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ORIGINAL PAPER

Sainfoin (*Onobrychis viciifolia* L.) protein isolate as a new source of alternative plant-based protein: cytotoxicity, immunoreactivity, nutritional and functional properties

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Abstract

The objective of this research was to develop an alternative plant-based protein isolate using sainfoin (*Onobrychis viciifolia* L.) seeds. The extraction process was optimized using response surface methodology (RSM) based on the Box-Behnken Design, which examined the effects of key parameters: solvent/solid ratio (10–50 mL/g), pH (8–11), temperature (20–50 °C), and extraction time (30–120 min), aiming to maximize protein yield. The optimal extraction conditions identified were a solvent/solid ratio of 49.96 mL/g, pH of 10.99, temperature of 20 °C, and a duration of 38.55 min, achieving a protein yield of 56.36%. Additionally, the amino acid composition, cytotoxicity, immunoreactivity, and functional properties of the sainfoin seed protein isolate (SPI) were evaluated. SPI exhibited a high crude protein content of 91.44%, with arginine being the most abundant amino acid at 158.20 mg/g. The protein isolate comprised a remarkable value of 50.26% essential amino acids. Additionally, SPI demonstrated desirable functional properties, including solubility of 53.95% at neutral pH, water holding capacity of 2.36 g/g, and oil binding capacity of 4.68 g/g. Its emulsifying performance was notable, with emulsion activity and stability values of 66.67% and 77.50%, respectively. Moreover, in vitro cell culture studies demonstrated that sainfoin seed protein exhibited no adverse effects on cellular toxicity or immunoreactivity. This study highlights the potential of SPI as a novel, high-quality plant protein source with promising nutritional and functional properties and demonstrates its potential as a functional ingredient in the formulation of plant-based foods, meat analogs, and dietary supplements.

Keywords Sainfoin seed · Alternative plant protein · Amino acid composition · Toxicity · *In vitro* digestion simulation

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Abbreviations

SPI	Sainfoin seed protein isolates
RSM	Response surface methodology
CV	Coefficient of variation
PS	Protein solubility
WHC	Water holding capacity
OBC	Oil binding capacity
EA	Emulsion activity
ES	Emulsion stability
FC	Foaming capacity
FS	Foam stability
LGC	Least gelation concentration
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
LPS	Lipopolysaccharide
TNF-α	Tumor Necrosis Factor- Alpha

IL-6	Interleukin-6
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate buffered saline

Introduction

As the world population rises, it is critical to identify sustainable protein sources that not only offer strong nutritional and functional profiles but are also safe for consumption. Ideal protein alternatives should be non-allergenic, non-toxic, highly digestible, and bioavailable. Sources like legumes, grains, and seeds, as well as newer options such as insect protein and lab-grown meats, provide diverse amino acids while helping to reduce environmental footprints and the demands on conventional animal farming. With a history of extensive cultivation, economic production, and global availability, plant-based proteins stand out as an environmentally sustainable choice [1].

Sainfoin, a perennial legume traditionally used as green fodder for livestock, has recently attracted attention due to its drought resistance and its rich content of condensed tannins and other phenolic compounds. These compounds provide numerous health and environmental benefits, including the prevention of bloat in cattle, enhanced protein digestion, reduced methane and nitrogen emissions, control over nematode parasitism in cattle and sheep, and anthelmintic properties [2–5].

Sainfoin is conventionally utilized in its green form exclusively as animal feed. Nevertheless, if the plant is left unharvested in the field, its seeds present an uninvestigated potential for human consumption [6]. Very few studies have examined the nutritional value of green sainfoin [3, 5, 7, 8], and almost none have explored the food potential of its seeds [9]. In a recent study, it was demonstrated that sainfoin seeds contain approximately 42% protein, as well as notable levels of dietary fiber and fat. Their lack of cellular toxicity and high phenolic content highlight their potential as a valuable plant-based protein source [6, 9].

A variety of techniques are employed to produce protein concentrates and isolates from plant sources, with alkaline extraction followed by isoelectric precipitation being the most widely adopted due to its simplicity and ability to yield high-purity proteins [10]. However, the efficiency and quality of the final product are highly dependent on key extraction parameters, including the solvent/solid ratio, pH, temperature, and extraction time. These factors can significantly influence not only the protein yield and purity but also the structural and functional characteristics of the isolates. Therefore, optimizing these extraction parameters is a crucial step in the process [11].

This study aims to optimize the extraction conditions for sainfoin seed protein using a Box-Behnken Design and to evaluate its potential as an alternative plant-based protein source. The evaluation includes analyzing its proximate and amino acid composition, as well as its functional properties, such as protein solubility (PS), water holding capacity (WHC) and oil binding capacity (WHC), emulsion activity (EA) and emulsion stability (ES), foaming capacity (FC) and foam stability (FS), and least gelation concentration (LGC). Additionally, in vitro simulated digestion was employed to assess the protein profile, along with in vitro cytotoxicity and immunoreactivity, both before and after digestion.

Materials and methods

Material and sample preparation

Sainfoin (*Onobrychis viciifolia* L.) seeds of the Lütfi Bey variety were sourced from local markets in Çanakkale, Türkiye. The seed coat and cotyledons were separated in two stages using a laboratory rice milling system (Yaşar Makina, CRM-1252T, Türkiye). Afterwards, the seeds were ground with a grinder (Kiwi, KSGP-4812, China) and sieved using a 1 mm sieve. The proximate composition of the seed flour has been previously documented by Andaç et al. [9]. The sainfoin seed flour was then defatted with hexane (1:4, w/v) using an orbital shaker (Jeio Tech, open-air shaker OS-4000, Korea) for 1 h at 25 °C, and the solvent was recovered by filtration. After repeating this process three times, the seed flour was left to dry overnight at room temperature and stored at 4 °C until the extraction process. The protein, ash, and fat content of defatted sainfoin flour were determined as 48.96 ± 0.14%, 4.12 ± 0.09%, and 2.52 ± 0.04%, respectively, based on dry weight.

Preparation of Sainfoin seed protein isolates

Protein extraction and optimization

In brief, defatted sainfoin seed flour was dispersed in deionized water (10, 30, 50 mL/g), and the pH (8, 9.5, 11) was adjusted using 2 M NaOH. The dispersions were shaken in a water bath (Nüve, ST 30, Türkiye) at 125 rpm at specific temperatures (20, 35, 50 °C) and times (30, 75, 120 min), followed by centrifugation (NF 800R, Nüve, Ankara, Turkey) at 9,400 x *g* at 4 °C for 10 min. The supernatant was filtered, and the soluble protein content was determined using the Lowry assay [12].

RSM was employed to optimize alkaline extraction conditions, including solvent/solid ratio (X_1), pH (X_2), temperature (X_3), and time (X_4), and experimental conditions were

constructed using a Box-Behnken Design to determine the highest protein yield. According to Box-Behnken Design, a set of 29 experimental runs, which consisted of 24 factorial points and 5 repetitions at the center point, were performed (Table 1). Protein extraction yield was selected as the response variable (Y) and calculated following the equation (Eq. 1):

$$Protein\ Extraction\ Yield\ (\%) = \frac{Weight\ of\ \ protein\ in\ extract}{Weight\ of\ protein\ in\ defatted\ sainfoin\ flour} \text{x}\ 100 \quad (1)$$

To estimate the optimum point and correlate the responses with independent variables, a quadratic model (Eq. 2) was used to fit the experimental data as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_{i=1}^{k-1} \cdot \sum_{\substack{j=2 \\ i<j}}^{k} \beta_{ij} X_i X_j \quad (2)$$

where Y represents the dependent variables, affected by independent variables Xi and Xj (i=1–4, j=1–4, where $i \neq j$); β_0, β_i, β_{ii}, and β_{ij} are constant, linear, quadratic, and interaction regression terms, respectively. Also, k represents the number of variables within the model.

Isoelectric precipitation

The isoelectric point of sainfoin proteins was determined by adjusting the pH of the extract obtained under optimum conditions (as determined in 3.1) to a range of 3.5 to 6.5 using 2 M HCl. After adjusting the pH to 3.5, 4, 4.5, 5, 5.5, 6, and 6.5, the extracts were centrifuged at 9,400 x g for

Table 1 Experimental design of independent variables and the response of protein yield for protein extraction from Sainfoin seed using RSM

Run	Independent variables								Response
	Coded variables				Uncoded variables				
	X_1	X_2	X_3	X_4	Solvent/solid ratio (mL/g)	pH	Temperature (°C)	Time (min)	Protein yield (%)
1	-1	0	0	+1	10	9.5	35	120	40.44
2	0	0	0	0	30	9.5	35	75	46.42
3	0	0	0	0	30	9.5	35	75	46.05
4	+1	0	0	+1	50	9.5	35	120	53.96
5	0	+1	0	-1	30	11	35	30	48.57
6	0	0	0	0	30	9.5	35	75	45.17
7	0	0	-1	-1	30	9.5	20	30	44.95
8	+1	0	+1	0	50	9.5	50	75	51.64
9	0	-1	+1	0	30	8	50	75	46.36
10	0	-1	0	-1	30	8	35	30	40.71
11	0	+1	+1	0	30	11	50	75	55.03
12	-1	+1	0	0	10	11	35	75	40.24
13	0	0	0	0	30	9.5	35	75	47.23
14	+1	0	-1	0	50	9.5	20	75	54.33
15	0	0	+1	+1	30	9.5	50	120	53.58
16	0	+1	-1	0	30	11	20	75	46.96
17	0	0	0	0	30	9.5	35	75	47.28
18	-1	0	0	-1	10	9.5	35	30	37.21
19	0	0	-1	+1	30	9.5	20	120	49.56
20	-1	0	+1	0	10	9.5	50	75	45.43
21	-1	-1	0	0	10	8	35	75	36.53
22	-1	0	-1	0	10	9.5	20	75	34.27
23	+1	+1	0	0	50	11	35	75	54.76
24	+1	0	0	-1	50	9.5	35	30	45.29
25	0	-1	0	+1	30	8	35	120	44.50
26	+1	-1	0	0	50	8	35	75	42.43
27	0	0	+1	-1	30	9.5	50	30	45.97
28	0	+1	0	+1	30	11	35	120	47.87
29	0	-1	-1	0	30	8	20	75	46.03

*X_1: solvent/solid ratio, X_2: pH, X_3: temperature, X_4: time

5 min, and the soluble protein content in the supernatants was measured using the Lowry assay [12]. The pH at which the soluble protein content was lowest was identified as the isoelectric point of the sainfoin protein. The protein in the precipitate was calculated by subtracting the protein content in the supernatant from the protein content of the protein extract obtained under optimum conditions.

Drying

The precipitated protein was dispersed in deionized water at a ratio of (1:4, w/w) and neutralized to pH 7 using 0.2 M NaOH. Firstly, the protein solution was placed into petri dishes and kept at -18 °C for 12 h. Then, it was dried with a freeze-dryer (Teknosem, TRST 4/4 DS, Türkiye) under 0.0033 mbar vacuum at -90.6 °C for 48 h. The lyophilized SPI was stored at -18 °C until analysis.

Proximate composition

The moisture, ash, and fat contents of defatted sainfoin flour and the protein isolates were determined using official AACC methods 44–15 A, 08 − 01, and 30 − 25, respectively [13]. The total crude protein contents of the samples were quantified by the Kjeldahl method according to AACC method 46 − 12 [13].

Amino acid composition

A total of 40 mg of the sample was mixed with 4 mL of 6 N HCl and hydrolyzed by standing at 110 °C for 24 h to determine the amino acid composition. The composition was analyzed using an LC/APCI-MS system (Agilent, 1100 HPLC, Germany). A 2 µL sample was injected into a C18 Phenomenex column (250 mm×4.6 µm×3 µm), connected to an Agilent 6120 quadrupole in SIM positive mode (Agilent Technologies, Germany). The peak areas were compared to an amino acid standard mix (Ref# NCI0180. 20,088, Thermo ScientificPierce, USA) and quantified using Agilent MassHunter Qualitative software. Results were expressed as mg of amino acid per g of sample protein [14].

Functional properties

Protein solubility

PS was assessed following the method of Mutlu and Korkmaz [15] across a pH range of 2.0 to 12.0. In brief, 0.2 g of protein was dispersed in 20 mL of deionized water, and the pH was adjusted using 1 M HCl or NaOH. The protein dispersion was mixed using an orbital shaker at 200 rpm for 30 min, followed by centrifugation (NF 800R, Nüve, Ankara, Turkey) at 6,600 x *g* for 15 min. The protein content in the supernatant was determined using the Lowry assay [12], and PS was calculated using the following equation (Eq. 2):

$$\text{PS}\ (\%) = \frac{Protein\ content\ of\ the\ supernatant}{Total\ protein\ content\ of\ the\ protein\ sample} \text{ x } 100 \tag{3}$$

Water holding capacity and oil binding capacity

WHC was analyzed by dispersing 0.25 g of the sample in 5 mL of distilled water in a pre-weighed centrifuge tube. The dispersion was vortexed for 5 min, followed by centrifugation at 5,000 x *g* for 10 min. After discarding the supernatant, the residue was weighed. For OBC analysis, sunflower oil was used in place of distilled water, with all other steps remaining the same [11]. WHC and OBC were calculated according to the following equation (Eq. 3):

$$\text{WHC or OBC}\,(\text{g/g}) = \frac{Final\ weight\ of\ centrifuge\ tube\ and\ residue - (weight\ of\ sample + weight\ of\ centrifuge\ tube)}{Weight\ of\ sample} \tag{4}$$

Emulsion properties

EA and ES were assessed following the method of Özdemir et al. [16]. The emulsion was prepared by mixing 10 mL of protein dispersion (5% w/v) with 10 mL of sunflower oil using a homogenizer (Witeg, HG-15D, Germany) at 14,000 x *g* for 30 s. The emulsion was then centrifuged at 1,200 x *g* for 5 min, and EA was calculated according to the following equation (Eq. 4):

$$\text{EA}\ (\%) = \frac{Volume\ of\ emulsion\ layer\ (\text{mL})}{Total\ volume\ (\text{mL})} \text{ x } 100 \tag{5}$$

Subsequently, the emulsion was kept in a water bath at 80 °C for 30 min and then re-centrifuged at 1,200 x *g* for 5 min. ES was determined by the following equation (Eq. 5):

$$\text{ES}\ (\%) = \frac{Volume\ of\ remaining\ emulsion\ layer\ (\text{mL})}{Volume\ of\ the\ original\ emulsified\ layer\ (\text{mL})} \text{x } 100 \tag{6}$$

Foaming properties

FC and FS were measured using the method previously developed by Kasapoğlu et al. [17]. In brief, 20 mL of a 1% (w/v) protein dispersion was mixed using a homogenizer (Witeg, HG-15D, Germany) at 16,000 x *g* for 2 min. The volumes were recorded before and after homogenization, as well as after 30 min of standing. FC and FS were calculated using the following equations (Eqs. 6,7):

$$\mathrm{FC}\ (\%) = \frac{Volume\ after\ whipping\,(\mathrm{mL}) - Volume\ before\ whipping\,(\mathrm{mL})}{Volume\ before\ whipping\,(\mathrm{mL})}\ \mathrm{x}\ 100 \tag{7}$$

$$\mathrm{FS}\ (\%) = \frac{Volume\ of\ foam\ after\ 30\ min\,(\mathrm{mL})}{Initial\ foam\ volume\ (\mathrm{mL})}\ \mathrm{x}\ 100 \tag{8}$$

Least gelation concentration

The method of Sethi et al. [18] was used to determine the LGC. In brief, protein dispersions were prepared at concentrations ranging from 2% to 20% (w/v) with two-unit intervals. These dispersions were heated in a water bath at 100 °C for 1 h and then stored at 4 °C for 2 h. The concentration at which the sample did not fall or exhibit fluidity when the sample tubes were inverted was recorded as the LGC.

In vitro digestion

The in vitro gastrointestinal digestion method was adapted from Minekus et al. [19] with slight modifications. Simulated oral, gastric, and intestinal digestion steps were carried out using salivary α-amylase (75 U/mL), pepsin (2000 U/mL), and pancreatin (100 U/mL trypsin activity), respectively. The protein sample was loaded at 5 mg/mL, and the pH values were adjusted to 7.0 (oral), 3.0 (gastric), and 7.0 (intestinal) for each phase using 1 M HCl or NaOH. A blank sample (the same amount of distilled water instead of samples) was also subjected to the same conditions to eliminate any interference from fluids used in the simulated in vitro digestion. Samples obtained after gastrointestinal digestion were immediately placed into an ice bath for the inactivation of digestion enzymes and then centrifuged at 4 °C and 23,000 x *g* for 5 min. The supernatant was stored at -20 °C until further analysis. The collected supernatant was subsequently used in SDS-PAGE, cytotoxicity, and immunoreactivity analyses.

SDS-Gel electrophoresis

The protein profile of SPI before and after in vitro digestion was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) separating and 4% stacking gel according to the Laemmli method with slight modifications [20, 21]. 30% acrylamide and bis-acrylamide premixed solution (Bio-Rad 1610158) was used while preparing gels. The sainfoin seed flour sample (Fig. 4, c) used for gel electrophoresis was prepared as described in Sect. 2.1 and directly loaded onto the gel. SPI before and after digestion (Fig. 4, a, b) samples used for gel electrophoresis were obtained following the extraction, isoelectric precipitation, and freeze-drying procedures described in Sect. 2.2. All samples were dissolved in sterile water to a final concentration of 2.5 mg/mL (75 µg in 30 µL) for each sample group. Samples were mixed with 6X loading buffer (0.5 M Tris–HCl, pH 6.8; 2% (v/v) SDS; 2.5% (v/v) glycerol; 0.2% (v/v) bromophenol blue; 0.5% (v/v) 2-mercaptoethanol) and heated at 95 °C for 4 min (SimpliAmp Thermal Cycler, The Applied Biosystems) to load 30 µl each well. Digested and non-digested samples were loaded as two replicates. Running was performed at 80 V for separating and 100 V for stacking gels using a polyacrylamide gel electrophoresis system (Mini-Protean Tetra cell, Bio-Rad). After running, the gel was fixed in a 40% (v/v) methanol and 10% (v/v) acetic acid solution for 30 min. The gel was rinsed for 5 min 3 times with distilled water, and the Coomassie G-250 premixed staining solution (Bio-Safe™ 610786) was added and shaken for 1 h. The gel was rinsed for 2 h and stored in water until imaging. Molecular mass was determined by Precision Plus Protein Dual Color Standard 12–102 kDa (Bio-Rad 1610374). Images were obtained from the Molecular Imager® VersaDoc™ MP Imaging Systems.

Protein and peptide toxicity

Cell culture

Rat intestinal epithelium cells (IEC-6, CRL-1592), human colonic epithelium cells (Caco-2, HTB-37), and murine macrophage cells (RAW 264.7, TIB-71) were purchased from American Tissue Culture Collection (ATCC®). IEC-6 cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco™ 11965092) supplemented with 10% Fetal Bovine Serum (FBS, HyClone™ SV30160.03), 1% penicillin-streptomycin solution (Capricorn, PS-B), and 0.1 Unit/mL human insulin (Sigma-Aldrich I9278). Caco-2 cells were maintained in Minimum Essential Medium (MEM, Gibco™ 11095080) with 15%

FBS, 1% penicillin-streptomycin, 1% sodium pyruvate (Capricorn NPY-B), and 1% non-essential amino acids (Capricorn NEAA-B). RAW 264.7 cells were maintained with RPMI1640 Medium (Gibco™ 11875093) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin solution. Caco-2 cells were kept between 25 and 35 passage numbers, and IEC-6 and RAW 264.7 cells were 15–25 passage numbers in all experiments. All cells were maintained in 75 cm^2 flasks at a 37 °C incubator with 5% CO_2.

In vitro cytotoxicity

To assess the cellular toxicity of protein samples and non-toxic concentrations for further assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) assay was applied [21]. Briefly, IEC-6 Caco-2 and RAW 264.7 cells were seeded 2×10^4 cells/well density in 96 well plates and incubated for 24 h to provide optimum attachment. The same amount digested and non-digested SPI samples were diluted from 10 mg/mL stock solutions (prepared in sodium phosphate dibasic buffer, pH 9.0; Sigma-Aldrich S0876) to final concentrations of 125, 250, 500, and 1000 µg/mL in the cell culture medium. The culture medium was buffered at pH 7.4, and the dilution ensured that the final pH remained within the physiological range.100 µL of fresh medium containing different samples was added to each well. Untreated wells were used as a control by adding only fresh medium. After 24 h incubation at 37 °C, cells were washed with Phosphate Buffered Saline (PBS) (Capricorn PBS-1 A), and the medium was changed. Cell morphology was examined under a trinocular inverted microscope (VWR, 89404-462) at 10× magnifications to assess potential cytotoxic effects. Observations focused on changes in cell shape, detachment, granularity after 24 h treatment. Afterward, the MTT (Biomatik A3338) solution was dissolved in PBS 5% (v/v), and 10 µL was added to each well, followed by 4 h incubation at 37 °C. Finally, the supernatant was discharged, and the crystals were dissolved in 100 µL of DMSO (Merck 1029521000) in each well. Absorbance at 570 nm was measured by a microplate reader (ThermoskanGO, Thermo Scientific), and % cell viability was calculated as follows (Eq. 8)

$$\text{Cell viability } (\%) = \frac{absorbance\ of\ the\ cells\ treated\ by\ samples}{absorbance\ of\ untreated\ cells} x\ 100 \quad (9)$$

Table 2 Estimated regression coefficients and ANOVA results of the quadratic model for protein extraction of Sainfoin seed

Parameters	Estimated coefficients	Standard error	df *	*p* value **
Constant	46.43	0.65	1	
Linear				
X_1 - solvent/solid ratio	5.69	0.42	1	< 0.0001
X_2 - pH	3.07	0.42	1	< 0.0001
X_3 - temperature	1.83	0.42	1	0.0007
X_4 - time	2.27	0.42	1	< 0.0001
Interaction				
X_1X_2	2.16	0.73	1	0.0106
X_1X_3	-3.46	0.73	1	0.0003
X_1X_4	1.36	0.73	1	0.0840
X_2X_3	1.93	0.73	1	0.0192
X_2X_4	-1.12	0.73	1	0.1471
X_3X_4	0.75	0.73	1	0.3222
Quadratic				
X_1^2	-2.26	0.57	1	0.0015
X_2^2	-0.58	0.57	1	0.3318
X_3^2	2.44	0.57	1	0.0008
X_4^2	-2.25	0.57	1	0.6664
Model *p* value	< 0.0001			
Lack of fit	0.1206			
R^2	0.96			
Adjusted R^2	0.93			
Predicted R^2	0.81			
CV%	3.17			

* Degrees of freedom

** $p<0.05$ is statistically significant.

Protein and peptide immunoreactivity

Enzyme-Linked immunosorbent assay (ELISA)

Raw 264.7 cells were used to assess immunoreactivity in terms of immune reactions. The cells were seeded in 5×10^5 cell/well 24-well cell culture plates and allowed to adhere overnight. The cells were treated with a starving medium containing 3% FBS to eliminate the effects of FBS on cytokine release [22]. Then, 125 µg/mL of non-digested and digested protein isolates were given to the cells for 12 h. Cells were treated with a medium containing 0.5 µg/mL lipopolysaccharide (LPS) to create inflammatory conditions as a positive control [23]. Medium was collected for each group. Tumor Necrosis Factor- Alpha (TNF-α) and Interleukin-6 (IL-6) cytokine releases were analyzed with enzyme-link immunosorbent assay (ELISA) according to the kit instructions (ElabScience E-EL-M3063, E-EL-M0044).

Statistical analysis

Response Surface Methodology (RSM) was employed to optimize the protein extraction yield from sainfoin seeds, using a Box–Behnken design. Design-Expert 13 software (Stat-Ease Inc., USA) was utilized for RSM. Results are presented as mean ± standard error, and all analyses were conducted in triplicate.

For cytotoxicity and immunoreactivity analysis, all data were expressed as the mean ± standard error from at least three experiments ($n \geq 3$). The samples were compared using a One-Way Analysis of Variance (ANOVA). Tukey's post hoc test was applied to compare the results. Statistical analysis was performed using GraphPad Prism 9.0 software, and a p value of <0.05 was considered statistically significant.

Results and discussion

Optimization of the Sainfoin protein extraction conditions

RSM was employed to optimize the protein extraction yield from sainfoin seeds. The effects of solvent/solid ratio (X_1), pH (X_2), temperature (X_3), and time (X_4) on protein extraction yield (Y) were investigated by a Box-Behnken Design. A quadratic regression model was applied to estimate protein extraction yield; the results of analysis of variance, lack of fit test, and model adequacy are summarized in Table 2. The model demonstrated a highly significant p-value of less than 0.0001, along with an insignificant lack of fit, indicating its suitability for the experiment. The R^2 and adjusted R^2 values, 0.96 and 0.93, respectively, confirmed the model's strong fit to the experimental data and its suitability for accurate prediction. A model is typically deemed successful if the R^2 value exceeds 0.75, indicating a strong alignment with the experimental data, and an adjusted R^2 value above 0.80 further confirms the model's quality and suitability for optimization [24]. The minimal difference between the R^2 and adjusted R^2 values indicated a strong agreement between the experimental and predicted data [25]. Moreover, a coefficient of variation (CV) below 10% confirmed the high precision and reliability of the experimental values [26, 27]. These evaluations clearly showed that the model was suitable for optimization and accurate for prediction within the range of experimental variables. The quadratic model (Eq. 9) for protein yield is presented below, along with the regression coefficient values detailed in Table 2.

$$Y = 46.43 + 5.69X_1 + 3.07X_2 + 1.83X_3 + 2.27X_4 + 2.16X_1X_2 - 3.46X_1X_3 + 1.36X_1X_4 + 1.93X_2X_3 - 1.12X_2X_4 + 0.75X_3X_4 - 2.26X_1^2 - -0.58X_2^2 + 2.44X_3^2 - 2.25X_4^2 \quad (10)$$

The protein extraction yield of sainfoin proteins was found to be in the range of 34.27–55.03%, depending on the extraction conditions (Table 1). Table 2 illustrates that all factors, including solvent/solid ratio, pH, temperature, and time, significantly impact protein extraction yield ($p < 0.05$). The protein extraction yield of sainfoin protein rose from 38.48% to 49.87% as the solvent/solid ratio was increased from 10 to 50 mL/g, with pH, temperature, and time held constant at zero levels (Fig. 1). Similar results were found by Hadidi et al. [25] for alfalfa protein, Akyüz and Ersus [24] for sugar beet leaf protein, and Chouaibi et al. [27] for jujube seed protein. This phenomenon may result from the solvent serving as the driving force for protein mass transfer [25]. Likewise, pH was observed to positively influence protein extraction yield, with an increase in pH from 8 to 11 leading to a rise in protein extraction yield from 42.78% to 48.93%, while maintaining the solvent/solid ratio, temperature, and time at zero levels. It has also been reported that protein yield improves with increasing pH during protein extraction from lentils [28], grass peas [18], jujube seeds [27], and safflower [11]. At elevated pH levels, enhanced electrostatic repulsion between proteins can result in increased protein solubility and, consequently, greater extractability [26]. Additionally, a positive coefficient for $X_1 \times {}_2$ indicated that an increase in both the solvent/solid ratio and pH synergistically enhanced the protein extraction yield (Fig. 1a). An increase of up to 54.76% in protein extraction yield was observed with rising pH and solvent/solid ratio, while maintaining time and temperature at zero levels (Fig. 1a; Table 1).

Although the temperature had a significant positive effect on protein extraction efficiency ($p < 0.05$), increasing the temperature from 20 °C to 50 °C resulted in only a 3.66% increase in extraction yield when the solvent/solid ratio, pH, and time were held constant at zero levels. This increase may be attributed to the enhancing effect of temperature on protein solubility [24]. However, it has been noted that lower temperatures are more suitable for extraction to avoid protein denaturation [11, 18]. Additionally, a significant negative effect resulting from the interaction between extraction temperature and the solvent/solid ratio was observed (Fig. 1b). A similar trend was noted in a previous study by Pasrija and Sogi [29] for the extraction optimization of muskmelon seed protein concentrate. Moreover, the contribution of the interaction term between the solvent/solid ratio and temperature ($X_1 \times {}_3$), which was − 3.46, was considerably higher than the contribution of the linear temperature term, which was 1.83. This indicates that the combined effect of the solvent/solid ratio and temperature on protein extraction yield has a more substantial negative impact compared to the direct influence of temperature alone. Therefore, the highest protein extraction efficiency can be achieved at low temperatures combined with high solvent/solid ratios (Fig.

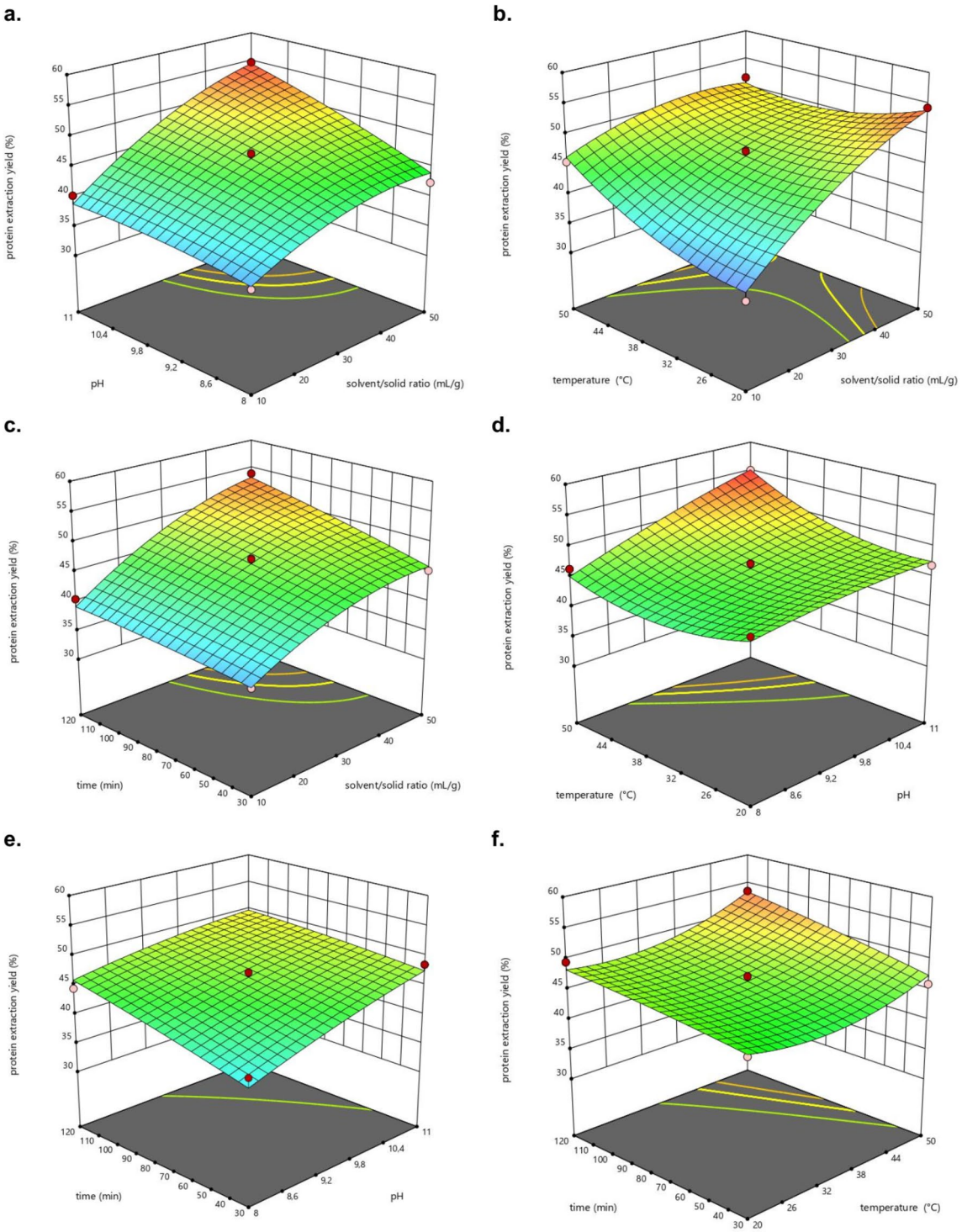

Fig. 1 Response surface plots showing the effects of solvent/solid ratio, pH, temperature, and time on the extraction of sainfoin protein

1b). Additionally, a slight increase in protein extraction yield was noted with longer extraction times, likely due to enhanced mass transfer [30]. However, none of the interaction or quadratic terms for time were found to be significant ($p < 0.05$) (Fig. 1c, e, f; Table 2).

Taking all these results into account, the optimal extraction conditions to maximize protein extraction yield were estimated using the desirability approach, which yielded the following parameters: a solvent/solid ratio of 49.96 mL/g, a pH of 10.99, a temperature of 20 °C, and an extraction time of 38.55 min. Desirability value of 1.00 was obtained in the

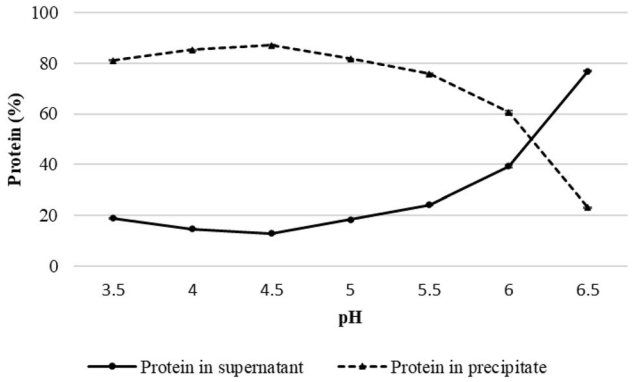

Fig. 2 Protein amounts in the supernatant and precipitate

Table 3 Proximate and amino acid composition of Sainfoin protein isolate (based on dry weight)

Component		
Proximate composition (%)	Moisture	6.11 ± 0.17
	Ash	4.19 ± 0.11
	Fat	2.66 ± 0.14
	Protein	91.44 ± 0.25
Amino acids (mg/g protein)	Lysine	68.75 ± 1.08
	Histidine	54.93 ± 0.89
	Arginine	158.20 ± 1.32
	Glycine	48.22 ± 0.43
	Cysteine	5.22 ± 0.02
	Serine	59.90 ± 0.50
	Alanine	2.26 ± 0.19
	Threonine	40.84 ± 0.29
	Isoleucine	141.37 ± 0.48
	Aspartic acid	141.27 ± 2.72
	Glutamic acid	28.56 ± 0.14
	Proline	53.39 ± 0.55
	Valine	43.39 ± 1.03
	Methionine	11.03 ± 0.46
	Tyrosine	24.11 ± 0.14
	Leucine	80.55 ± 0.36
	Phenylalanine	37.31 ± 0.72

optimization model. Under the established optimal conditions, the predicted protein extraction yield was 55.04%. When these optimal extraction conditions were applied, the experimental yield was measured at 56.36%. It was also confirmed that the predicted and experimental values were in agreement and fell within the 95% confidence interval. Therefore, it was concluded that the model adequately represented the optimization of extraction conditions for sainfoin proteins.

Determination of isoelectric point of Sainfoin protein

The pH of the sainfoin protein extract, obtained under optimal conditions, was adjusted to induce protein precipitation, after which the remaining protein in the supernatant was measured. Protein extracts form a colloidal system influenced by repulsive and attractive forces that affect stability. These interactions, determined by particle surface charge, depend on the pH. Depending on the net charge, repulsive forces keep particles suspended, while attractive forces cause aggregation and precipitation [31]. The protein content in the supernatant was found to range from 0.73 ± 0.01 to 4.34 ± 0.01 mg/mL, representing 12.95 ± 0.09% to 76.80 ± 0.22% of the extracted protein, respectively. As shown in Fig. 2, the lowest protein concentration in the supernatant occurred at pH 4.5. At this pH, 87.05 ± 0.09% of the proteins precipitated, indicating that pH 4.5 was the isoelectric point of sainfoin proteins (Fig. 2). At the isoelectric point, proteins are electrically neutral, with equal positive and negative charges, reducing electrostatic repulsion and solubility, which promotes aggregation and precipitation [11]. Similarly, most plant proteins have an isoelectric point between pH 4.0 and 5.0 [32].

Proximate and amino acid composition

The proximate composition of the freeze-dried protein sample following extraction and precipitation is presented in Table 3. The sainfoin seed protein sample had a high protein content of 91.44 ± 0.25%, based on dry weight. Protein fractions with a purity above 90% are classified as protein isolates [33]. Therefore, the sainfoin seed proteins obtained in this study can be identified as a protein isolate. This result also demonstrates that aqueous alkali extraction combined with isoelectric precipitation is an effective method for producing protein isolates from sainfoin seeds. Naturally, protein isolates were not completely pure, containing 4.19 ± 0.11% ash and 2.66 ± 0.14% fat, based on dry weight (Table 3).

The amino acid composition of the SPI is shown in Table 3. Of the 17 amino acids identified, arginine was the most abundant, with a concentration of 158.20 ± 1.32 mg/g. Arginine is classified as a conditionally essential amino acid because it may become essential during periods of stress or catabolic conditions when endogenous synthesis is insufficient. It plays a crucial role in metabolic, immune, and reparative responses to trauma and is also necessary for optimal neonatal growth and embryonic survival, making it essential during infancy [34]. The SPI also contained elevated amounts of isoleucine and aspartic acid. Isoleucine, an essential amino acid, plays a beneficial role in muscle development. Additionally, aspartic acid is crucial for synthesizing amino acids like arginine, lysine, methionine, threonine, and isoleucine [24]. In contrast, methionine, cysteine, and alanine were recognized as limiting amino acids, with alanine having the lowest concentration at 2.26 ± 0.19

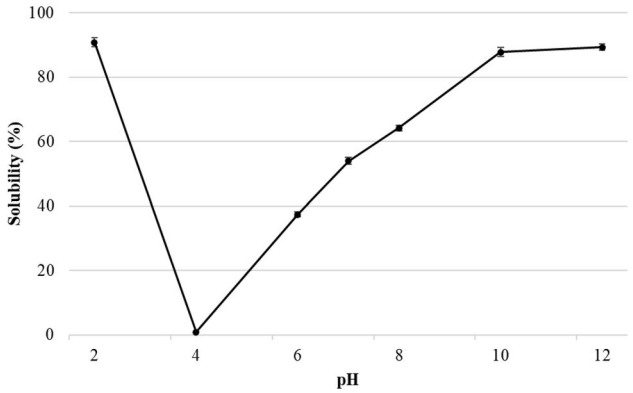

Fig. 3 pH solubility of sainfoin protein isolate

Table 4 Functional properties of Sainfoin protein isolate

Functional properties	
Water holding capacity (g/g)	2.36 ± 0.06
Oil binding capacity (g/g)	4.68 ± 0.04
Emulsion activity (%)	66.67 ± 0.17
Emulsion stability (%)	77.50 ± 0.87
Foaming capacity (%)	39.17 ± 0.83
Foam stability (%)	68.52 ± 1.85
Least gelation concentration (%)	16.00 ± 0.01

mg/g. Together, methionine and cysteine, which are sulfur-containing amino acids, make up 1.63% of the total amino acid content. Research indicates that legume proteins typically have low levels of sulfur amino acids [35]. Additionally, SPI was rich in essential amino acids, accounting for 50.26% of the total amino acids, emphasizing its potential as a new protein source.

Amino acid profiles significantly influence the physicochemical and functional properties of protein isolates [36]. Aromatic amino acids, such as tyrosine and phenylalanine, which enhanced the protein's antioxidant capacity [37], made up 6.15% of the total amino acids in the SPI. Additionally, hydrophobic amino acids, including alanine, isoleucine, leucine, methionine, phenylalanine, proline, and valine, constitute 36.96% of the total amino acids. These hydrophobic amino acids are essential for stabilizing protein conformation, with a higher proportion of these amino acids associated with increased heat resistance in protein [38]. Conversely, 13.02% of the identified amino acids exhibited hydrophilic characteristics, including cysteine, serine, threonine, and tyrosine.

Functional properties

Protein solubility

PS is a key functional property of protein that affects other important characteristics, such as emulsification and foaming abilities [39]. Additionally, PS is a good marker for the evaluation of the protein isolates' performance in the creation of new food products [38]. As PS is influenced by pH, the PS of the SPI across a pH range of 2.0–12.0 is illustrated in Fig. 3. The SPI exhibited V-shaped PS curves within this pH range. The highest PS was recorded at pH 2.0 (90.78 ± 1.39%) and within the range of pH 10.0–12.0 (87.77 ± 1.35–89.32 ± 1.00%), while the lowest PS of 0.91 ± 0.14% was observed at pH 4, which is close to the isoelectric point of the SPI at pH 4.5. The low PS near the isoelectric point can be attributed to the balanced positive and negative charges, which reduce electrostatic repulsion between protein molecules, leading to aggregation and precipitation [40]. Furthermore, the PS of the SPI at pH 7.0 was 53.95 ± 1.13%, which is lower than that of sunflower protein isolates (73.04%) [32] and pea protein isolates (56.5%) [41], but higher than that of Chinese quince seed protein (42.38%) [40], panda bean protein (50%) [35], as well as soybean and wheat protein isolates (less than 40%) [42]. Also, the protein solubility of soybean at pH 7 was reported in a wide range of 26.06%-64.00% [17, 42, 43]. Given that the pH of food systems is typically neutral or slightly acidic [44], it is crucial for SPI to exhibit relatively high solubility at these pH levels for effective use in food applications. The PS of the SPI increased further from pH 7.0 to 10.0 ($p < 0.05$), peaking at pH 10.0, while no significant change in PS was observed within the pH range of 10.0–12.0 ($p > 0.05$) (Fig. 3). This behavior is attributed to alterations in the net charge of the proteins as a function of pH [35].

Water holding capacity and oil binding capacity

WHC, which refers to the ability of proteins to retain water against gravity, is closely linked to the viscosity of food products. This is because water can bind to proteins without causing their dissolution, thereby enhancing thickening and viscosity [32]. The WHC of the SPI was measured at 2.36 ± 0.06 g/g (Table 4), which is lower than that of mung bean (2.62 g/g) [45], soybean (3.55 g/g) [46] and pea (2.52 g/g) [46] protein isolates. However, the SPI demonstrated a higher WHC compared to pinto bean (1.65 g/g) [47], grass pea (1.12 g/g) [18], wheat (1.38 g/g) [42], pine nut (1.15 g/g) [26], sunflower (0.98 g/g) [32], and safflower (1.78 g/g) [11] protein isolates. WHC is determined by the formation of hydrogen bonds between the hydrophilic groups of proteins/amino acids and water [33]. Based on this, the amino acid profile, conformational structure, and surface hydrophobicity/hydrophilicity ratio may affect the WHC of protein [45]. Moreover, it has been indicated that proteins with WHC values between 1.49 and 4.72 g/g are ideal for use in viscous foods [40]. Thus, SPI could be a beneficial ingredient in products such as soups, confections, and baked goods.

OBC value indicates a protein's ability to absorb oil and is closely related to shelf life, flavor retention, and emulsifying properties [40, 45]. Additionally, OBC highlights the hydrophobic characteristics of protein and its ability to connect fat and water in food products [48]. As presented in Table 4, the OBC of SPI was measured as 4.68 ± 0.04 g/g, which is lower than that of pine nut protein isolate (6.66 g/g) [26]. In contrast, the OBC for grass pea, pinto bean, soybean, wheat, sunflower, and safflower protein isolates ranged from 1.08 to 2.57 g/g, indicating that SPI has a higher capacity [11, 18, 32, 42, 47]. OBC is influenced by the protein's conformational structure and amino acid composition. Furthermore, amino acids with longer non-polar side chains, such as leucine, isoleucine, and valine, interact physically with fat, resulting in increased OBC [33]. A high OBC is advantageous in formulated foods such as mayonnaise, salad dressings, cake batters, and sausages because it helps to preserve and enhance flavor [16]. This suggests that sainfoin protein isolates could serve as promising plant-based protein options for use in high-fat food applications.

Emulsion properties

The emulsifying properties of the SPI were evaluated by measuring EA and ES, which indicate the protein's adsorption capacity at the interface and the characteristics of the adsorbed film, respectively [32]. As shown in Table 4, the SPI exhibits a high EA of 66.67 ± 0.17%, surpassing the results for soybean (64.45%), sunflower (50.75%), and safflower (53.17%) reported in previous studies [11, 17, 32]. Furthermore, the ES of SPI was measured at 77.50 ± 0.87%, falling within the range of 49.11–84.3% reported for the noted plant proteins [11, 17, 32].

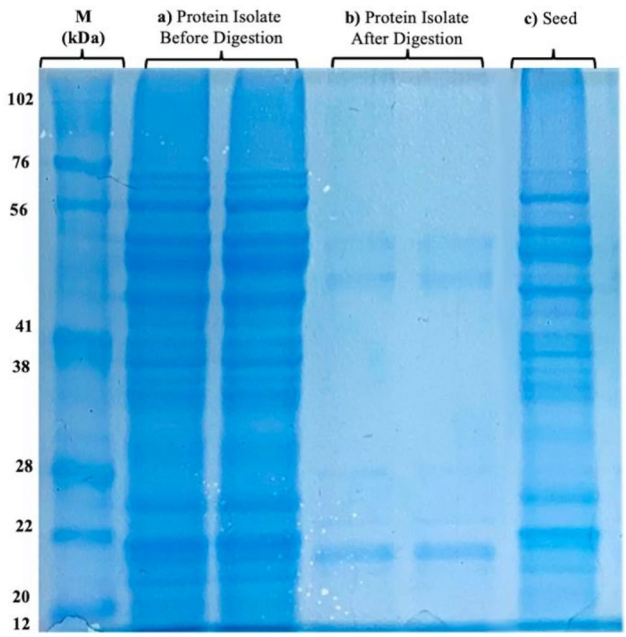

Fig. 4 Protein profile in SDS-PAGE. Protein isolates before **(a)** and after **(b)** in vitro test tube digestion were loaded as two replicates, and sainfoin seed before protein isolation **(c)** was shown. M means molecular weight marker. Equal volumes (30 µL) were loaded in all wells

Protein emulsion properties are significantly influenced by their conformational stability, the arrangement of hydrophobic and hydrophilic residues on the protein surface, and the protein molecule's capacity to fold and unfold at the oil-water interface [38]. Hydrophobic amino acid residues tend to migrate toward the oil phase during emulsification, allowing the protein to adsorb at the interface, reduce interfacial tension, and stabilize oil droplets via a viscoelastic film [49]. Additionally, solubility plays a crucial role in determining the emulsifying properties of proteins [11]. A protein needs to have good solubility to rearrange in the interfacial phase and form a film with desirable viscoelasticity and stability [33]. Thus, the greater solubility of SPI likely contributes to its enhanced emulsifying properties. In conclusion, the high EA and ES values of SPI suggest its potential as an ideal ingredient for formulating emulsion-based foods, such as meatballs, sausages, and hot dogs.

Foaming properties

The foaming properties, characterized by FC and FS, refer to the ability of proteins to form and maintain interfacial films [33]. For the SPI, the FC was recorded at 39.17 ± 0.83%, while the FS reached 68.52 ± 1.85% (Table 4). In comparison, soybean protein isolates demonstrated a similar FC of 39.39% but a significantly higher FS of 92.42% [17]. Adenekan et al. [50] reported FC and FS values of 33.3% and 54.57%, respectively, for pigeon pea protein isolates. For safflower protein isolate, previous findings indicated FC and FS values of 35.83% and 83.81%, respectively [11]. Dabbour et al. [32] observed a FC of 34.62% with a notably low FS of 8.60% in sunflower protein isolates.

The capacity to form foam is contingent upon the rapid diffusion and reorientation of proteins at the interface [33], making FC closely related to protein solubility. Greater solubility facilitates enhanced water-protein interactions, promoting protein unfolding, which improves air encapsulation [40]. Conversely, FS is affected by protein-protein interactions, the rheological properties of the protein membrane, and various environmental conditions [39]. These findings suggest that SPI exhibits favorable FS, indicating its potential to stabilize foam effectively. Such excellent foaming properties present a promising alternative to commercial protein ingredients in food formulations that require aeration and volume, including ice creams, whipped creams, and bakery products.

Least gelation concentration

The LGC refers to the minimum concentration needed to form a stable and self-supporting gel that does not flow when the tube is turned [51]. As indicated in Table 4, the LGC of SPI was measured at 16.00 ± 0.01%, which is comparable to the LGC values of pinto bean protein and grass pea protein isolates [18, 47]. The LGC values for protein isolates of pea [46], safflower [11], and soybean [17] were all recorded at 12%. The gelation properties of proteins are affected by the availability of functional groups, such as amide groups and hydrophobic regions [17]. A lower LGC reflects a strong gel-forming capacity in proteins, as less protein is necessary to achieve gel formation [17]. The gel-forming characteristics of proteins are crucial as they demonstrate the ability to create a robust structural matrix capable of retaining water, sugars, flavors, and other food components [40].

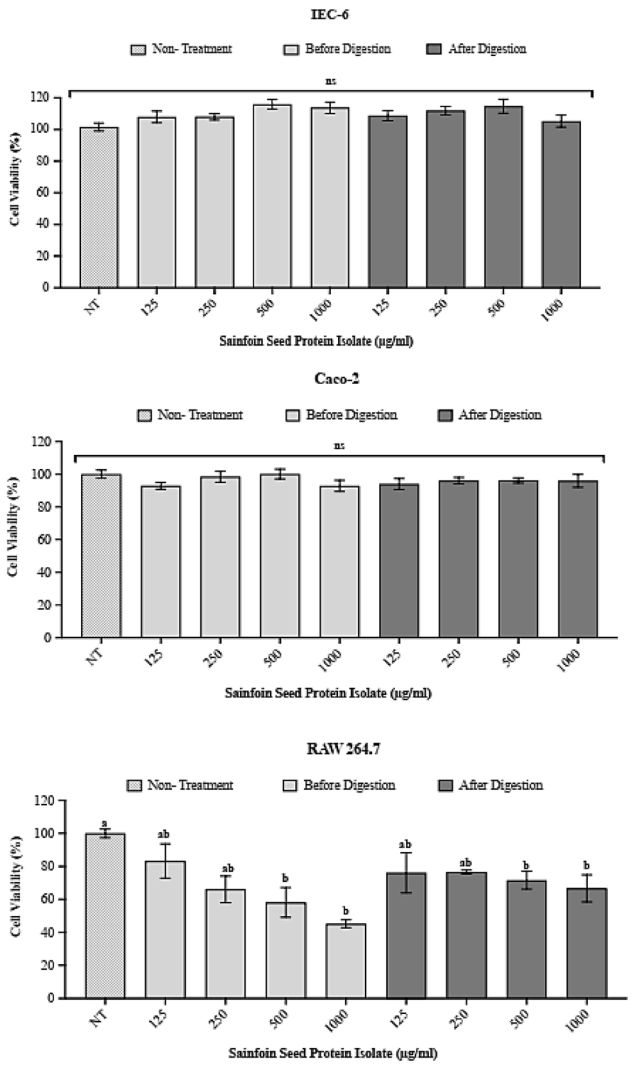

Fig. 5 In vitro cytotoxicity of sainfoin seed protein isolates. Protein isolates were prepared before and after in vitro test tube digestion in IEC-6 **(a)**, Caco-2 **(b)**, and RAW 264.7 **(c)** cells. **a**, **b** Statistically different from one another within each panel ($p<0.05$). Bars (A–I) depict mean ± SE

Analysis of molecular weight distribution by SDS-PAGE

The protein profile of sainfoin seed and SPI before and after in vitro test tube digestion was shown by SDS-PAGE in Fig. 4. Several clear bands were seen at 76 − 20 kDa in undigested sainfoin seed replicates and protein isolates. However, band distribution significantly changed after the digestion process. Lighter bands were observed below the 56 kDa and 28–20 kDa regions in the electrophoretic lanes. This shift and gradual disappearance of bands indicate that the proteins were hydrolyzed into lower molecular weight fragments as a result of enzymatic digestion [52, 53]. Furthermore, bands observed in digested samples may include digestion enzyme proteins or their peptides, as distinct protein band patterns were evident between digested and non-digested groups, confirming SPI's digestibility. Subsequent studies are crucial to identify the specific SPI-derived amino acids and potential peptides.

Potential toxicity of protein and peptide

To show the impact of protein isolates on cell viability before and after digestion and determine non-toxic concentrations for further in vitro analysis, an MTT assay was applied to 24 h sample-treated Caco-2, IEC-6, and RAW 264.7 cells. After spectrophotometric measurements and observations under the microscope, 125–1000 µg/mL concentrations of SPI did not cause cytotoxicity in Caco-2 and IEC-6 cells. as shown in Fig. 5. Microscopic evaluation supported these findings, as no observable alterations in cell morphology, attachment, or monolayer integrity were detected after treatment. In Caco-2 and IEC-6 cells, there were no differences in viability between the non-treated control group and digested/non-digested protein isolate treatments ($p>0.05$). In RAW 264.7 cells, there was a significant reduction in 250, 500, 1000 µg/mL concentrations of non-digested and 500, 1000 µg/mL concentrations of digested samples ($p<0.05$). These findings suggest that while SPI is non-toxic to intestinal epithelial cells, its effects on macrophage-like cells may vary at higher doses. Our results showed that SSPI could safely be used to expand further studies about the novel potentials in vitro.

Potential immunoreactivity of protein and peptide

RAW 264.7 cells were used to investigate inflammatory responses involved in immunoreactivity. Due to the immunoreactivity of certain molecules, the immune system can be

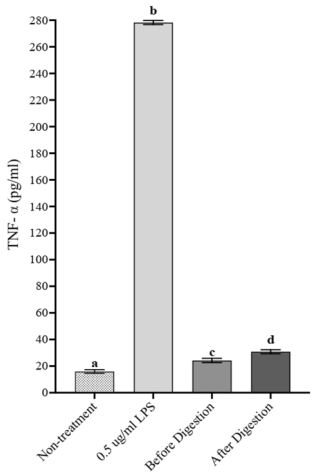

Fig. 6 Relative TNF- α cytokine production in RAW264.7 cell line. The cells were treated with 0.5 µg/mL LPS for 12 h as a positive control. Sainfoin seed protein isolates (125 µg/mL) were prepared before and after in vitro test tube digestion and given without LPS. Different letters indicate statistical significance between groups (p<0.05). Bars (A–D) depict mean ± SE

activated, leading macrophages to secrete specific protein known as cytokines as part of the immune response [54–56]. TNF-α and IL-6 cytokines are critical multifunctional inflammatory agents that have a significant role in developing diseases and acute phase immune responses [57–60]. To test the potential effects on allergy-induced inflammatory reactions of sainfoin seed protein, TNF-α and IL-6 were used as biological markers. LPS is an endotoxin component found in gram-negative bacteria that can cause inflammation in macrophage cells. LPS has significantly increased TNF-α protein levels in RAW 264.7 and induced inflammation [23, 61]. This study used LPS as a positive control to confirm the inflammatory condition. Its concentration was determined based on a previous study [23]. After 0.5 µg/mL LPS treatment, the TNF-α protein level increased significantly compared to the non-treatment control (Fig. 6) ($p < 0.05$). IL-6 levels were undetectable in all conditions (i.e., below the assay's lower detection limit of 31.25 pg/mL); therefore, the results were not presented. As a result of the finding on the MTT assay, the concentration of digested and undigested samples was determined to be 125 µg/mL for immunoreactivity studies on macrophages. When the macrophages were treated with 125 µg/mL undigested and digested seed protein isolates, TNF-α levels were significantly lower than the LPS-treated control ($p < 0.05$). Although there was a difference between the non-treatment control and protein isolates groups, it was considered a stress response related to pH and other changes in the medium because of protein density in samples. Studies showed macrophage cells are susceptible to stimulation from pH changes and environmental factors as they have pro-inflammatory secretion and macrophage differentiation specialty against novel conditions [62–64]. Our results suggested that undigested and digested SPI did not induce the immune system by TNF-α and IL-6 cytokine secretion, indicating that sainfoin seed did not have immunoreactivity potential in vitro.

Conclusions

This study employed RSM to optimize protein extraction yield from sainfoin seeds. Using a Box-Behnken Design, the effects of different extraction parameters were systematically analyzed. The desirability approach identified optimal conditions, resulting in a solvent/solid ratio of 49.96 mL/g, a pH of 10.99, a temperature of 20 °C, and an extraction time of 38.55 min. Under these conditions, the predicted protein extraction yield was 55.04%, while the experimental yield reached 56.36%. The resulting SPI had a high protein content of 91.44%. Among the 17 amino acids identified, arginine was most abundant at 158.20 mg/g, along with substantial levels of isoleucine and aspartic acid. Notably, essential amino acids made up 50.26% of the total amino acid profile. The SPI also displayed favorable functional properties. In vitro cell culture studies for toxicity and immunoreactivity indicated no physiological risk associated with the consumption of SPI, with further in vivo studies recommended for deeper immunoreactivity assessment. In conclusion, SPI shows strong potential as an alternative protein source, offering high nutritional quality and functional attributes suitable for diverse food applications. Further research may focus on product applications, incorporating a holistic approach to product development that includes sensory evaluation, functional properties, and overall consumer acceptability.

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Data availability Data will be available upon request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Not applicable.

Consent for publication Not applicable.

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