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Optimizing Barley Flour Protein Extraction via Response Surface Methodology: Characterization and Anti-inflammatory Activity

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Abstract

One possible source of bioactive proteins has been identified: barley flour protein (BFP). An extraction process was optimized through the use of a Box-Behnken Design (BBD). The results showed that an extraction temperature of 54.5^{o}C, a buffer-to-sample ratio of 41.8 mL/g, as well an extraction period of 120 minutes were the ideal parameters for achieving the greatest protein yield (17.8%). For these settings, the computed desirability value was 0.47 %. The BFP electrophoresis analysis showed that two different protein types were present. One kind was made up of low molecular weight proteins, known as globulins of the (2S) type, that ranged in size from 6 to 98 kDa. Furthermore, the presence of lectins was confirmed by a noticeable protein band of 27.6 kDa. Furthermore, in lipopolysaccharide (LPS)-induced RAW264.7 macrophages, the proteins isolated under optimal circumstances showed action in suppressing the release of nitric oxide (NO) and pro-inflammatory inducers such IL-6, IL-1β, and TNF-α. These results imply that BFP may be used as a useful nutraceutical, offering proteins that are both functional and health-promoting.

***Keywords**: Barley flour protein; optimisation; anti-inflammatory impact; Box-Behnken design*

1. Introduction

Proteins with particular biological activity that can improve human health are known as bioactive proteins. Many times, these proteins come from organic sources including microbes, plants, and animals. Their potential to promote health has led to a great deal of interest in the field of nutrition and functional foods. One of the most crucial factors used by the food and brewing industries to determine the functional and nutritional attributes of grains is their protein content [1]. Hordeum vulgare L., or barley, is a cereal grain crop that has been grown for a long time and is ranked fourth globally in terms of production [2]. It has been discovered that seed proteins offer defense and prevention against a number of illnesses, such as diabetes, obesity, cancer, heart disease, and high cholesterol. These proteins have drawn interest due to their possible health advantages and capacity to enhance general well-being [3].

The health benefits of unmasked proteins and their hydrolysates made from natural bioresources have been extensively studied. In macrophages activated with lipopolysaccharide (LPS), a number of dietary proteins have demonstrated significant anti-inflammatory effects by regulating the synthesis of iNOS, inflammatory cytokines, and COX-2. [4, 5], implying that multifunctional proteins with anti-inflammatory properties may be extracted. The definition of inflammation is a normal physiological response to outside stimulants, chemicals, or microbiological toxins [6, 7]. Type 2 diabetes (T2D) can progress due to excessive and persistent inflammation caused by the uncontrolled release of pro-inflammatory cytokines like tumour necrosis factor (TNF-α), interferon-gamma (INFγ), interleukin

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6 (IL-6), interleukin 1 (IL-1), chemokines (e.g., CCL2, CCL5), reactive oxygen species (ROS), adhesion molecules (e.g., ICAM-1, VCAM-1), as well as high levels of nitric oxide (NO) and nitrogen intermediates [8].

The use of frequently prescribed drugs, such as immunosuppressants, has been limited for a considerable amount of time because of their assessment of cost and gastrointestinal side effects in addition to effectiveness [9]. To minimize the negative side effects of industrial medicines, it is a good idea to use extraction procedures for natural bioactive substances. Response surface methodology (RSM) is an extraction technique used for process optimization that requires complex computations. Using this method results in an experimental plan that is well-designed and covers every variable under test. RSM creates a set of formulas that can improve extraction yields, improve the extraction function, and reduce time by using the data gathered throughout the experiment [10-12].

In many scientific and research applications, including protein purification and analysis, protein extraction is an essential first step. Protein extraction performance and efficiency can be influenced by a number of factors. Protein stability is a crucial aspect to take into account during extraction. Moreover, protein yield can be impacted by the protein concentration and the precipitant utilized in the extraction process. Furthermore, maintaining the stability and activity of proteins is greatly influenced by the buffer system that is utilized during protein extraction. The buffer should try to replicate the protein's in vivo environment as much as feasible. A perfect buffer environment for the target protein should be created by carefully balancing variables including pH, ionic strength, and temperature [13]. The present investigation examined the use of barley flour as the principal material in order to maximize the extraction of functional protein. In order to accomplish this goal and create a viable extract that might lessen the effects of inflammation in place of commercial treatments, the response surface technique was used. A perfect buffer environment for the target protein should be created by carefully balancing variables including pH, ionic strength, and temperature.

2. Materials and methods

2.1. Materials

We acquired the Giza 123 barley cultivar from the Sakha Agricultural Research Station. The source of the murine RAW264.7 cells was Nanjing, China's Nanjing University of Chinese Medicine. The ELISA kits used to measure TNF-α, IL-6, IL-1β, and nitric oxide (NO) were acquired from Jiancheng Bioengineering Institute, which is also situated in Nanjing, China. We purchased MTT, LPS, and penicillin from Sigma Chemical Co. Cell Signalling Technology, situated in Danvers, Massachusetts, USA, provided the primary and secondary antibodies that were directed towards anti-inflammatory mediators, albumin marker, and β-actin. Analytical-grade chemical reagents were all that were employed in the investigation.

2.2. Methods

2.2.1. Extraction of barley flour protein (BFP)

With a few minor adjustments, the BFP extraction procedure was carried out in accordance with the stated method by Segura-Campos et al. (2013) [14] A particular quantity of barley flour was suspended in a pH 8 phosphate buffer solution in order to maximize the extraction process. After that, the suspension was incubated in various settings, such as with variable time, temperature, and buffer-to-sample ratio. To determine the most efficient extraction technique, these factors were changed and put to the test. The resultant solution was then centrifuged for an hour at 3000 rpm and 10°C after the incubation, and the supernatant was taken out. The supernatant's pH and was brought to 4 by adding 2N HCl, and mixing the mixture for 20 minutes. Under the identical circumstances, the centrifugation stage was carried out again. In order to obtain BFP, the precipitate was finally collected and freeze-dried at -50°C.

2.2.2. Determination of protein content

Using a UV/VIS spectrophotometer 722 (Shanghai Jinghua Science & Technology Instruments Co., Ltd., China) set to 595 nm in wavelength, the protein concentration of BFP was determined using the Bradford technique [15]. Bovine serum albumin (BSA) was used to create the standard curve.

2.2.3. Response surface experimental design

Response Surface Methodology (RSM) experimental design was carried out after single-factor studies to ascertain the impact of different parameters on protein yield. These variables included duration (from 40 to 160 minutes), temperature (from 30 to 60°C), and buffer-to-sample ratio (20 to 50 mL/g). These single-factor experiment results were utilized to create appropriate indications for the ensuing Box-Behnken design (BBD), [16]. As shown in Table 1, the three independent variables were used in the BBD at

three levels. The whole BBD featured an experimental design with five core points to evaluate the method's dependability, along with 17 suggested runs that were randomly executed (Table 1). A second-order polynomial equation was used to examine the connection between the independent variables and the responses in the BBD:

$$Y=\beta_o+\sum_{i=0}^{n}\beta_i X_i+\sum_{i\neq o}^{n}\beta_{ii} X_i^2+\sum_{i\neq i=1}^{n}\beta_{ij} X_i X_j \quad (1)$$

where Xi and Xj are the points of the independent parameters; Y is the evaluated response; βo denotes the intercept; n is the number of components tested; and βi, βii, and βij show the linear (principal effect), quadratic, and cross product model coefficients, respectively.

Table 1. BBD and response values for Protein yield

Run	Temperature X1 (°C)	Buffer-to-sample X2 (mLg^{-1})	Time X3 (min)	Protein yield (mg/g)
1	50	35	60	17.78
2	45	42.5	120	19.8
3	55	42.5	60	21.41
4	50	42.5	90	23.03
5	45	42.5	60	19.28
6	55	50	90	21.95
7	50	42.5	90	21.98
8	50	50	60	22.85
9	55	35	90	21.02
10	55	42.5	120	25.33
11	45	35	90	18.01
12	45	50	90	20.32
13	50	42.5	90	22.91
14	50	42.5	90	23.35
15	50	50	120	21.95
16	50	35	120	21.36
17	50	42.5	90	22.53

2.2.4. SDS-PAGE analysis of BFP

The resultant BFP (barley flour protein) was electrophoresed on a 10% polyacrylamide SDS gel after the parameters were optimized. A Mini-Protean II electrophoresis cell from Bio-Rad, which is based in Hercules, California, USA, was used to perform the electrophoresis. After the electrophoresis, the gel was cleaned and stained for three hours using a solution that contained 10%, 0.1%, and 20% of glacial acetic acid, Coomassie blue, and methanol, respectively. After that, a distaining procedure was carried out with a 50% methanol and 10% glacial acetic acid solution. Sigma-Aldrich model PS 500-2 generator was used to perform the electrophoresis migration operation at a strength of 20 mA per plate. Lastly, a digital camera was used to take a picture of the gel.

2.3. Anti-inflammatory effect of BFP

2.3.1. Determination of the cell viability of BFP on RAW 264.7 cells

In the current investigation, a battery of tests was conducted to assess the anti-inflammatory activity of the BFP (optimized with the maximum yield). According to Liu, Luo, Ye, et al. (2010), the MTT-colorimetric approach was used to evaluate the anti-inflammatory activity. The experiment involved the cultivation of murine cells at a density of 1 × 105 cells/mL in a modified environment (37°C and 5% CO2) using DMEM supplemented with 10% (v/v) newborn calf serum and 1% (v/v) antibiotic solution (containing streptomycin at 100 IU/mL and penicillin at 100 IU/mL). After adding 100 µL of the murine cell suspension to a 96-well culture plate, it was cultured for 12 hours. Next, a fresh medium with different BFP final concentrations was added to the culture medium in an amount of 100 µL. The blank control was DMEM on its own. For a full day, the plates were incubated once more. After that, the plates were incubated for a further 4 hours with 50 µL of MTT solution (1.0 mg/mL) added in place of the cell culture medium. Each well was then filled with 150 µL of DMSO following the removal of the MTT solution. With the use of a microplate reader, the absorbance of the wells was determined at 570 nm. The following formula was used to get the proportion of viable cells:

$$Cell\ viability = \frac{Ab_{sample}}{Ab_{control}} * 100 \quad (2)$$

2.3.2. Assay of nitrite oxide

As per our earlier protocol [17], following 150 minutes of incubation of RAW 264.7 cells with varying BFP concentrations in 96-well plates, LPS (1µg/mL) was added to each well, and the plates were then placed in an incubator for an additional 24 hours. Using an ELISA kit and the Griess reagent, the presence of nitrite (µmol/L) was determined in accordance with approved guidelines.

2.3.3. Assay of cytokine production

2.3.3.1. Western blot analysis

Picograms per milliliter (Pg/mL) of the tested cytokine were measured using the Invitrogen ELISA kit. Cells were cultivated in a 96-well plate at a density of 2 × 105 cells per well and incubated overnight to prepare for the ELISA experiment. After treating the cells with BFP for 60 minutes, LPS (1 µg/mL) was injected to cause inflammation, which lasted for a whole day. Following the incubation period, the cytokine-secreted supernatant was extracted. The ELISA assay was carried out in compliance with the

instructions provided by the manufacturer. To perform the assay precisely and consistently, the precise measures and guidelines specified in the protocol were adhered to. With the appropriate modifications, the western blot method was carried out in accordance with Wan et al. 2019 [18]. The cells were centrifuged after being exposed to BFP for predetermined amounts of time. After that, each sample's supernatant—which contained the same quantity of protein—was electrophoresed on a 10% SDS-PAGE gel. Using a semi-dry electrophoretic transfer cell, the proteins on the gel were moved to polyvinylidene difluoride (PVDF) membranes once the gel running procedure was finished. Subsequently, the PVDF membranes were incubated for 120 minutes in a solution that contained 5% skim milk and tris-buffered saline Tween 20 (TBST). The purpose of this procedure was to prevent any non-specific binding sites on the membranes. The membranes were blocked and then left overnight to be incubated with particular primary antibodies. To get rid of any unbound primary antibodies, the membranes were cleaned five times with TBST solution the next day. The membranes were then subjected for 120 minutes to a secondary antibody, namely HRP-conjugated goat anti-rabbit secondary antibody at a dilution of 1:50000. Amersham Biosciences, UK, supplied enhanced chemiluminescence reagents (ECL), which were utilized to see the protein bands. The resultant bands on the membranes were examined and analyzed using the Tanon 6000 imaging equipment.

2.4. Statistical analysis

The findings for the single-factor experiment, which included both cell viability and anti-inflammatory efficacy, were analyzed using SPSS software through a one-way analysis of variance (ANOVA). The investigation of any significant differences ($P < 0.05$) between the groups was made possible by this statistical analysis. The Response Surface Methodology (RSM) data analysis and experimental design were carried out using the Design Expert program. This software made it easier to create and carry out the experimental design and analyze the data that was produced. It was feasible to investigate the link between several factors and how they affected the relevant response variables by utilizing RSM.

3. Results and discussion

3.1. Single factor investigation

3.1.1. Effect of temperature on protein yield

Figure 1A illustrates how temperature affected the amount of protein in barley flour throughout the extraction process. The proportion of BFP increased significantly ($p < 0.05$) when the barley flour was heated to 50 °C. Nevertheless, a significant ($p < 0.05$) drop in BFP was noted above 50 °C. According to Yu, Ahmedna, and Goktepe (2007) [19], denaturation brought on by increased temperatures may be the source of this drop in BFP. Moreover, raising the temperature may facilitate the interaction of protein complexes with other constituents in the seeds. Protein solubility is subsequently reduced as a result of these protein complexes [20]. As a result, depending on the steepest ascent approaches, the extraction temperature was chosen for the ensuing optimization study to be between 45 and 55 °C.

3.1.2. Effect of percentage of buffer-to-sample

Different buffer-to-sample ratios from 20 to 50 mL/g were investigated in Figure 1B. It was found the protein production considerably increased to its maximum value at a ratio of 40 mL/g. But when the ratio was raised to 50 mL/g, no discernible ($p < 0.05$) changes were seen. Proteins' hydrophobic surfaces, which encourage protein-protein interactions, and the protein solvent's hydrophilic surface interact to affect how extractable proteins are (Kristinsson & Hultin, 2004) [21]. Additionally, an increase in the solvent-to-solid ratio results in an improvement in the solute-solvent contact area, which supports the solubility of bioactive substances found in plant cells [10]. The study's main goal was to maximize BFP extraction in order to maximize yield. As a result, the experimental design that followed focused on the ratios between 35 and 50 mL/g, with ratios below 35 mL/g being disregarded.

3.1.3. Effect of time

Figure 1C indicates that there was a significant ($p < 0.05$) increase in the extracted protein content is between 40 and 80 minutes. Nonetheless, yield did not alter in a way that was statistically significant ($p > 0.05$) after the extraction duration was increased to 120 minutes. Conversely, if the extraction time was extended beyond 120 minutes, the BFP content decreased significantly ($p < 0.05$). These results imply that increasing the extraction time does not increase BFP extraction efficiency. As a result, the extraction period for additional optimization experiments was selected to be between 60 and 120 minutes, which is consistent with other research in this field [20] that isolated bioactive protein from cumin seed.

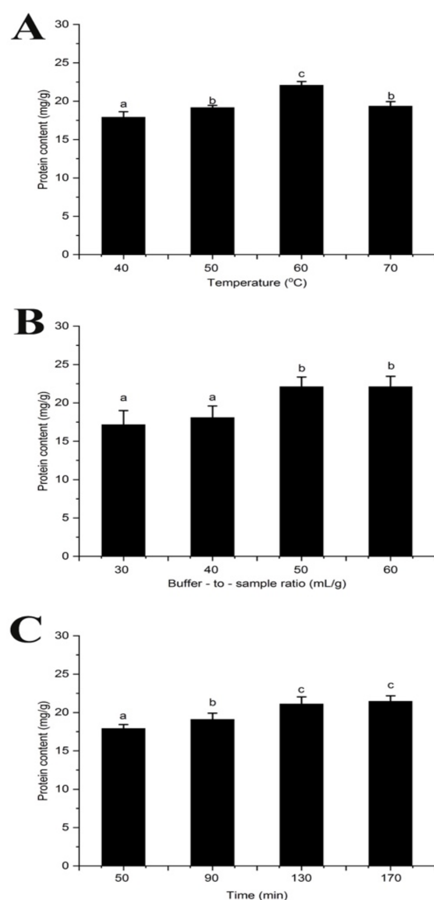

Fig. 1. Effects of temperature (A), buffer-to-sample ratio (B), and time (c) on protein content.

3.2. Designing of BBD and response values

As can be seen in Table 1, three extraction parameters: temperature (X1), buffer-to-sample ratio (X2), and duration (X3), were studied in relation to the yield of BFP using a BBD (Box-Behnken Design). The findings of the earlier single-factor studies were used to establish the range for each factor, including the minimum and maximum values. With three levels (-1, 0, and +1) and five runs at the center, the design called for a total of 17 runs. Depending on the particular extraction conditions used, the protein yield varied. Notably, the experimental conditions of X1 at 55 °C, X2 at 42.5 mL/g, and X3 at 120 minutes resulted in the greatest protein yield of 45.5 mg/g. When it came to extracting protein from highly functioning plant seeds, the BBD approach turned out to be a great option [22].

3.2.1. Analysis of ANOVA and model fitting

An analysis of variance (ANOVA) was performed to ascertain the significance of the model's coefficients. The current model's protein content F-value (13.42) was determined to be significant, suggesting that the model's equation had a substantial impact on forecasting the BFP extraction yield. Furthermore, in comparison to the pure errors, the P-value (0.1780) for the lack of fit was determined to be non-significant. This suggests that for different combinations of temperature, buffer-to-sample ratio, and time, the existing model equation was enough for correctly forecasting the extraction yield of BFP [23]. Furthermore, if the absolute F-value increases and the P-value decreases [24], both of which are consistent with the data acquired in Table (2), the identified variables will become more significant. Equation (3) produced the fitted quadratic model for the BFP yield in coded variables. The current model's results highlight that the linear terms of temperature X1, buffer-to-sample X2, time X3, quadratic term, and cooperation between temperature X1 & time X3, buffer-to-sample X2 & time X3, X12, and X22 had the most effects on BFP yield. There was a strong degree of correlation between the observed and predicted values, as indicated by the convergent values of the adjusted coefficient of ($R^2_{adj.}$) and coefficient of (R^2) of the projected model for BFP yield.

Table 2. ANOVA for response surface quadratic mode

Source	Sum of Square	Mean square	*F-value*	*P-value*	Significant
Protein content					
Model	57.82	6.45	13.42	0.0012	*Significant*
X_1-Temperature	20.41	18.91	39.35	0.0005	
X_2-Buffer—to sample	8.7	9.9	20.6	0.0022	
X_3-Time	6.43	6.34	13.19	0.0075	
X_1X_2	0.86	0.48	0.99	0.3453	
X_1X_3	2.63	2.89	6.01	0.053	
X_2X_3	6.04	5.02	10.44	0.0153	
Residual	3.42	0.48			
Lack-of-fit	2.26	0.75	0.98	0.1780	*Not significant*
Pure error	1.05	0.28			
Adj R-Squared	0.8653				
R-Squared	0.9642				
C.V.	1.08				

p values lower than 0.05 are statistically significant; yield values obtained by the subsequent evaluation:

$$\textbf{Yield} = 66.40 + 1.54 X_1 + 1.11 X2 + 0.89 X3 - 0.35 X1X2 + 0.85 X1X3 - 1.12 X2X3 - 0.98 X1^2 - 1.45 X2^2 - 0.32 X3^2 \quad (3).$$

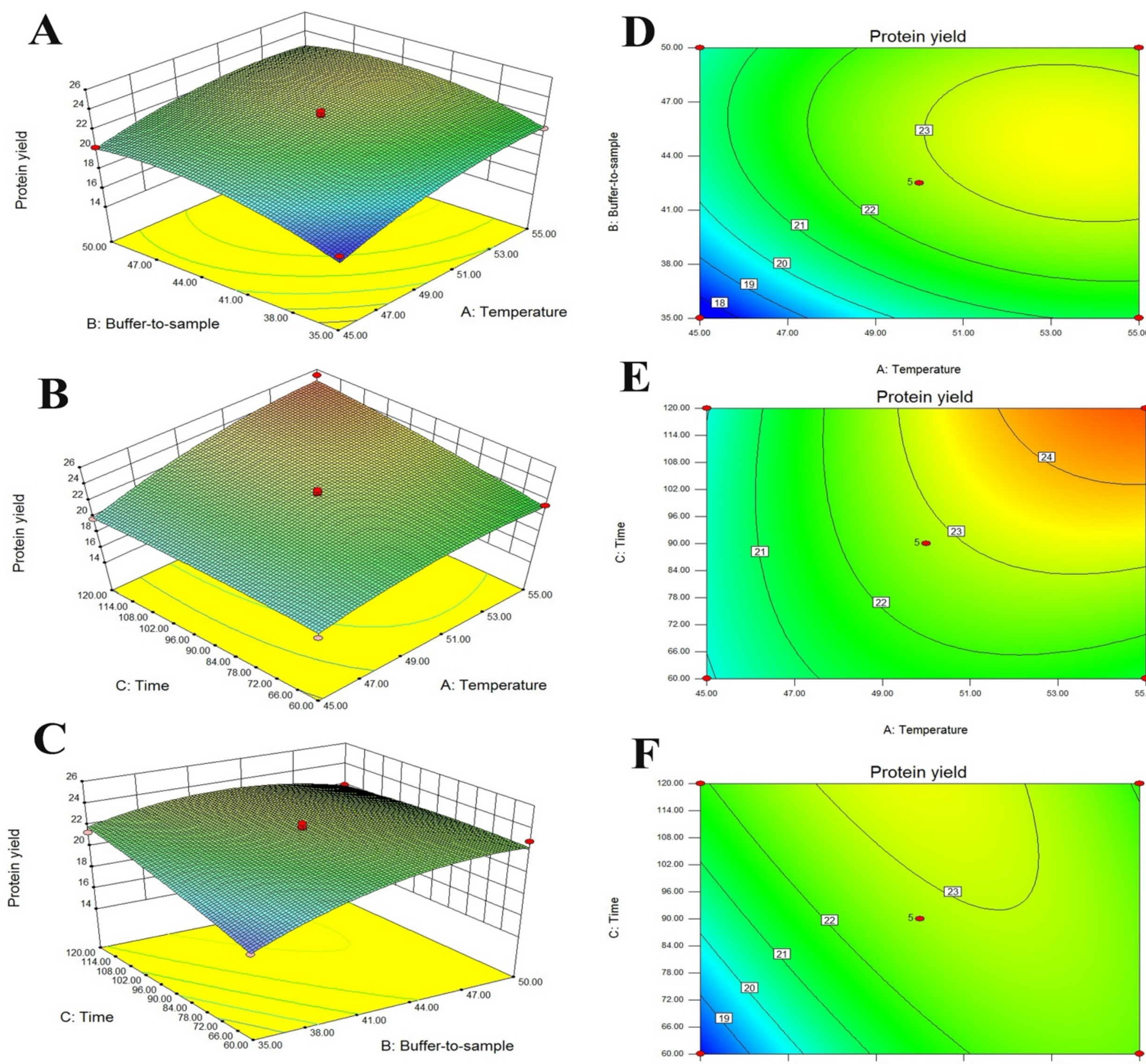

Fig. 2. Response surface plots (A-C) and contour plots (D-F) showing the effect of extraction variables on protein content.

3.2.2. Analysis of response surfaces

To display the interaction effects between the factors under examination and the protein content, two-dimensional contour plots (Figures 2 (D-F) and three-dimensional shapes (Figures 2 (A-C) were made. It is implied by the circular contour plots that there is no statistically significant interaction between the examined variables. However, according to Muralidar, Chirumamila, Marchant, and Nigam (2001) [25], the elliptical contour shape indicates that the related variables have a considerable impact on the protein concentration.

The circular shape seen in Figures 2 (D-F) suggests that there is a considerable interaction or collaboration between the components under test. These outcomes are consistent with the ANOVA analysis results shown in Table 2, which further emphasizes the importance of the interactions between the variable components and their significant influence on the BFP yield (temperature, buffer-to-sample ratio, and time). After taking into account a range of tested parameter values, it was found that the following extraction parameters were ideal for optimizing the protein content of BFP: an extraction temperature of 54.53 °C, a buffer-to-sample ratio of 41.79 mL/g, and

an extraction period of 120 minutes. Based on calculations, the desirability value for these ideal conditions came out to be 0.471%. A practical experiment was carried out using the optimized values with minor alterations to confirm their practical applicability. A temperature of 54.5 °C, a buffer-to-sample ratio of 42 mL/g, and a duration of 120 minutes was established as the realistic extraction conditions. An extraction yield of 25.67 ± 0.52% (n = 3) was obtained under these realistic extraction conditions, which is consistent with the expected value and shows the dependability of the optimization procedure.

3.2.3. SDS-Page characterization of BFP

As seen in Figure 3, the protein electrophoretic mobility was examined and protein bands were seen using the SDS-PAGE technique. The MSKP electrophoresis patterns showed that the proteins' molecular weights (MW) varied from 6 to 98 kDa. The electrophoretic examination revealed and emphasized the presence of globulin, which is known as the primary storage protein in seeds [26]. The lowest bands in MW may account for globulin's attendance with type (2S). Furthermore, the presence of lectins, which were commonly discovered in seeds, was explained by a different primary protein band measuring 27.6 kDa [27]. The outcomes attained aligned with the findings of Siow et al. (2014) [20].

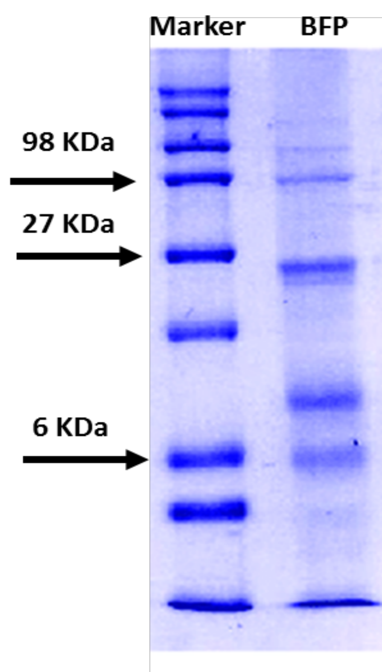

Fig. 3. SDS-PAGE profile of BFP compared to standard protein marker.

3.2.4. Anti-inflammatory effect of BFP

To assess BFP's anti-inflammatory impact on RAW264 produced by LPS. Numerous indicators, including cell viability, IL-1β, IL-6, TNF-α, and nitric oxide (NO) generation, were evaluated in 7 cells. The MTT test was used to measure the cytotoxic concentration of BFP in order to evaluate cell viability before making the remaining calculations. Figure 4 shows that at the chosen concentrations, BFP had no cytotoxic effects on RAW264.7 macrophages, with the exception of 900 μg/mL. Consequently, BFP concentrations of 300, 500, and 700 μg/mL were employed for the next measurements. As illustrated in Figure 5 (A-C), the introduction of varying doses of BFP into the cultured media resulted in a dose-dependent decrease in the activity of proinflammatory mediators, including IL-1β, IL-6, and TNF-α, in contrast to the application of LPS alone. These results imply that BFP might be helpful in reducing inflammatory conditions. Similar outcomes were seen when proteins from Salvia hispanica L. seeds were added to the culture media to inhibit the expression of genes encoding IL-1β, IL-6, and TNF-α, as well as the expression of nuclear factor κB (NF-κB) or other transcription factors [28].

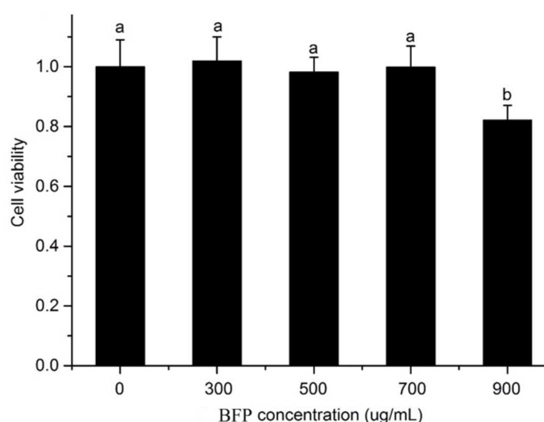

Fig. 4. Effects of BFP concentrations on cell viability of RAW264.7.

In addition, it was shown that the protein isolated from Myristica fragrans seeds could prevent RAW 264.7 murine macrophages from producing interleukin (IL)-6, IL-10, interferon-inducible protein-10, monocyte chemotactic protein (MCP)-1, and MCP-3 [29]. Prior to LPS stimulation, the cells were treated with BFP, and the concentration of NO was determined in order to evaluate the effect of BFP on NO concentration in the cultured medium after LPS stimulation. Pretreatment with BFP considerably decreased the NO levels caused by LPS treatment in a dose-dependent manner, as shown in Figure 5D. The concentration of BFP employed in the cell culture

media at 700 µg/mL produced the lowest NO concentration. Higher concentrations of NO in response to inflammatory stimuli are mediated by iNOS, which can result in a variety of inflammatory diseases including endotoxemia. NO is a cellular mediator that is produced at sites of inflammation upon stimulation by LPS [30]. Consequently, it is thought that preventing NO from being produced is a useful strategy for reducing inflammatory reactions. By downregulating mRNA and protein levels, the extracted proteins from seeds may affect the expression of iNOS and COX-2, which would ultimately lead to a decrease in NO production [31].

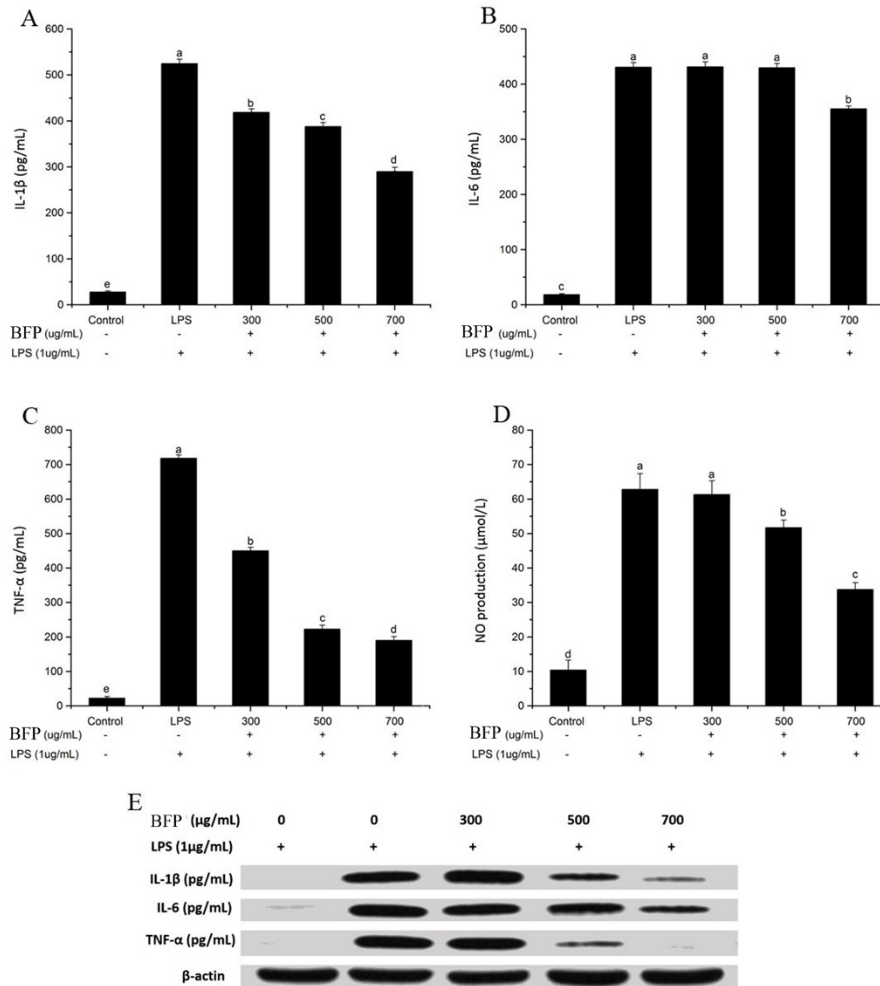

Fig. 5. Effects of BFP concentrations on production of IL-1β (A), IL-6 (B), TNF-α (C), NO (D), and Western blot analysis (E).

To validate the impact of BFP on the protein levels of the analyzed inflammatory cytokines, a Western blot experiment was conducted using phosphorylated versions of the antibodies after LPS stimulation. Electrophoresis and subsequent Western blot analysis are essential techniques for examining biochemical alterations in cells and tissues exposed to the components under inquiry [32]. Consistent with the findings of the anti-inflammatory experiments, cells treated with BFP showed a concentration-dependent pattern of reduced TNF-α, IL-1β, and IL-6 protein expression levels in comparison to the LPS treatment group alone, as shown in Figure 5E. At the highest BFP concentration detected (700 µg/mL), there was a near-total suppression of these protein expressions.

4. Conclusion

In conclusion, the current investigation showed that each of the parameters under investigation significantly affected the yield of BFP. The

optimisation method for BFP extraction was successfully validated by applying Response Surface Methodology (RSM) based on the findings of the single-factor tests. With a desirability value of 0.471%, the ideal extraction conditions were found to be an extraction temperature of 54.53 ^{o}C, a buffer-to-sample ratio of 41.79 mL/g, and an extraction duration of 120 minutes. The results of the SDS-PAGE examination confirmed that the isolated BFP included functional proteins. Additionally, the BFP showed anti-inflammatory properties against proinflammatory cytokines and NO. Further research into the chemical and biological characteristics of BFP is advised.

Acknowledgments

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