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RESEARCH ARTICLE

Anti-inflammatory effects of eggshell membrane hydrolysates on carrageenan-induced rat

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Abstract

Background: Eggshells are one of the most common culinary wastes, yet using eggshell membrane detritus in medical treatments remains largely unexplored. **Objective:** This study aims to assess the anti-inflammatory potential of eggshell membrane hydrolysate. **Method:** Eggshell membrane hydrolysate was prepared using acid-base hydrolysis. The intraplantar carrageenan-induced inflammation model was used to test the anti-inflammatory effect. The experimental group consisted of a control, comparison (4.5 mg/kg BW diclofenac-Na, 4.5 mg/kg BW hyaluronic acid, and 45 mg/kg BW collagen), and eggshell membrane hydrolysates (9, 22.5, 45 and 90 mg/kg BW). Observations of inflammation, percentage of inflammation, and percentage of inhibition of inflammation were measured every 30 minutes for 180 minutes. Statistical analysis was performed using the *t*-test and also trendline analysis. **Result:** The findings revealed that collagen and hyaluronic acid demonstrated the ability to suppress inflammation compared to controls ($p < 0.05$). Compared to the control group, the group that received eggshell membrane hydrolysate at doses of 9, 22.5, 45, and 90 mg/kg BW also exhibited inhibition of inflammation ($p < 0.05$). Furthermore, increasing the dosage of eggshell membrane hydrolysate correlated with an increase in the percentage of inflammation inhibition in carrageenan-induced rats. **Conclusion:** Eggshell membrane hydrolysate has the potential to act as an anti-inflammatory agent.

Introduction

Inflammation is one of the immune system's primary reactions to infection. It is characterised by redness (rubor), pain (dolour), heat (calour), swelling (tumour), and loss of function (functio laesa) (Oronsky *et al.,* 2022). Depending on the onset, inflammation is classified as acute or chronic. Immediate vascular alterations, the influx of inflammatory cells, and the extensive effects of inflammatory mediators indicate an acute inflammatory response. These changes result in vascular alterations, including vasodilation and increased capillary permeability, leading to a reddened area. Neutrophils and white blood cells play a central role in this response. During an infection, plasma protein fluid from white blood cells is released into the injured tissue, resulting in exudate or tissue oedema. Tissue oedema and the presence of prostaglandins in the exudate contribute to the experience of pain and fever. The widespread impact of inflammatory mediators causes fever and other systemic symptoms. Chronic inflammation, on the other hand, is caused by the infiltration of macrophages, lymphocytes, and fibroblasts, which leads to persistent inflammation, fibroblast proliferation, and scarring (Oronsky *et al.,* 2022; Silva, 2016).

The eggshell serves as the outermost protective layer of the egg, safeguarding its contents. Eggshells, a byproduct of the poultry industry, are notably rich in calcium, making them a valuable mineral source. Within the eggshell exists a thin white layer known as eggshell membrane (ESM). ESM possesses a fibrous structure due to gastric protease-resistant collagen and

keratin. ESM is composed of several varieties of proteins and peptides, such as lysozyme, ovo transferrin, and ovalbumin (Makkar *et al.,* 2013; Shi *et al.,* 2021).

It has been demonstrated that the addition of ESM in poultry diets leads to increased IgM levels, which serve as natural antibodies preventing infection, reducing inflammation, reacting to foreign antigens, activating the complement system, and stimulating IgG responses (Makkar *et al.*, 2013). Furthermore, adding ESM powder to human monocytic cell line U937–3xB-LUC cells induced by lipopolysaccharide (LPS) demonstrated that ESM powder has an anti-inflammatory effect by decreasing the activity of transcription factor NF-κB, down-regulating the expression of immune regulating receptors toll-like receptor 4 (TLR4) and ICAM-1, cell surface glycoprotein CD44, and increasing secretion of the anti-inflammatory cytokine IL-10 (Suso *et al.,* 2017). In vivo studies using mice, rats, and rabbits have extensively explored the use of ESM. It is proven to have effects on peripheral nerve regeneration, skin and bone enhancement, and inflammatory diseases, as well as promoting functional recovery, either when used alone or in combination with lycopene. Additionally, ESM has demonstrated the ability to repair open wounds in the first phase of wound healing by enhancing angiogenesis and facilitating the formation of a new epidermis. ESM has anti-ageing effects and improves skin health. It can also repair bone and is a potential graft candidate material. ESM can be used in the treatment of inflammatory bowel disease (Herman *et al.,* 2023).

According to the present study, it appears that there is a lack of fundamental research on the anti-inflammatory properties of ESM hydrolysate produced by acid-base hydrolysis. Therefore, the aim of this study is to evaluate the anti-inflammatory effect of ESM hydrolysate in rats using the in vivo experimentation using the carrageenan induction method.

Methods

Materials

The eggshells were acquired from a culinary company in Bandung, West Java, Indonesia. Subsequently, the ESM was separated from the eggshells, rinsed, and air-dried for three days before being pulverised.

Other materials used for this study were diclofenac-Na (Kimia Farma®), collagen (Amigen, Vihn Hoan Corp), hyaluronic acid (Primahyal 10, Givaudan), and λ-Carrageenan (Sigma Aldrich®).

Preparation of eggshell membrane hydrolysates

The eggshell membrane hydrolysate (ESMH) was prepared using a modified Park procedure. ESM powder was immersed for six hours in 50% ethanol at pH 12 and 60 ^{o}C, with pH adjustment carried out using 2N NaOH. The solution was then filtered and neutralised using 99% acetic acid. The filtrate obtained was then dried (Park *et al.,* 2012).

Determination of protein content

Protein analysis of ESM and ESMH was carried out at the Saraswanti Indo Genetech (SIG) Laboratory in Bogor using the Kjeldahl method with a Kjeltec instrument according to protocol 18-8-31/MU/SMM-SIG.

Anti-inflammatory assay

This research was conducted with the approval of the Jenderal Achmad Yani University with certificate number 8024/KEP-UNJANI/II/2021 institutional ethics committee. The Wistar rat used in the experiment was obtained from the Animal laboratory of the research center for biogenetics and biosciences, Institut teknologi, Bandung.

Prior to the experiment, the animals underwent a one-week acclimatisation period. A total of 40 animals were divided into eight groups, each consisting of five animals: control, comparison (4.5 mg/kg BW diclofenac sodium, 4.5 mg/kg BW hyaluronic acid, and 45 mg/kg BW collagen), and eggshell membrane hydroly***s***ate (9, 22.5, 45, and 90 mg/kg BW). The initial measurement of the hind paw's normal volume was conducted using a mercury plethysmometer. Subsequently, each animal was administered the group-specific preparations orally. 30 minutes following the test preparation, the animals were stimulated intraplantarly with 0.05 mL 1% λ-carrageenan***.*** After the induction, measurements of the hind paw's oedema were obtained at 30 minutes intervals for a duration of 180 minutes. The percentage of inflammation was determined by comparing the amount of oedema to the normal volume of the rear paw. Meanwhile, the percentage of inflammation inhibition was calculated by comparing the percent inflammation in the test group to the control group (Patil *et al.,* 2019; Akpinar, 2021).

Data analysis

Inflammation percentage data expressed as mean ± standard deviation. The *t*-test was used to compare the percentage of inflammation in the test and control groups. A value of $p < 0.05$ was deemed statistically

significant. SPSS software was used to conduct a *t*-test analysis. In addition, trendline analysis was performed in Microsoft Excel on the data regarding the percentage of inflammation.

Results

The results of total protein content in ESM and ESMH is displayed in Table I below. Based on these results, there was a change in total protein content before and after.

Table I: Total protein content of ESM and ESMH

Sample	Protein (%)
ESM	80.42
ESMH	25.55

Swelling is a symptom of inflammation. In this study, the carrageenan-induced *o*edema of the footpad was utilised as the parameter for the acute anti-inflammatory assay. The observations were carried out over a period of three hours, as this is the optimal timeframe for using carrageenan to induce inflammation, typically resulting in inflammation within 3 hours after injection (Akpinar, 2021). The results of the ESMH's acute anti-inflammatory effect are presented in Table II-III.

Calculation of percent inflammation inhibition is presented in Table III. It reports that increasing the dosage of eggshell membrane hydrolysate increased the percentage of inflammation inhibition in carrageenan-induced rat.

Table II: Percent inflammation of the test group (control, comparison and eggshell membrane hydrolysate) for 180 minutes

Groups	Inflammation (%) at minutes					
	30	60	90	120	150	180
Control	29.14±8.35	65.67±11.51	73.86±16.36	92.14±7.21	94.64±5.89	98.74±8.65
Diclofenac sodium 4.5 mg/kg BW	26.25±7.60	37.99±6.03*	36.83±7.62*	34.83±3.50*	33.78±5.13*	32.60±5.37*
Collagen 45 mg/kg BW	27.25±12.09	31.06±13.00	32.71±11.21	44.48±13.10*	41.73±19.80*	44.35±10.85*
HA 4.5 mg/kg BW	17.07±6.30*	21.80±7.84*	26.89±5.92*	31.86±4.24*	43.01±3.61*	49.08±5.14*
ESMH 9 mg/kg BW	15.68±2.56*	20.85±10.17*	37.59±14.07*	51.74±13.67*	61.68±9.66*	70.89±13.10*
ESMH 22.5 mg/kg BW	13.18±6.74*	23.51±11.05*	29.09±12.98*	32.25±10.64*	42.22±7.89*	38.56±10.77*
ESMH 45 mg/kg BW	12.33±3.60*	19.00±18.08*	31.83±14.23*	31.83±14.23*	33.17±13.93*	39.67±9.14*
ESMH 90 mg/kg BW	8.62±6.06*	16.10±11.60*	26.30±9.89*	31.43±15.22*	37.68±7.90*	49.98±9.18*

n=5, *$p < 0.05$ compared to the control group using the *t*-test

Table III: Percentage inflammation inhibition of the test group against the control group

Groups	Inhibition of inflammation (%) at minutes					
	30	60	90	120	150	180
Diclofenac sodium 4.5mg/kg BW	12.81	42.69	44.94	62.83	64.98	67.59
Collagen 45mg/kg BW	-8.82	39.32	35.00	45.72	45.52	49.52
HA 4.5mg/kg BW	37.56	68.61	60.93	64.47	53.34	47.08
ESMH 9mg/kg BW	42.64	65.16	41.42	42.29	33.08	26.29
ESMH 22.5mg/kg BW	51.77	63.08	54.67	62.64	54.19	59.90
ESMH 45mg/kg BW	54.88	68.19	50.39	64.50	64.01	57.02
ESMH 90mg/kg BW	68.48	74.71	56.62	62.32	59.12	48.03

The rate at which carrageenan induces *o*edema will increase over time. The administration of test substance is expected to control the rate of swelling . Table II indicates that the comparator (diclofenac-sodium, collagen, and hyaluronic acid) and the group that received eggshell membrane hydrolysate at

dosages of 9, 22.5, 45, and 90 mg/kg BW were able to reduce inflammation when compared to controls group ($p < 0.05$). While all groups exhibited anti-inflammatory effects as compared to controls group, the onset of these effects varied. Diclofenac sodium had the ability to inhibit inflammation as early as the 60th minute, collagen as early as the 120th minute, hyaluronic acid and ESMH as early as the 30th minute.

In addition to statistical analysis using the *t*-test, a simple linear trendline analysis was conducted on the percent information data. A trendline analysis is an approximation of a calculation model based on historical data, in which the data tends to increase or decrease continuously in a relatively repetitive pattern (Wan Ahmad *et al.,* 2021). On the basis of the obtained data (Table II), it is presumed that, under constant conditions *for* 180 minutes observation, the percent of inflammation increases in steady rate. *To* validate the findings, a trendline analysis was performed due to the fluctuating values of the anti-inflammatory calculation outcomes. The results of the trendline analysis are illustrated in Figure 1.

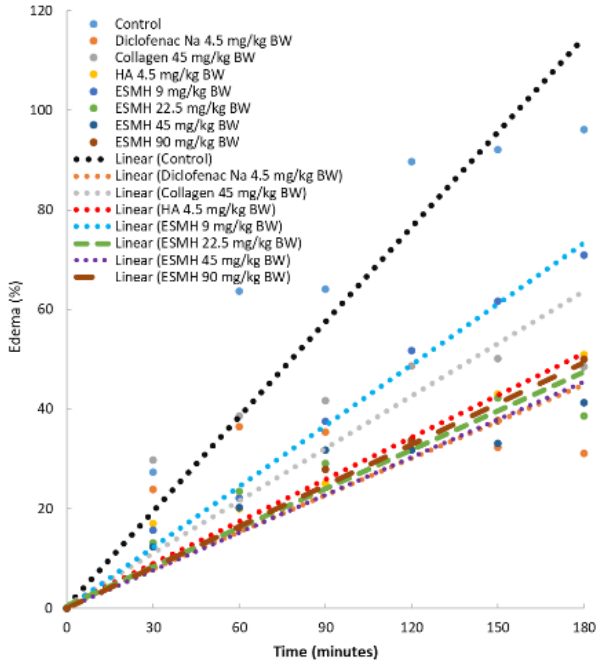

Figure 1: Trendline analysis graph of anti-inflammatory assay

The results of the trendline analysis revealed that all test groups had an anti-inflammatory effect, as evidenced by a lower percentage of inflammation compared to the control group. Specifically, trendline analysis found that in the comparison group, diclofenac-Na had the highest anti-inflammatory effect, followed by hyaluronic acid and collagen. The anti-inflammatory effect of ESMH at a dose of 9 mg/kg BW was less than that of collagen. In comparison to hyaluronic acid and collagen, the anti-inflammatory effect of ESMH at dosages of 22.5, 45, and 90mg/kg BW was better, with ESMH 45mg/kg BW presenting a similar tendency pattern to that of diclofenac sodium.

Discussion

Serum contains two main types of proteins: albumin and globulin. The relationship between albumin levels and inflammation is reciprocal. Inflammation can lead to hypoalbuminemia, and conversely, hypoalbuminemia can also cause inflammation-related diseases (Pathania, 2021). ESMH is rich in protein, suggesting that this protein content may play a role in mitigating inflammation.

During the ESMH making, a hydrolysis process was carried out, resulting in the production of several peptides with potential anti-inflammatories properties. Bioactive peptides and hydrolysed proteins, as opposed to integrative proteins, have demonstrated a potential anti-inflammatory and immunomodulatory effects. This could be attributed to their structures having a low molecular weight, which enhances their digestibility and bioavailability (De Medeiros *et al.,* 2022).

Collagen, osteopontin, fibronectin, keratin, cysteine-rich eggshell membrane proteins (CREMPs), histones, beta defensins, ovocalyxin, apolipoprotein, protocadherin, chondroitin sulphate, and hyaluronic acid are the main components of ESM. Collagen, fibronectin, and CREMPs accelerate wound healing, in which inflammation is one of the phases (Shi *et al*., 2021). Although this study did not examine the composition of ESMH, it suggests that the collagen, fibronectin, and hyaluronic acid compounds in ESMH play a crucial anti-inflammatory role.

Collagen serves as one of the extracellular matrix's constituent elements. Tissue injury and inflammation can lead to collagen degradation. Exogenous collagen can help remodel the extracellular matrix and suppress the release of pro-inflammatory mediators (Schwarz *et al.,* 2022). Collagen hydrolysate has demonstrated the ability to inhibit ear inflammation in test animals induced by zymosan in both skin and ears. Additionally, hydrolysed collagen can also reduce IL-6 levels in whole blood cells stimulated with LPS, presumably by acting as a glycine receptor (GlyR) antagonist (Hartog et al., 2013). Collagen peptide testing on LPS-induced human skin fibroblasts (CCD-1072Sk) and human keratinocytes (hKT-nh-skp-KT0026) revealed that collagen peptides could induce fibroblast and keratinocyte proliferation

and pro-collagen-1 expression, as well as increased TGF-β and VEGF expression and suppression of an inflammatory response induced by LPS (Brandao-Rangel *et al.,* 2022).

Fibronectin is a glycoprotein essential for migration, differentiation, signalling, adult wound healing, and overall tissue health. When fibronectin binds to TLR4, inflammatory responses are generated. Fibronectin can also increase metalloproteinase (MMP) activity, cyclooxygenase 2 expression, and prostaglandin E2 expression through the NF-κB and ERK1/2 pathways; as well as cytokine activity through the p38 and MK-2 pathways (Dalton & Lemmon, 2021). Vascularisation, one of the earliest stages of inflammation, involves laminin and collagen IV attenuating inflammatory signalling in part via integrin 21, while fibronectin mediates inflammatory signalling via integrin α5 in vascular remodelling (Budatha *et al.,* 2021).

Aside from that, another ESMH component deemed to have anti-inflammatory potential in this research is hyaluronic acid. Hyaluronic acid is capable of modulating tissue hydration, balancing the osmotic and physical properties of the extracellular matrix, and constructing a hydrated and stable extracellular space. Hyaluronic acid interacts with particular cytokines during inflammation and acute damage, modulating immune cell activity (Marinho *et al.,* 2021).

This fundamental research has demonstrated that ESMH has anti-inflammatory properties. These findings may provide a rationale for the use of ESM/ESMH in the treatment of a variety of diseases for which anti-inflammatory drugs are prescribed. Among the conditions requiring anti-inflammatory medication are asthma, rheumatism, and arthritis. ESM has been shown to have an effect in preclinical animal trials of potassium oxonate-induced hyperuricemia. ESM can stimulate uric acid secretion and regulate its transport (Sung & Kim, 2021). ESM also influenced collagen-induced arthritis animal models. ESM can reduce inflammatory markers: collagen type II C-telopeptide (CTXII), cartilage oligomeric matrix protein (COMP), and alpha-2-macroglobulin (A2M) (Wedekind *et al.*, 2017).

However, the proteins included in ESMH were not identified in this research, making it challenging to pinpoint the precise active ingredient responsible for its anti-inflammatory properties. Therefore, a comprehensive analysis of ESMH content is recommended. Furthermore, In vitro testing, including protein denaturation tests, proteinase activity inhibition tests, membrane stabilisation tests, and related studies, can be conducted to elucidate the mechanism of ESMH action as anti-inflammatory agent.

Conclusion

Eggshell membrane hydrolysate has the potential to be an anti-inflammatory agent, likely due to its protein content and its ability to counteract hypoalbuminemia. Eggshell membrane hydrolysate can be an alternative for treating inflammation-related diseases. Nevertheless, further study is needed to validate these findings and delve deeper into the mechanisms underlying its anti-inflammatory effects.

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