *S. aureus* and MRSA of 8 synthetic peptides at a final concentration of 1000 μg/mL (A). The EE9, RP8 and PK8 peptides were selected for thermal stability testing by incubation at 90 °C for 15 min (B) and 121 °C for 30 min (C). After cooling, the antimicrobial activity of the thermally treated peptides was measured. The distinct letters (a–n) presented on the bar graphs indicate significant differences in the mean values ($P < 0.05$).

also showed a significant preference for *S. aureus* over MRSA (45.66 and 17.89 % inhibition respectively) (Fig. 5A).

The thermostability of antibacterial peptides is a critical factor in their potential application in various fields, including medicine and food preservation [44]. In this study, the thermostability of the most potent antibacterial peptides EE9, RP8 and PK8 was investigated by incubating them at 90 °C for 15 min (Fig. 5B), autoclaving at 121 °C for 30 min (Fig. 5C) and finally using them in

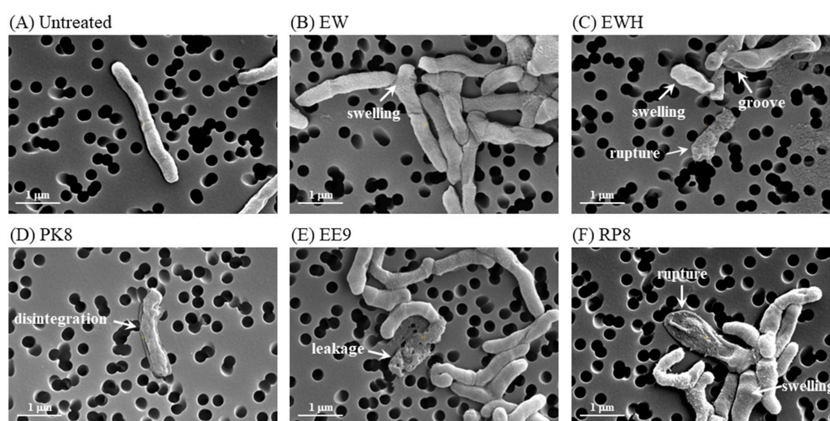

Fig. 6. Scanning electron micrographs showed the morphology of *C. acnes* after incubation with the active peptides at 1000 μg/mL for 18–22 h. (A) Untreated *C. acnes*; (B and C) *C. acnes* treated with native egg white (EW) and egg white hydrolysate (EWH); (D–F) *C. acnes* treated with synthetic peptides PK8, EE9 and RP8.

growth inhibition assays. All the tested peptides exhibited good thermal stability. EE9 was the most promising showing a consistent inhibitory effect irrespective of heat treatment across all bacteria with inhibition ranging from 33.76 to 38.29 % at 90 °C and from 37.35 to 45.49 % at 121 °C; levels that are comparable to the unheated control. RP8 maintained a similar inhibitory effect on bacterial growth after heating at 90 °C (38.28–54.22 % inhibition) compared to the unheated control. However, its activity decreased after autoclaving at 121 °C (21.05–26.18 % inhibition). Finally, for PK8, heating at both 90 °C and 121 °C gave similar activity to the unheated control against *S. aureus* (23.73–28.31 % inhibition) and MRSA (31.68–28.10 % inhibition), but its activity decreased against *C. acnes* (34.12–27.04 % inhibition) compared to the unheated control (Fig. 5A; 60.08 % inhibition). These observations highlight the importance of considering specific bacterial strains and intended applications when assessing the thermostability of antibacterial peptides.

Finally, having shown the activity of the peptides against bacteria, we investigated their mechanism of action. Most AMPs directly disrupt microbial cell membranes [45]. Prior research has described the mechanism of action of AMPs derived from the egg whites of *Crocodylus siamensis* in disrupting bacterial cell membrane structures [17]. As a result, the effect of the most potent inhibitors PK8, EE9 and RP8 on the *C. acnes* bacterial cell membrane was evaluated by SEM.

C. acnes, a gram-positive anaerobic bacterium, displayed distinctive rod-shaped cells measuring 0.5–1.5 μm in width and 1.0–1.5 μm in length (Fig. 6A). The exposure of cells to both EW and EWH distinctly revealed structural abnormalities in the cell membrane, particularly in the case of EWH. Cells treated with EW exhibited evident cell membrane swelling (Fig. 6B), whereas EWH treatment resulted in more pronounced cellular damage, including swelling, the formation of furrows, and ultimate cell membrane disruption leading to cell lysis (Fig. 6C). Furthermore, it was observed that the cell membrane of *C. acnes* was significantly disrupted by three synthetic peptides, PK8, EE9 and RP8, confirming the antibacterial properties of these AMPs against *C. acnes* (Fig. 6D–F). This is especially interesting for negatively charged EE9. Cells treated with EWH exhibited more extensive damage to the cell membrane compared to the use of individual synthetic peptides. This observation was consistent with the findings of our antibacterial assays.

The results of this study demonstrate egg white peptides are potentially a gentler natural alternative for acne treatment compared to conventional treatments such as benzoyl peroxide [33] and clindamycin [46]. They are more suitable for individuals with sensitive skin [47,48] who are less tolerant of traditional treatments that cause side effects such as dryness, irritation, and photosensitivity. Moreover, the antimicrobial activity of egg white peptides against acne-causing bacteria, including *C. acnes*, *S. aureus* and MRSA,

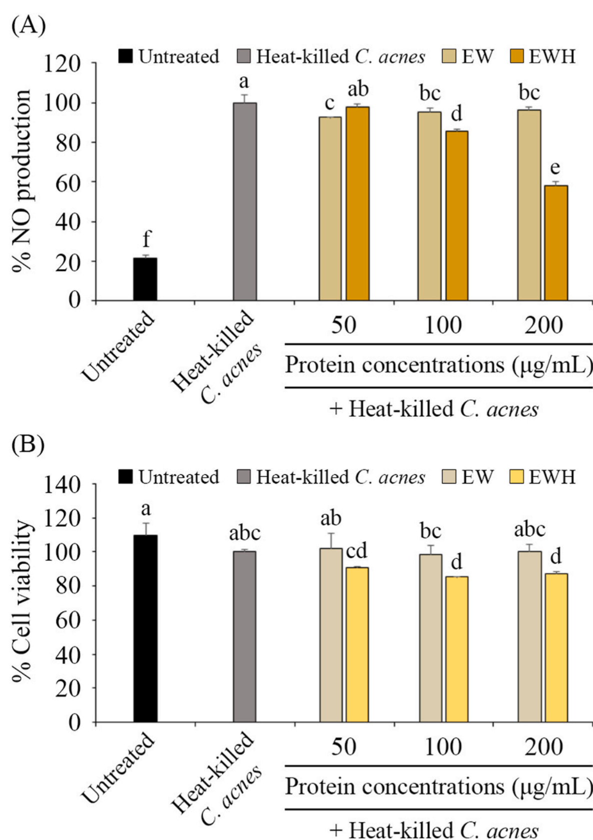

Fig. 7. Evaluation of anti-inflammatory activities (A) and cytotoxicity (B) of intact egg white (EW) and egg white hydrolysate (EWH) on RAW 264.7 cells treated with heat-killed *C. acnes*. RAW 264.7 cells were co-incubated with 100 μg/mL of heat-killed *C. acnes* and different concentrations of EW and EWH for 24 h. The nitric oxide (NO) production was assessed using Griess reagent (A), and the viability of remaining RAW 264.7 cells was determined via MTT assay (B). The distinct letters (a–e) presented on the bar graphs indicate significant differences in the mean values ($P < 0.05$).

suggests they can bypass bacterial drug resistance that is traditionally developed over time. They achieve this by acting on bacteria through diverse mechanisms, most notably by directly disrupting microbial cell membranes. This multifaceted approach makes it challenging for bacteria to develop resistance compared to conventional antibiotics, which typically target specific pathways.

3.6. Anti-inflammatory activities of EW and EWH and selected synthetic peptides

Acne vulgaris is primarily characterized by inflammation, which is evident at all stages of lesion formation. Excessive growth of *C. acnes* leads to the formation of keratin plugs and heightened sebum production. Severe acne starts with the disruption of follicular epithelium by *C. acnes*, allowing bacteria to spread to skin and immune cells such as keratinocytes and macrophages [49]. Nitric oxide (NO) is an inflammatory mediator synthesized by nitric oxide synthase in various cell types including macrophages. Upon bacterial infection or stimulation by pro-inflammatory cytokines, the expression of inducible nitric oxide synthase (iNOS) is upregulated, resulting in increased production of NO [50]. The NO released affects the inflammatory mechanism in several ways. For instance, it can act as an antimicrobial agent by directly killing bacteria and stimulating phagocytosis of phagocytes through a positive feedback regulation mechanism [51]. Therefore, a reduction in NO secretion by macrophages serves as an indicator of subsequent reduction in inflammation.

In this study, RAW 264.7 cells were stimulated with heat-killed *C. acnes* to induce the production of nitric oxide (NO). Subsequently, the efficacy of EW, EWH, and peptides PK8, EE9 and RP8 in reducing NO production was examined. NO production by heat-killed *C. acnes*-treated RAW 264.7 in the absence of any peptides was considered as 100 % NO production (i.e. positive control), while NO production in the absence of heat-killed *C. acnes* and peptides was considered the negative control (RAW 264.7 gave low levels of NO production ranging from 18.31 to 21.61 %; Figs. 7A and 8A, C, E).

While EW was unable to suppress NO production, EWH was able to reduce NO production in a dose-dependent manner (50–200 μg/mL; Fig. 7A). At 200 μg/mL, EWH reduced NO production to approximately 58.31 % (Fig. 7A). Of the selected peptides from EWH (PK8, EE9 and RP8), all were capable of inhibiting NO synthesis in cells in a dose-dependent manner (125–1000 μg/mL; Fig. 8A, C, E). Notably, PK8 and RP8 demonstrated significant reduction of NO production at lower concentrations than EE9. At 1000 μg/mL, NO production for PK8, EE9 and RP8 were 44.85 %, 50.92 % and 60.02 % respectively. Cytotoxicity analysis of EW, EWH and the selected peptides on the residual RAW 264.7 cells using the MTT assay indicated high cell viability with low cytotoxicity at the tested concentrations (Figs. 7B and 8B, D, F). This confirms that any observed reduction in NO levels was not a consequence of cell death.

Anti-inflammatory peptides are normally rich in hydrophobic and positively charged amino acids with the position of the amino acids also being important. It has been proposed that hydrophobic amino acids enhance the interaction of the peptides with cell membranes [52,53], while positively charged residues might act as functional mimics of chemokines enabling the peptides to modulate the immune response by binding to specific chemokine receptors [52,53]. In terms of the position of these amino acids, the N-terminus tends to have hydrophobic amino acids, while the C-terminus has charged amino acids; the combination of which are thought to reduce inflammation through inhibition of a cascade reactions of crucial inflammatory signaling pathways [52].

In this context, our selected peptides share common characteristics with those found in anti-inflammatory peptides. However, it is interesting to note that the combination of these peptides in EWH is more potent than the individual peptides. This might have been due to synergistic interactions of peptides in EWH that no single peptide alone can induce.

4. Conclusion

Alkaline hydrolysis of hen egg white protein yields short peptides (<14 kDa) with dual antibacterial and anti-inflammatory properties. These peptides are effective at inhibiting the growth of *C. acnes*, *S. aureus* and antibiotic-resistant MRSA, while also reducing NO production in RAW 264.7 cells treated with heat-killed *C. acnes*. Interestingly, the complex mixture of peptides in EWH was more potent than the individual synthetic peptides, suggesting that the peptide mixture exhibits synergistic effects. The simple and safe preparation method as well as the good antimicrobial and anti-inflammatory activity suggest there is further scope for EWH to be considered for the treatment of acne and/or antibiotic-resistant bacteria.

Data availability

Data included in article/supplementary material/referenced in article.

CRediT authorship contribution statement

Anuwat Wanthong: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Chanapat Boonmark:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Nichakamol Vaisopha:** Investigation, Formal analysis. **Sittiruk Roytrakul:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Anupong Tankrathok:** Writing – review & editing, Visualization, Data curation. **Lapatrada Taemaitree:** Writing – review & editing, Visualization, Data curation. **Sakda Daduang:** Writing – review & editing, Supervision, Resources, Methodology. **Sophon Boonlue:** Writing – review & editing, Supervision, Methodology. **Watcharee Khunkitti:** Writing – review & editing, Supervision, Resources, Methodology. **Sompong Klaynongsruang:** Supervision, Resources, Methodology. **Nisachon Jangpromma:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

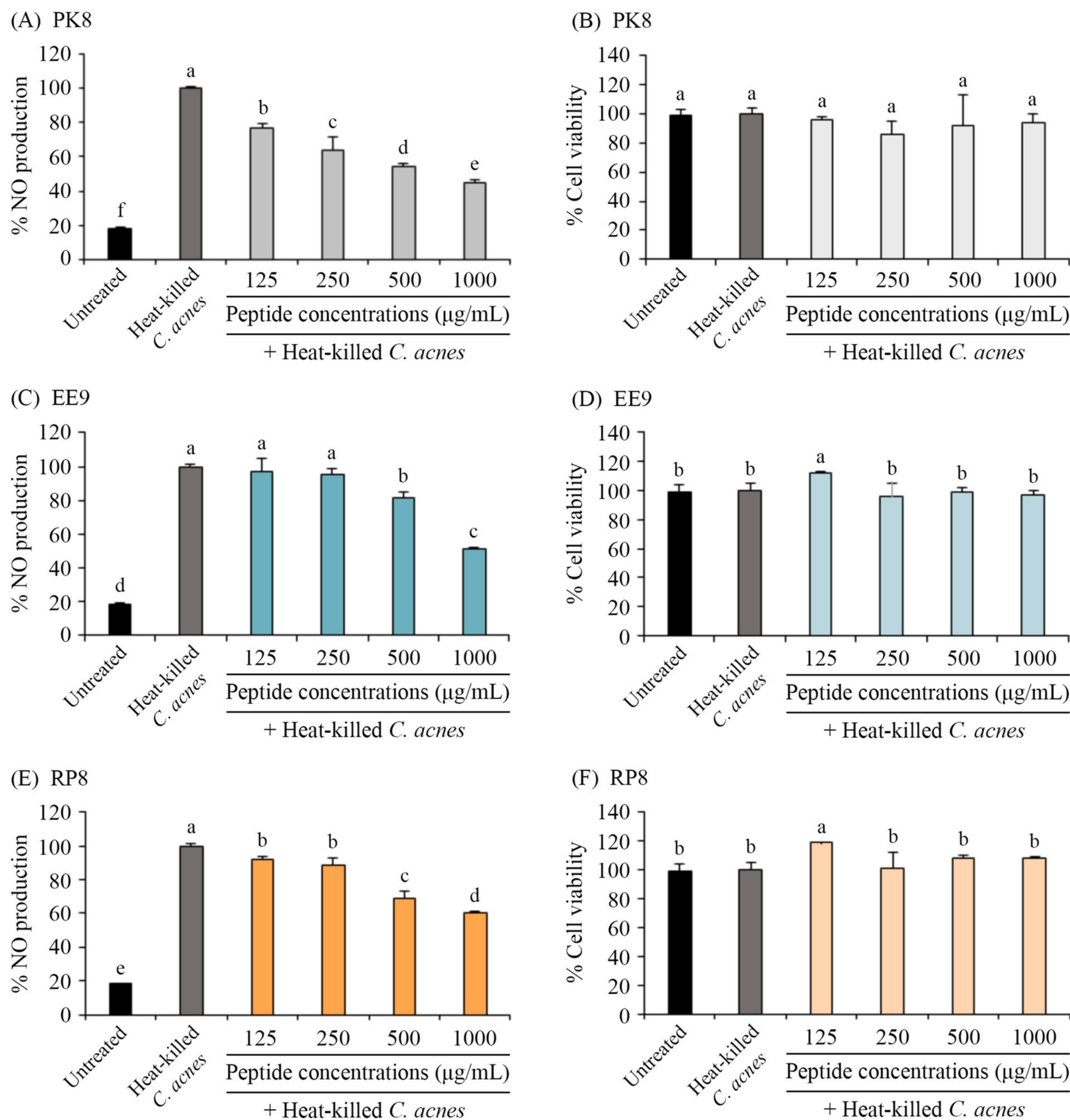

Fig. 8. Evaluation of anti-inflammatory activities (A, C, E) and cytotoxicity (B, D, F) of peptides on RAW 264.7 cells treated with heat-killed *C. acnes*. RAW 264.7 cells were co-incubated with 100 μg/mL of heat-killed *C. acnes* and different concentrations of PK8 (A, B), EE9 (C, D), and RP8 (E, F) peptides for 24 h. The nitric oxide (NO) production was assessed using Griess reagent (A, C, E), and the viability of the remaining RAW 264.7 cells was determined via MTT assay (B, D, F). The distinct letters (a–f) presented on the bar graphs indicate significant differences in the mean values ($P < 0.05$).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32468.

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