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Original paper

Extraction optimization, characterization and anti-inflammatory activity of functional protein from *Mangifera indica* seeds kernel

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Abstract Mango seed kernel (MSK) was observed to be a likely source of bioactive protein. Box-Behnken Design (BBD) was employed to optimize the protein extraction conditions. The optimum extraction conditions for maximum protein yield (68.336%) were corresponding to extraction temperature 54.53 ^{o}C, buffer-to-sample values 41.79 mL/g and extraction time 120 min with desirability value of 0.471%. The results of electrophoresis patterns of MSKP revealed two types of proteins; low molecular weight between 6 to 98 kDa explained the presence of globulin with type (2S) and another principal protein band of 27.6 kDa explained the presence of lectins. The extracted proteins under optimized conditions showed inhibition activity of nitric oxide (NO) release and pro-inflammatory inducers like (IL-6), (IL-1β) and (TNF-α) in lipopolysaccharide (LPS)-induced RAW264.7 macrophages. These findings revealed that MSK could be used as a promising nutraceutical of functional and health-promoting proteins.

Keywords Mango seed kernel; Box-Behnken Design; Optimization; Anti-inflammatory effect

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Introduction

The accumulation of vast quantity of wastes created by food industry produces major environmental issues as well as economical financial losses. These wastes have been discovered to be good sources of potentially important bioactive compounds. Mango by-products, particularly peels and seeds, are thought to be low-cost sources of nutritious foods and nutraceuticals (JAHURUL et al., 2015). Mango (*Mangifera indica*) is a popular tropical fruit cultivated mostly for its pulp. Mango seed (20-60% of the whole fruit) is an abundant waste of the food industry and is increasing due to the development of fruit production (DA SILVA MEIRELES et al., 2010). The seeds represent 30–45% of each mango's weight, contingent on the variety, and remain part which is commonly burned or discarded (ALENCAR et al., 2012). Mango seed kernels (MSK) contain 85-80% of carbohydrates, 6-13% of proteins and 6-16% of fats. Mango seed kernels (MSKP) contain essential amino acids and its oil has a considerable amount of essential fatty acids (DIARRA, 2014). Plant-derived proteins are becoming more popular in the nutraceutical field as parts of functional foods or substitutes of the environmentally costly production of animal protein (LIMA-CABELLO et al., 2018).

Seed proteins may have preventative and protective benefits against a variety of diseases such as obesity, cancer, cardiovascular disease, hypercholesterolemia and diabetes (VILLARINO et al., 2016). Furthermore, the health benefits of uncovered proteins and their hydrolysates derived from natural bioresources have been widely studied.

Various food proteins displayed distinguished anti-inflammatory effect in LPS-stimulated macrophages through regulation the production of iNOS, inflammatory cytokines, and COX-2 (AHN et al., 2012; UDENIGWE et al., 2013), suggesting the possibility of extraction of multifunctional proteins with anti-inflammatory activity.

Inflammation has been defined as a natural physiological reaction to external factors such as physical or chemical stimulation or microbial poisons (KNALL, 2015; LIU et al., 2018). Continued and excessive inflammation promoted by the uncontrolled production of pro-inflammatory cytokines like tumor necrosis factor (TNF-α), interferon gamma (INFγ),interleukin 6 (IL-6), interleukin 1 (IL-1), chemokines (i.e. CCL2, CCL5) ,reactive oxygen species (ROS), adhesion molecules (i.e. ICAM-1, VCAM-1), and the overproduction of nitric oxide (NO) and nitrogen intermediates might elicit a characteristic situation of T2D progression (TURNER et al., 2014).

Most commonly used medications, such as immunosuppressants, have not only been evaluated for efficacy, but also for gastrointestinal side effects and cost, which has limited its use as a medical remedy for a long time (SIEGEL, 2011). Thus, the extraction techniques of natural bioactive compounds as alternatives for industrial remedies are a good choice to avoid their unfavorable side effects.

Response surface methodology (RSM) is an extraction technique used for optimising a process that includes complex calculations. This method creates suitable experimental design that incorporates all of the tested variables and utilizes the data entry from the experiment to produce a set of formulas which can provide outcomes to enhance extraction yields, extraction function and save time (ABDIN et al., 2011; MOTA et al., 2003). In this work, use of MSK as principal material to optimize extraction of functional protein by using response surface technique that might be exploited as potential extract to reduce inflammation effects instead of industrial remedies and to reduce accumulation of wastes in environment.

Materials and methods

Materials

The Murine RAW264.7 cells were procured from Nanjing University of Chinese Medicine (Nanjing, China). The (ELISA) kits for nitric oxide (NO), TNF-α, IL-6, and IL-1β were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Penicillin, LPS and MTT were obtained from Sigma Chemical Co. The Primary and secondary antibodies against anti-inflammatory mediators and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). All other chemical reagents utilized were of analytical grade.

Methods

Preparation of Mango seeds kernel

Mango seeds were exposed to tap water for washing twice and air fan was directed to seeds for drying for 10 h on 20 °C. After that, the dried seeds were hammered to facilitate obtaining the kernels by removing the outer cover by hand. Then, an amount of warm sulphited tap water at 50 °C was mixed with resulted kernels for 48 h and dried by tray drier at 23 °C. The dried kernels were milled using a laboratory electronic mill (BRAWN, Model 2001 DL, Germany) to get soft powder. After that, the resulted fine powder was kept in polyethylene bags at 4 °C until further analysis.

Extraction of Mango seeds kernel protein

The extraction process of Mango seeds kernel protein (MSKP) was performed depending on reported method by (SEGURA-CAMPOS et al., 2013) with minor modification. Briefly, an amount of MSKP was suspended in phosphate

buffer solution (pH 8) and subsequently incubated at tested parameters (Time, temperature and buffer – to-sample ratio) to investigate the optimization technique. The resulted solution was centrifuged for 1 h at 3000 rpm and 10°C and the supernatant was obtained. The supernatant pH was adjusted to 4 by using 2N HCl and the solution was mixed for 20 min and the centrifuging step was repeated on the same conditions. Finally, the precipitated was collected and freeze dried on -50°C to afford MSKP.

Determination of protein content

The protein concentration of MSKP was calculated by Bradford method (BRADFORD 1976) using UV/VIS spectrophotometer 722 (Shanghai Jinghua Science & Technology Instruments Co., Ltd, China) at the wavelength of 595 nm and the standard curve was generated by using Bovine Serum Albumin (BSA).

Response surface experimental design

The experimental design of RSM was conducted based on the results of single – factor experiments. The single factor experiments were performed by studying the effects of the following parameters (temperature 30 – 60 °C, buffer-to-sample 20-50 mL/g and time 40 -160 min) on protein yield to generate suitable indicators for the subsequent Box–Behnken design (BBD). The BBD was done with the three independent variables at three levels based on results of single factor experiments. As indicated in (Table 1), the complete BBD was composed of 17 suggested runs carried out randomly, and the experimental design with five central points was conducted to indicate the frequency of the method. The following second order polynomial equation was utilized to investigate the linkage between the independent variables and the responses in BBD.

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

where Y is assessed response, βo indicates the intercept, n belongs to the number of factors investigated, βi, βii and βij showing the linear (main effect), quadratic and cross product model coefficients, respectively; Xi and Xj are the points of the independent parameters.

SDS-PAGE analysis of MSKP

After applying optimized conditions, the resulted MSKP was transferred for electrophoresis technique on a 10% polyacrylamide SDS gel by using Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). After the application of the washing gel, the staining process was performed by using staining solution composed of glacial acetic acid, Coomassie blue, methanol with percentages of 10%, 0.1% and 20%, respectively for 3 h followed by additional distaining using solution of 50% methanol and 10% glacial acetic acid. The migration procedure was done under strength of 20 mA per plate using a generator (PS 500-2 Sigma-Aldrich). The image was taken using a digital camera.

Anti-inflammatory effect of MSKP. Determination the cell viability of MSKP on RAW 264.7 cells

In the current study, MSKP optimized with the highest yield was utilized to test the anti-inflammatory activity in the following experiments. The MTT-colorimetric method was used as indicated by method of LU et al. (2011). Briefly, a density of 1×10^5 cells/mL from murine cells were cultured under modified atmosphere (37°C and 5% CO_2) in DMEM consisted of 1% (v/v) antibiotic solution contained streptomycin (100 IU/mL) and penicillin (100 IU/mL) with 10% (v/v) newborn calf serum. A 96-well culture plate was filled with amount of (100 µL/well) from suspension of murine cells and incubated in incubator for 12 h. A volume of 100 µL of new medium containing various final concentrations of MSKP was added to culture medium. DMEM alone was represented as a blank control. Then, the plates were incubated for extra 24 h and the suspension was replaced by 50 µL of MTT in concentration of 1.0 mg/mL, and the plates were incubated for additional 4 h. After discarding of MTT, an amount of 150 µL from DMSO was pipetted to well. Then, the absorbance was recorded at 570 mm by microplate Reader, and the percentage of the viable cells were determined by the subsequent equation.

$$\text{Cell viability} = \frac{\text{Ab}_{\text{sample}}}{\text{Ab}_{\text{control}}} * 100 \qquad (2)$$

Assay of nitrite oxide

According to our previous procedure (ABDIN et al., 2020), after incubation of RAW 264.7 cells and different concentrations of MSKP in 96-well plate for 150 min, the LPS (1µg/mL) was transferred to each well and the plates were transferred to incubator for further 24 h. The attendance of nitrite (µmol/L) was calculated using the Griess reagent of ELISA kit which recommended by suggested protocols.

Assay of cytokine production

The ELISA kit (Invitrogen) protocol was utilized to determine the amount of tested cytokine (Pg/mL). The preparation method to apply the protocol was done depending on culture of cells into 96-well plate in density of 2×10^5 cells/well and incubation overnight. Then, the cells were mixed with MSKP for 60 min followed by the addition of LPS (1 µg/mL) for one day to induce inflammation. After collecting the supernatant, the assay was performed typically according to guidelines of protocol manufacturing.

Western blot analysis

The western blot method was performed according to (WAN et al., 2019) with suitable modification. Briefly, after treating cells with MSKP for interval times, the cells were centrifuged and the supernatant of each sample with equal protein content was electrophorized with 10% (SDS-PAGE). After finishing running process, the protein on gel was transferred to polyvinylidene difluoride membranes by Semi-Dry Electrophoretic Transfer Cell. Then, the gel membranes were exposed to solution of 5% skim milk in the presence of tris buffered saline Tween 20 (TBST) for 120 min. After that, the blocked membranes were incubated with specific primary antibodies. The next day, the membranes were washed five times with TBST solution and exposed to secondary antibody type (HRP-conjugated goat anti-rabbit secondary antibody (1:50000)) for 120 min. The enhanced chemiluminescence reagents (ECL, Amersham Biosciences, UK) was utilized to visual each band and the Tanon 6000 imaging system was used to investigate the resulted bands.

Statistical analysis

The data of single factors experiment, cell viability and anti-inflammatory activity were statistically analyzed by one-way analysis of variance (ANOVA) by SPSS software. The design expert software was exploited to perform experimental design and analysis the data of RSM.

Results and discussion

Single factor investigation

Effect of temperature on protein yield

The extraction temperature had cleared effect on the protein content of mango seed kernel (MSK) as illustrated from Figure 1A. The percentage of mango seed kernel protein (MSKP) was significantly ($p < 0.05$) increased by heating during the extraction process up to 50 °C. After that, significant ($p < 0.05$) decrease was observed in MSKP. The raising of temperature more than 50 °C could lead to occurrence of denaturation and eventually reduced the protein solubility (YU et al., 2007). Additionally, the raising of temperature could stimulate the interaction between protein and other components inside seeds to form protein complexes in which decreased the solubility of the protein (SIOW & GAN 2014). Hence, the extraction temperature was selected within the ranges of 45 to 55 °C for the subsequent optimization study depending on steepest ascent techniques.

Effect of percentage of buffer-to-sample

As indicated in Figure 1 B, the buffer -to-sample ratios from 20 to 50 mL/g were tested. At ratio of 40 mL/g, the protein yield reached to the maximum value significantly. However, by increasing the ratio to 50 mL/g there was non-significant ($p < 0.05$) differences. The extractability of protein depends on the interactions between protein-protein hydrophobic surface and protein solvent hydrophilic surface (KRISTINSSON & HULTIN 2004). Additionally, with the increase of solvent/solid ratio, the contact area between solute and solvent is also improved to stimulate the solubility of bioactive components within the plants cells (ABDIN et al., 2019).The main objective of the study was to maximize the MSKP yield and as a result the ratios before 35 mL/g were discarded and the ratios in ranges of 35 to 50 mL/g were selected for the subsequent design.

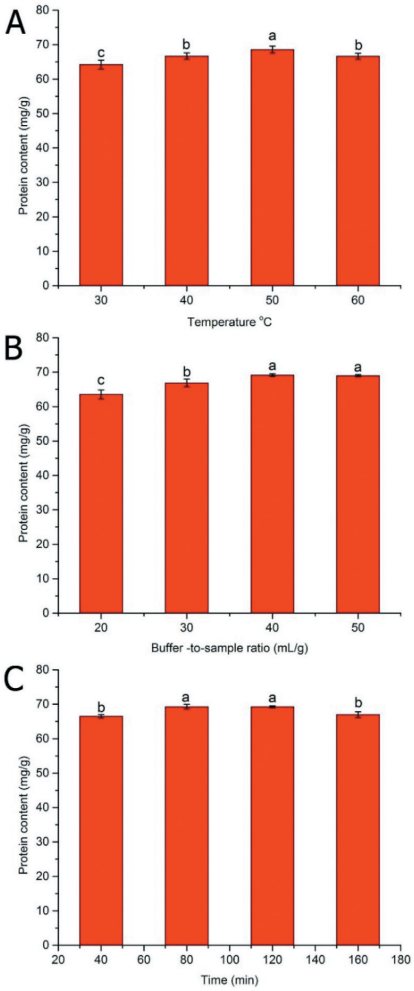

Fig. 1. Effects of temperature (A), buffer -to- sample ratio (B) and time (c) on protein content.

Bars with different letters are significantly different ($P \leq 0.05$).

Effect of time

As shown in Figure 1C, the extracted protein content was significantly ($p < 0.05$) increased from 40 to 80 min. Further extraction to 120 min did not illustrate any significant ($p > 0.05$) changes in yield. Furthermore, the increasing of extraction time more than 120 min caused significant ($P < 0.05$) decrease in MSKP content. The obtained results suggested that extended extraction time did not enhance MSKP extraction efficiency. Thus, the extraction time within the range of 60 to 120 min was selected for further optimization study. The selected extraction time was in the same trend of previously work (SIOW & GAN 2014) who extracted bioactive protein from cumin seed.

Designing BBD and response values

As observed from Table 1, the influences of three extraction parameters: temperature (X_1), buffer – to - sample ratio (X_2) and time (X_3), on the MSKP yield were investigated using a BBD design. The ranges of each factor (maximum and minimum) were selected depending on the previous results of single factor experiment. The design suggested 17 runs with 5 runs in central point at three levels (minimum -1, central 0 and maximum +1). The protein yield seemed to be varied depending on the extraction conditions given. It could be noticed that the maximum protein yield (45.5 mg/g) was obtained under the experimental conditions of X_1 55 °C, X_2 42.5 mL/g and X_3 120 min. The BBD technique was an ideal choice to extract protein from plant seeds with high functionality (KUMAR et al., 2021).

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	55	42.5	60	65.05
2	50	42.5	90	66.55
3	50	42.5	90	66.67
4	45	42.5	120	63.44
5	50	50	120	65.59
6	55	35	90	64.66
7	50	42.5	90	65.62
8	50	42.5	90	66.17
9	45	50	90	63.96
10	50	35	60	61.42
11	45	35	90	61.65
12	50	50	60	66.49
13	55	42.5	120	68.97
14	55	50	90	65.59
15	45	42.5	60	62.92
16	50	35	120	65.00
17	50	42.5	90	66.99

Analysis of ANOVA and model fitting.

The analysis of ANOVA was performed to investigate the significance of model's coefficient. In the current model, the *F*-value (13.42) of protein content was significant and the *P*-value of the lack of fit (0.1785) was not significant relative to the pure errors. The non-significant values of lack of fit implied that the current model equation was adequate for predicting the extraction yield of MSKP under any combination of temperature, buffer-to-sample and time (WANG et al., 2016). Moreover, the determined variables would be more significant if the absolute *F*-value becomes greater and the *P*-value becomes smaller (ATKINSON & DONEV, 1992) which are in agreement with obtained data in Table (2) .

Table 2. ANOVA for response surface quadratic mode

Source	Sum of Square	Mean square	*F-value*	*P-value*	Signif.
Protein content					
Model	58.04	6.45	13.42	0.0012	Signif.
X_1-Temperature	18.91	18.91	39.35	0.0004	
X_2-Buffer—to sample	9.9	9.9	20.6	0.0027	
X_3-Time	6.34	6.34	13.19	0.0084	
X_1X_2	0.48	0.48	0.99	0.3527	
X_1X_3	2.89	2.89	6.01	0.044	
X_2X_3	5.02	5.02	10.44	0.0144	
Residual	3.36	0.48			
Lack-of-fit	2.26	0.75	0.98	0.1785	Not signif.
Pure error	1.1	0.28			
Adj R-Squared	0.8748				
R-Squared	0.9452				
C.V.	1.06				

p values lower than 0.05 are statistically significant

yield value was obtained by the subsequent euation:

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

The fitted quadratic model for MKSP yield in coded variables was generated in Eq. (3). The results of current model emphasize that, the largest effect on MSKP yield was in linear term of temperature X_1, buffer-to-sample X_2, time X_3, quadratic term and collaboration between temperature X_1 & time X_3, buffer-to-sample X_2 & time X_3, X_1^2 and X_2^2. The values of the coefficient of (R^2) and the adjusted coefficient of (adj.R^2) of the predicted model for MSKP yield was convergent, which suggested that there was a high degree of correlation between the observed and predicted values.

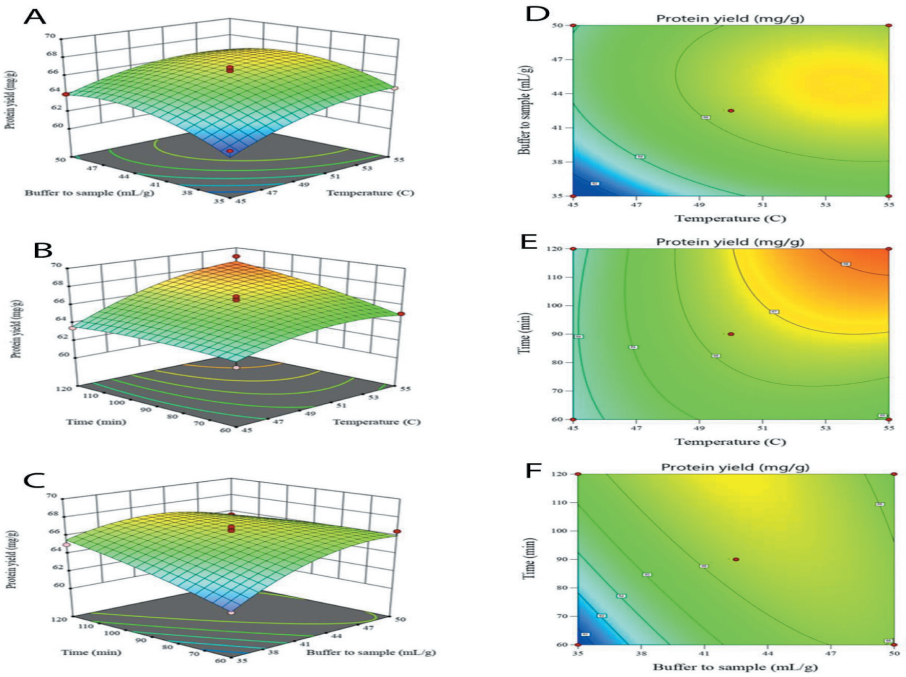

Figure 2. Response surface plots (A-C) and contour plots (D-F) showing effect of extraction variables on protein content.

Analysis of response surfaces

The three-dimensional shape in (Figure 2 A-C) and the two-dimensional contour plots in (Figure 2 D-F) are generated to describe the interaction effects between tested variables and protein content. Circular contour plot expresses that the interactions between the tested variables are non-significant, while the elliptical contour shape suggests that the corresponding variables are significant (MURALIDHAR et al., 2001).

Actually, the elliptical shape in (Figure 2 D-F) suggests that the co-operation between the tested factors is significant. The results are consistent with the results of ANOVA in (Table 2), which indicated that interactions between the variable factors (Temperature, buffer-to-sample and time) were significant and have great effect on MSKP yield.

It was summarized that the optimal settings for the extraction of MSKP while using in range values of tested parameters and maximized value of protein content (68.336%), were extraction temperature 54.53 °C, buffer-to-sample values 41.79 mL/g and extraction time 120 min with desirability value of 0.471%. The additional verification of the practical experiment was conducted based on the optimized predicted values of tested parameters with slight modification in optimized values to facilitate the practical experiment to be temperature 54.5 °C, buffer-to-sample values 42 mL/g and time 120 min. As a result, a practical extraction yield of 68.72 ± 0.52% (n = 3) was attained under the optimal extraction conditions, which is in the same trend with the predict value.

SDS-Page characterization of MSKP

The SDS-page technique was utilized based on protein electrophoretic mobility to notice protein bands as indicated in (Figure 3). The electrophoresis patterns of MSKP revealed that the MW was between 6 to 98 kDa. Depend-

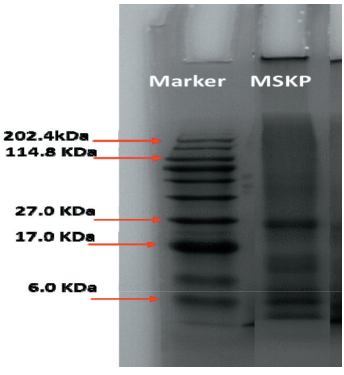

Figure 3. SDS-PAGE profile of MSKP compared to standard protein marker.

ing on the globulin considers the principal storage protein in seeds (ORRUNO & MORGAN, 2007) , the lowest bands in MW may explain attendance of globulin with type (2S). Additionally, another principal protein band of 27.6 kDa explained the presence of lectins , which was detected widely in seeds (NASI et al., 2009). The obtained results were in the same line of Siow et al. 2014 (SIOW & GAN, 2014).

Anti-inflammatory effect of MSKP

The anti-inflammatory effect of MSKP in LPS induced RAW264.7 cells was determined through five determinations; cell viability, production of IL-1β, IL-6, TNF-α and nitrite oxide (NO). Firstly, the cytotoxic concentration of MSKP was observed using MTT assay by calculation of cell viability. As shown in (Figure 4), MSKP showed no cytotoxic influence against RAW264.7 macrophages in all selected concentrations except of 900 µg/mL. Thus, the tested concentrations of 300, 500 and 700 µg/mL were utilized for the subsequent determinations.

As shown in (Figure 5 A – C), the attendance of different concentrations from MSKP in the cultured media caused re-

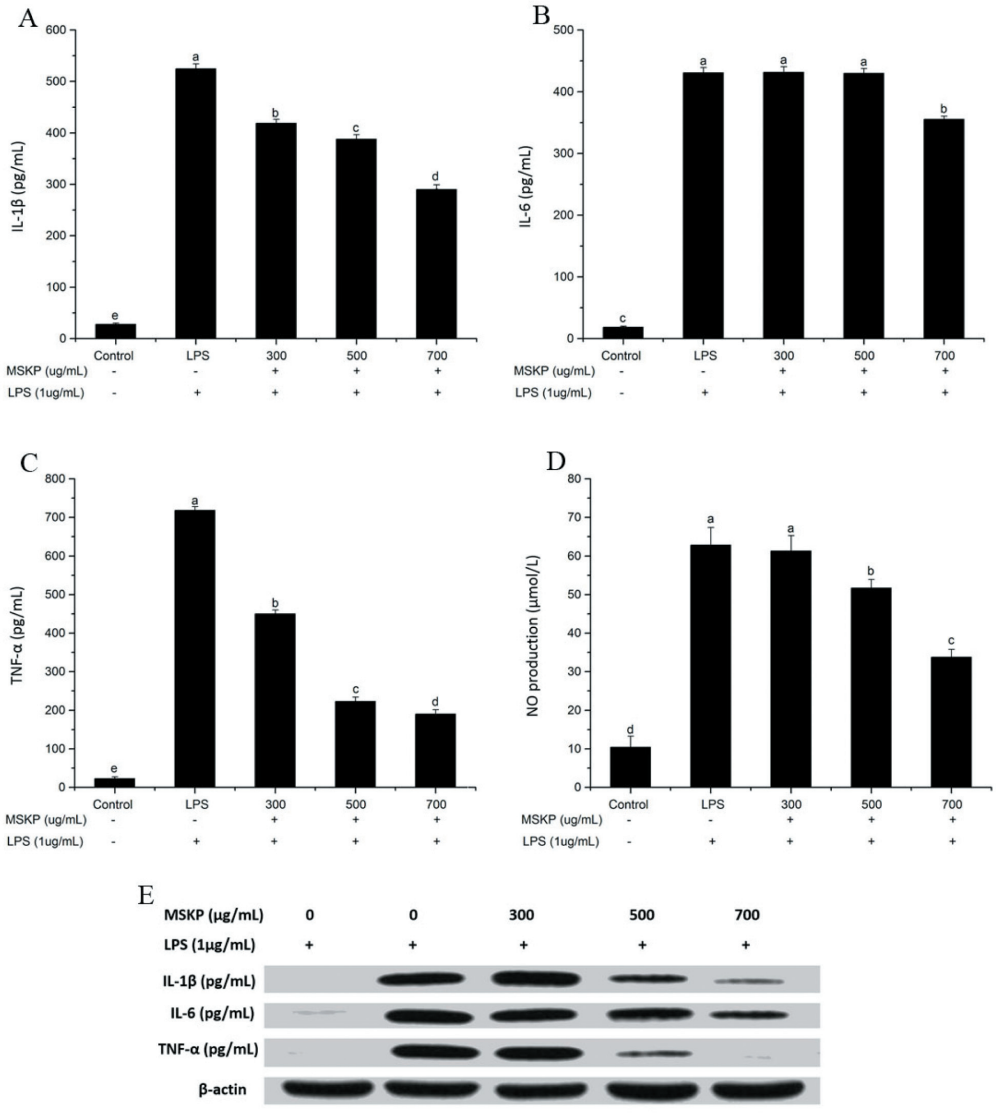

Figure 4. Effects of MSKP concentrations on cell viability of RAW264.7.

duction of proinflammatory mediators activity (IL-1β, IL-6 and TNF-α) in a dose-dependent style compared to those of LPS treatment alone. The current results revealed that MSKP may be beneficial for retarding inflammatory disorder. Similar results by the inhibition of the expression of nuclear factor κB (NF-κB) or another transcription factor, as well as the expression of genes for IL-1β, IL-6, and TNF-α was observed by attendance of *Salvia hispanica* L. seeds proteins inside culture media (CHAN-ZAPATA et al., 2019). Additionally, the extracted protein from seeds of *Myristica fragrans* showed inhibition of the production of interleukin (IL)-6, IL-10, interferon inducible protein-10 and monocyte chemotactic protein (MCP)-1, MCP-3 of RAW 264.7 mouse macrophages (LEE & PARK, 2011).

After treatment with MSKP followed stimulation of LPS, NO concentration in the cultured medium was investigated. As shown in (Figure 5 D), NO concentration by LPS treatment was significantly decreased by pretreatment with MSKP in a dose-dependent manner and it reached to the lowest value by using concentration of 700 µg/mL of MSKP in cell cultured media. NO is one of cellular mediators that is produced at inflammatory spots upon motivation by LPS and the higher concentrations of NO in response to inflammatory inducements is mediated by iNOS, leading to different inflammation and entotoxemia (MORO et al., 2012). Thus, the inhibition of NO is thought to be satisfied technique for retarding of inflammatory reaction. The iNOS and COX-2 might be affected by the extracted proteins from seeds through downregulation of the expressions of mRNA and protein and as a result lead to reduction of NO production (MOON et al., 2019).

Further, Western blot experiment with phosphorylated forms of antibodies following stimulation with LPS was performed to prove the effect of MSKP on protein levels of tested inflammatory cytokines. Electrophoresis and the subsequent western blot analysis are essential to investigate biochemical changes in cells and tissues exposed to tested components (HIRANO, 2012). In line with the results obtained from anti-inflammatory assays, clear decrescent in protein expression levels of TNF-α, IL-1β, and IL-6 were observed and it followed a concentration dependent pattern (Figure 5 E) when cells were treated with MSKP compared to LPS treatment group alone. At the highest concentration of MSKP examined (700 µg/mL) these protein expressions were almost totally inhibited.

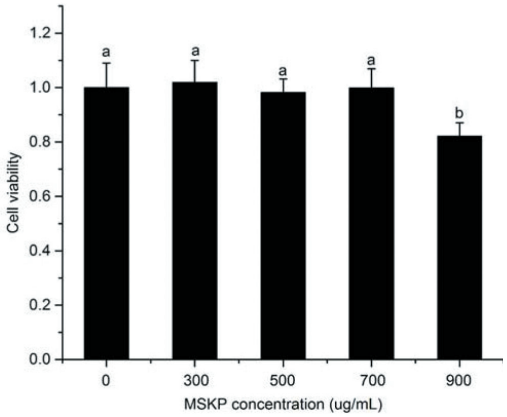

Figure 5. Effects of MSKP concentrations on production of IL-1β (A), IL-6 (B), TNF-α (C), NO (D) and Western blot analysis (E).

Conclusion

In conclusion, the current investigation proved that the single factors experiment had a significant influence on the yield of MSKP. The application of RSM depending on single factors results was successfully verified in the optimization technique of MSKP and expressed on the most efficient extraction conditions corresponding to extraction temperature 54.53 °C, buffer-to-sample values 41.79 mL/g and extraction time 120 min with desirability value of 0.471%. The SDS-PAGE results proved the findings of functional protein. The resulted MSKP showed anti-inflammatory effect against NO and proinflammatory cytokines. Further study of the chemical and biological properties of MSKP is recommended.

Acknowledgments

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Conflict of interest

The authors declared that they have no conflict of interest

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