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Optimization of Hyaluronic Acid Extraction from Eggshell Membrane using Ultrasonic-Assisted Enzyme Hydrolysis

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ABSTRACT

Eggshell membrane (ESM) from eggshell waste is a valuable source of hyaluronic acid (HA), a biomaterial that is in high demand in the medical and cosmetic fields. However, the extraction of HA from ESM using ultrasonic-assisted enzymatic hydrolysis has not yet been optimized. The study aimed to optimize the extraction of HA from ESM by using papain through the proposed method. The optimization process was conducted using the Box-Behnken design within the framework of Response Surface Methodology (RSM). The antioxidant and anti-inflammatory activity of HA were determined by DPPH radical scavenging, ferric reducing antioxidant potential (FRAP) and lipoxygenase inhibitor screening assays, respectively. The optimum processing conditions that contributed to the high content of HA (5.062%) were achieved at a hydrolysis time of 3.1 hours, an enzyme concentration of 9.7%, a pH of 7.5 and an ultrasonic treatment time of 2.1 minutes. The analysis of variance showed that the enzyme concentration was an important factor in the extraction of HA from ESM. In addition, the HA extract showed acceptable result of anti-inflammatory properties (9.4104%) suggesting

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its importance in attenuating inflammatory responses. These results indicated that ESM is a promising and sustainable source for HA production with desirable bioactivities. Future research should explore the mechanistic actions of HA thereby enriching our understanding of its therapeutic potential and expanding its commercial applications.

1. INTRODUCTION

Eggshell membrane (ESM) is rich in hyaluronic acid (HA), collagen, glucosamine, chondroitin and elastin with diverse bioactive properties [1,2]. ESM represents about 5% of the total eggshell weight [3]. HA is a type of polysaccharide and is highly valued in biomedical and cosmetic domains due to its exceptional properties such as viscoelasticity and water-binding capacity [4]. It plays a crucial role in inflammation modulation, wound healing, and skin moisture maintenance, thus earning its versatility in the applications of tissue engineering, wound care, and cosmetic procedures [5]. HA also has high efficacy as viscosupplementation for osteoarthritis and tissue regeneration [6]. Its diverse applications and biological functions make it a key player in advancing therapeutic and regenerative approaches in healthcare.

Enzymatic hydrolysis is an effective method for isolating pure HA, making it a preferred approach for HA extraction. Studies have successfully demonstrated the extraction, purification, and characterization of HA from rooster comb using enzymes [7]. Among the commonly used enzymes is papain, a cysteine protease derived from papaya latex which has been extensively researched for its effectiveness in various processes including protein hydrolysis. Its high proteolytic activity makes it particularly suitable for extracting HA from different sources including eggshell membrane [8]. Additionally, the combination of papain with other enzymes has proven to be a potent strategy in HA extraction further underscoring its versatility and efficiency [9].

Ultrasound-assisted extraction is an innovative method with numerous benefits over traditional extraction approaches. The advantages include minimal solvent usage, short extraction times, simplified equipment needs, and reduced costs and environmental footprint [10]. The synergistic effect of ultrasonication with enzymatic hydrolysis may assist in breaking down the complex structure of the ESM, making it more accessible to the enzymes involved in the hydrolysis process. This approach has shown promising effects in improving the extraction efficiency of HA and reducing processing times, making the overall extraction process more cost-effective and environmentally friendly.

By optimizing the hydrolysis processing condition, the efficiency and yield of HA extraction can be improved thus ensuring a higher quality product for various biomedical and cosmetic applications. Despite advancements in enzymatic hydrolysis techniques for extracting HA from eggshell membranes, there remains a need to optimize the extraction efficiency for high yield of HA while ensuring cost-effectiveness and environmental sustainability, considering factors such as enzyme selection, process optimization, and utilization of agricultural by-products for improved extraction outcomes. Thus, the study aims to extract

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and optimize the yield of HA from ESM by using papain enzymatic hydrolysis extraction process under varied enzymatic hydrolysis conditions.

2. EXPERIMENTAL METHODOLOGY

2.1 Materials

Eggshell wastes were collected from Zenxin Agriculture Sdn. Bhd, Johor, Malaysia. All chemicals used such as acetic acid, phosphoric acid, boric acid, sodium hydroxide, sulphuric acid, sodium tetraborate, distilled water, carbazole were analytical grades. Standard sodium hyaluronate was purchased from Sigma (St. Louis, MO). Food grade papain (100 KU/g) were purchased from Nanning Biotech, China and Lipoxygenase Inhibitor Screening Assay Kit (760700) was obtained from Cayman Chemical, USA.

2.2 Preparation of ESM Powder

The ESM was removed manually from the eggshell and washed with distilled water to remove impurities. Then, the ESM was dried and washed with distilled water to remove impurities. It was then dried in an oven at 30 ℃ for 5 hours. The dried ESM was then ground into powder using a blender and sieved through an 80-mesh sieve (<180 μm) to obtain a homogenous powder size. The ESM powder was stored in an airtight container at -20 ℃ freezer until further use.

2.3 Ultrasonic- assisted Enzymatic Extraction of HA

HA extraction was performed according to the method of Ürgeová and Vulganová [11] and Cocuľová & Krajcovic [12] with minor modifications. About 0.5 g of dried ESM powder was mixed with 20 ml of Britton and Robinson buffer solution in a ratio of 1:40 (w/v). The sample was pretreated with ultrasound at 40 kHz for 2 – 30 minutes. The sample was then preheated to the optimum temperature for papain enzyme (60 °C) for 10 minutes before the papain was added (w/w) and mixed until homogenous. The mixtures were incubated at 60 °C for 3 – 7 hours and shaken every 10 minutes to ensure homogenization. The samples were then heated to 100 °C to inactivate the papain and cooled. The hydrolysed samples were centrifuged at 5000 rpm for 20 minutes and the supernatant was kept as the HA sample.

2.4 Experimental Design and Optimization

Table 1. The independent variables, its coded and actual level in the Box-Behnken design

Independent variable	Notation	Factor levels		
		-1	0	1
Hydrolysis time (hours)	A	3	5	7
Enzyme concentration (w/w)	B	1	5.5	10
pH	C	7	7.5	8
Ultrasonic time (minutes)	D	2	16	30

The optimal processing conditions of ultrasound-assisted enzymatic hydrolysis for extracting HA from ESM were determined using the Box-Behnken design in Response Surface Methodology (RSM) (Design Expert Software Version 11, Stat-Ease Inc Minneapolis, MN, USA). The processing conditions including hydrolysis time, enzyme concentration, pH and ultrasonic time were optimized. Three replicates were

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performed at the central point, giving a total of 27 experimental runs. The total yield of HA (%) was analysed as a response. Table 1 presents the range of independent variables used in the optimization process.

2.5 Determination of HA Concentration

The HA content was analysed using the carbazole method. The quantification by the carbazole method involves two main steps, hydrolysis of glycosaminoglycan into uronic acid and glucosamine using sodium tetraborate in sulfuric acid, followed by staining of uronic acid with carbazole [13]. Approximately, 50 µL of a diluted HA sample (10 µL/1 mL) was added with 200 µL of 25 mM sodium tetraborate in sulphuric acid in a 96-well plate and heated for 10 minutes at 100 °C. After cooling to room temperature for 15 minutes, 50 µL of 0.125% carbazole in absolute ethanol was carefully added into the well and mixed thoroughly. The absorbance was measured at 530 nm using microplate reader spectrophotometer (Epoch, BioTek Instruments, USA). The total yield of HA in the ESM sample was determined using a calibration curve prepared from a sodium hyaluronate standard solution.

2.6 Evaluation of the Antioxidant Activity of HA

2.6.1 DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of HA was analysed according to Irshad et al. [14] with a slight modification with the positive reference from ascorbic acid. About 20 µL of the crude HA extract was diluted with 1 mL of methanol, and serial dilutions were performed with methanol to a final volume of 100 µL in a 96-well plate. Subsequently, 100 µL of the 0.1 mM methanolic DPPH solution was added to each sample including the blank. The plate was incubated at 37 °C for 20 minutes. The absorbance was read at 515 nm using a microplate spectrophotometer (Epoch, BioTek Instruments, USA). The percentage of inhibition was calculated using the following standard Eq. (1), with each sample analysed in triplicate to ensure measurement reliability.

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \quad (1)$$

where $Abs_{control}$ represent absorbance for control from ascorbic acid and Abs_{sample} represent absorbance for sample of HA measured.

2.6.2 Ferric Reducing Antioxidant Potential (FRAP) Assay

FRAP assay was analysed according to Schlesier et al. with minor modifications [15]. This method relies on the reduction of Fe^{3+}-TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) to produce Fe^{2+}-TPTZ by the antioxidants, forming a blue-coloured complex. About 2 mL of crude HA sample was diluted with 1 mL of distilled water, followed by serial dilutions to obtain a final volume of 10 µL. Then, 300 µL of FRAP reagent was added into the TPTZ blank and sample wells. The plate was incubated at 37 °C for 15 minutes and allowed to cool down for 5 minutes before reading. The absorbance of the samples was read at 593 nm using a microplate reader spectrophotometer (Epoch, BioTek Instruments, U.S.A). Finally, the amount of the Fe^{2+} in mmol was calculated using the calibration curve of the Trolox standard solution with the equation of $y = 0.5218x + 0.0142$.

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2.7 Anti-Inflammatory Activity

2.7.1 Lipoxygenase Inhibitor Screening Assay

To measure lipoxygenase activity of HA, 1 mL of concentrated assay buffer was diluted with 9 mL ultrapure water. Similarly, 1 mL of HA samples was diluted with 9 mL assay buffer. For the positive control, a 1.1 mM stock solution of nordihydroguaiaretic acid (NDGA) was prepared by adding 500 µL of assay buffer, while a 15-Lipoxygenase standard was prepared by mixing 10 µL of enzyme standard with 990 µL of assay buffer. The assay and sample preparations were carried out in a 96-well plate following the protocol outlined in Table 2. The plate was then incubated at room temperature for 5 minutes. Following the addition of 1mM linoleic acid substrate, the samples was shaken for 10 minutes, followed by an additional 5 minutes after the chromogen was added. The absorbance of the samples was finally measured at 495 nm using a microplate spectrophotometer (Epoch, BioTek Instruments, USA). The percentage of inhibition was calculated using Eq. (2).

$$\% \text{ inhibition} = (100\% \text{ Initial activity-inhibitor}) / (100\% \text{ Initial activity}) \times 100 \quad (2)$$

Table 2. Sample preparation for the lipoxygenase inhibitor-screening assay

Sample	Assay Buffer (µL)	15-LO (µL)	Solvent (µL)	Inhibitor (µL)	Sample (µL)	Linoleic acid substrate (µL)	Chromogen (µL)
Blank	100	-	-	-	-	10	100
Positive control	10	90	-	-	-	10	100
100% initial activity	-	90	10	-	-	10	100
Inhibitor (NDGA)	-	90	-	10	-	10	100
Sample	-	90	-	-	10	10	100

3. RESULTS AND DISCUSSION

3.1 Optimization of ultrasonic-assisted enzyme extraction

RSM was applied to optimize the processing conditions for ultrasonic-assisted enzyme. The effects of hydrolysis time, pH, enzyme concentration, and ultrasonic time on the yield of HA were observed using a Box-Behnken design. The results obtained from the 27 experimental runs are shown in Table 3, indicating a range of HA yields from 1.206% to 4.629%. The highest HA concentration (4.629%) was achieved in run 7 with 5 hours of incubation time, 10% enzyme concentration, pH of 7.5, and a 2-minute ultrasonic treatment time. Conversely, the lowest HA concentration (1.206%) was observed in run 13, under conditions of 5 hours of incubation time, 1% enzyme concentration, pH of 8, and 16-minute ultrasonic treatment. These results demonstrated a significant effect of enzyme concentration on HA extraction from ESM. Higher enzyme concentrations enhance the enzymatic hydrolysis process leading to increased HA yield.

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Table 3. HA production by ultrasonic-assisted enzyme extraction from ESM using Box-Behnken design

Run	Independent variables Hydrolysis time (A) (hours)	Enzyme concentration (B) (w/w)	pH (C)	Ultrasonic Time (D) (minutes)	Dependant variable Hyaluronic acid (%w/w)
1	3	5.5	8	16	3.400
2	5	1	7.5	30	1.747
3	7	1	7.5	16	1.353
4	5	5.5	7.5	16	3.539
5	5	5.5	8	2	3.859
6	7	5.5	7.5	30	4.151
7	5	10	7.5	2	4.629
8	5	10	7	16	4.114
9	3	10	7.5	16	4.557
10	5	10	7.5	30	4.481
11	7	5.5	7.5	2	3.670
12	3	1	7.5	16	1.416
13	5	1	8	16	1.206
14	7	5.5	8	16	4.035
15	5	1	7.5	2	1.383
16	5	5.5	7	30	3.346
17	7	10	7.5	16	3.796
18	5	5.5	7	2	3.758
19	5	5.5	8	30	3.499
20	5	1	7	16	1.370
21	7	5.5	7	16	3.353
22	5	5.5	7.5	16	3.521
23	5	10	8	16	4.394
24	5	5.5	7.5	16	3.710
25	3	5.5	7.5	2	4.091
26	3	5.5	7	16	3.879
27	3	5.5	7.5	30	4.020

(w/w): (weight of enzyme, g / weight substrate, g)

Regression analysis was performed to justify the validity of the model. The analysis of variance (ANOVA) results for the model are presented in Table 4. The model's *F*-value of 52.31 indicates that the model is highly significant, and the enzyme concentration (B) significantly influenced HA production. The *F*-value for the model's lack of fit is 4.54 indicating that it was insignificant to the pure error, with a 19.40% probability that the lack of fit was due to noise.

Table 4. ANOVA for the overall effect of independent variables on HA concentration (%)

Source	SS	DF	MS	*F*-Value	*p*-Value
Model	31.55	14	2.25	52.31	< 0.0001*
A	0.0843	1	0.0843	1.96	0.1873
B	25.51	1	25.51	592.2	< 0.0001*
C	0.0274	1	0.0274	0.6366	0.4404
D	0.0017	1	0.0017	0.0405	0.8439
AB	0.1219	1	0.1219	2.83	0.1183
AC	0.3369	1	0.3369	7.82	0.0161*
AD	0.0761	1	0.0761	1.77	0.2086
BC	0.0492	1	0.0492	1.14	0.3061
BD	0.0655	1	0.0655	1.52	0.2412
CD	0.0007	1	0.0007	0.0164	0.9003

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A^2	0.0612	1	0.0612	1.42	0.2562
B^2	3.43	1	3.43	79.66	< 0.0001*
C^2	0.0353	1	0.0353	0.8187	0.3834
D^2	0.2617	1	0.2617	6.07	0.0298*

A: Hydrolysis time (hours), B: Enzyme concentration (%), C: pH, D: ultrasonic time (minutes), DF: degree of freedom, SS: Sum of square, MS: Mean of Square. *Significant at $p<0.05$.

Multiple linear regression analysis of the experimental data was performed to obtain the coefficients of the empirical model. The model coefficients with positive sign represents a synergistic effect, whereas negative coefficients indicate an antagonistic effect. The most influential parameter in the model was enzyme concentration (B), which showed the highest positive coefficient (1.46) and F-value (592.2). Eq. (3) shows the second order polynomial equations obtained from the BBD model for the HA yield.

$$HA\ (\%) = +3.59 - 0.0838\ A + 1.46\ B + 0.0478\ C - 0.0121\ D - 0.1746\ AB + 0.2902\ AC + 0.1379\ AD + 0.1109\ BC - 0.1280\ BD + 0.0133\ CD + 0.1071\ A^2 - 0.8022\ B^2 - 0.0813\ C^2 + 0.2215\ D^2 \quad (3)$$

By applying the polynomial equation and the three-dimensional (3D) response surface plot, it was possible to obtain optimum HA concentration. Fig. 1 shows the interaction effect of hydrolysis time and enzyme concentration on HA yield. The areas in red and orange zones indicate higher HA yields, which are achieved at elevated enzyme concentrations. In the enzymatic extraction process, the maximum yield was achieved at enzyme concentration above 7 %.

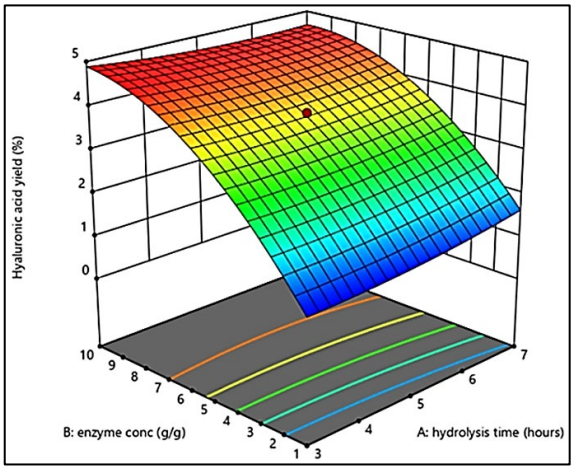

Fig. 1. Response surface plot showing the effect of hydrolysis time and enzyme concentration on HA concentration

Fig. 2 shows the actual and predicted plot of HA yield production. The actual versus predicted plot is essential for assessing the precision and reliability of the model in forecasting the response variable based on the input variables. The actual data aligned closely along a diagonal line, indicating a good fit, thus, the model is valid and can accurately predict the response under different conditions. Studies have shown that enzyme concentration significantly affects the efficiency of the extraction process, with higher enzyme concentrations generally leading to increased extraction yields [16]. The findings are similar to a study by Nishad et al., which indicates that enzyme concentration is a crucial factor in enhancing the extraction of

phenolic from *Citrus paradisi L.* peels [17]. Higher enzymes concentrations improve substrate accessibility, accelerate reaction rates, maximize yield, optimize enzyme-substrate interactions, and minimise inhibitory effects. Collectively, these factors contribute to a more efficient and effective extraction process which is crucial for industries aiming to maximize product yield and overall extraction efficiency.

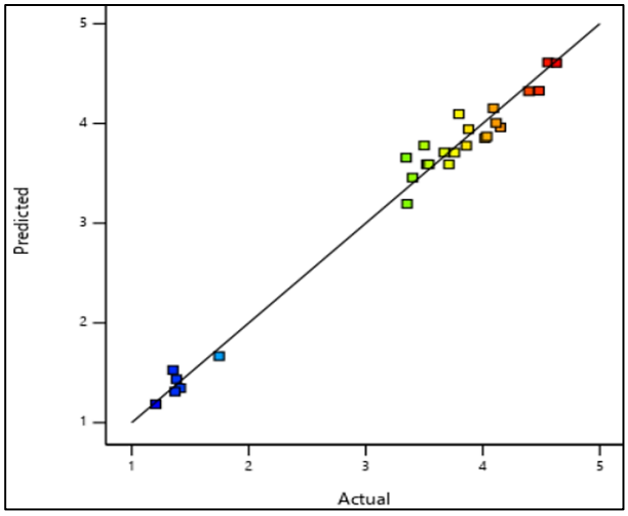

Fig. 2. Actual and predicted plot of hyaluronic acid yield production by papain hydrolysis with ultrasonic pre-treatment (%)

To verify the reliability of the model, the optimal processing conditions for achieving the highest HA concentration were suggested as a 3.1-hour hydrolysis time, an enzyme concentration of 9.7%, a pH of 7.5 and 2.1-minutes sonication time. The mean HA concentration (5.062%) was determined by three replicate experiments and compared with the predicted values (5.841%). The calculated error deviation was 7.64%, indicating that the models was suitable for optimal HA yield, as it agreed closely with the experimental results.

3.2 Antioxidant Activity

The DPPH radical scavenging activity for HA extracts at a concentration of 15 µL/mL was 41.602 ± 1.577%. The extracted HA showed relatively high percentage of DPPH radical scavenging activity. A study by Mohammed & Niamah has reported the radical scavenging activity of bacterial hyaluronic acid to be less than 25% at concentration 50 µL/mL [18]. The significant difference in radical scavenging activity suggests that the extracted HA possesses stronger antioxidant capacity compared to the bacterial-sourced HA, indicating its potential advantages as an antioxidant comparing the result with ascorbic acid as standard reference. Based on the results obtained, the IC_{50} value for the HA extract was 23.55 µg/mL. The relatively low IC_{50} value further underscores the strong antioxidant activity of the HA extract. While the FRAP values for HA extracts was 0.298 ± 0.0018 mmol Fe^{2+}/mL [19].

Recent spectroscopic studies have shown that HA can neutralise reactive oxygen species by mitigating the toxicity of free radicals [20]. It also plays a crucial role in preventing oxidative damage by reducing the

production of reactive oxygen species in cartilage subjected to mechanical stress [21]. HA has been reported to be able to enhance the antioxidant effect of HA-based formulations, such as HA−bilirubin nanoparticles (HABNs), which has been developed for the therapy of non-alcoholic steatohepatitis (NASH) and fibrosis [22].

In addition, HA has been used in the development of antioxidant hybrid hydrogels for wound healing that exhibited enhanced antioxidant activity to scavenging reactive oxygen species within the wound microenvironment [23]. HA-based tissue adhesive hydrogels have also been developed that possess antioxidant properties and provide both tissue adhesion and antioxidant benefits [24]. HA has also been studied in combination with other compounds to enhance antioxidant activity in health products. For example, HA co-gelatin cryogels have been developed for tissue engineering applications that utilizing the antioxidant properties of HA and the cell adhesion properties of gelatin to promote cell attachment and survival [25].

3.3 Anti-inflammatory Activity

The lipoxygenase inhibitor screening assay was used to assess the anti-inflammatory activity of the HA extract by measuring its ability to inhibit the enzyme 15-lipoxygenase (15-LOX). At a concentration of 15.625 µL/mL, the HA extract demonstrated a 9.4104% inhibition of 15-LOX. Although this percentage indicates some level of inhibitory activity, the result suggests that the HA extract exhibits a relatively moderate inhibitory effect on 15-LOX. The 15-LOX is an enzyme involved in the metabolism of polyunsaturated fatty acids that catalyses the oxidation polyunsaturated fatty acids to form pro-inflammatory mediators, such as leukotrienes, which play key roles in the inflammatory response [26]. Therefore, inhibiting this enzyme represents a potential mechanism for reducing inflammation. The moderate inhibition observed in this study suggest that, although the HA extract possesses some anti-inflammatory properties, it may be more effective when combined with other anti-inflammatory compounds or applied at higher concentrations. HA has also been studied for its wound healing capabilities, as promotes both anti-inflammatory and antibacterial effects [27, 28]. HA has been found to modulate macrophages-mediated inflammatory response and exert a strong anti-inflammatory effect, particularly during wound healing processes [29].

4. CONCLUSIONS

The present study demonstrated the optimization of ultrasonic-assisted enzymatic hydrolysis processing condition for obtaining a high yield of HA (5.062%) from ESM was successful. The optimum processing condition obtained were 3.1 hours hydrolysis time, 9.7% papain concentration, 7.5 pH and 2.1 minutes of ultrasonic pre-treatment. Papain concentrations was identified as the most important factor influencing HA yield. The extracted HA exhibited notable antioxidant and anti-inflammatory activities, indicating its potential in medical and cosmetic applications. These findings highlight ESM as a sustainable source of HA production, with implications for the pharmaceutical and cosmetic industries. Further research should explore the mechanistic anti-inflammatory effects of HA and compare its properties with HA derived from other sources to further enhance its therapeutic and commercial potential.

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6. CONFLICT OF INTEREST STATEMENT

All authors declare that there is no conflict of interest during the preparation of this manuscript.

7. AUTHORS' CONTRIBUTIONS

Siti Nor Azlina Abd Rashid: Conceptualisation, methodology writing - original draft and writing – review and editing; Muhammad Zulhelmi Nazri: Writing-original draft and writing – review and editing; Rui-Fang Wong: Formal analysis, methodology, investigation; Kian-Kai Cheng: Conceptualisation, supervision, and validation; Nor Zalina Othman: Conceptualisation, supervision, and validation; Hong-Yeng Leong: Conceptualisation, validation, funding acquisition, resources, supervision and writing – review and editing.

8. DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this work, the author(s) used **ChatGPT (OpenAI)** and **Perplexity AI** in order to support language refinement, improve clarity of expression and assists in structuring and summarizing relevant scientific information. After using these tools, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

9. DATA AVAILABILITY/SUPPLEMENTARY MATERIALS

Data included in the article: All data generated or analysed during this study are included in this published article.

10. ETHICS STATEMENT

This study did not involve human participants or animals; therefore, ethical approval was not required.

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