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FULL ARTICLE

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Optimizing the potential bioactivity of isoflavones from soybeans via ultrasound pretreatment: Antioxidant potential and NF-κB activation

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Abstract

Soybean consumption has been associated with health benefits. However, the effect of ultrasound (US) soybean pretreatment in terms of potential health benefits has not been investigated so far. Accordingly, the total phenolic content (TPC) and the total aglycone content (TAC) were optimized using the Box–Behnken design. Contrasting samples regarding isoflavones aglycones and TPCs were screened for their antioxidant and anti-inflammatory potentials using RAW 264.7 macrophages. US pretreated soybeans (55°C, 15 min, and 24 W/cm^2) showed greater TPC and TAC compared to the control and this translated to higher antiradical activity and reduction of nuclear factor kappa B (NF-κB) activation. The concentration of genistein in treated soybeans increased by 95%. Furthermore, US pretreated soybeans rendered phenolic extracts that reduced the NF-κB activation by 86%. Therefore, this contribution demonstrates the beneficial effects of US pretreatment of soybeans, which provides a better feedstock for the functional food industry.

Practical applications

Soybeans can be consumed as such or used as a feedstock to produce soy yogurt, fermented soymilk, tofu, and protein concentrate, among others. The greatest bioavailability of isoflavones compared to other flavonoids has recently been highlighted, and this has been explained by the relatively moderate lipophilicity of isoflavones as aglycones. The present contribution supports the use of US pretreatment of soybeans to obtain a feedstock with improved contents of isoflavones as aglycones. We have confirmed that phenolic extracts obtained from the US pretreated samples showed higher bioactivity as radical scavengers and by reducing the activation of nuclear factor kappa B (NF-κB) in a cell model, which is mediated by oxidative species. The clinical importance of NF-κB activation is derived mainly from its role in inflammatory responses. Therefore, our investigation may have a practical application in the procurement of soybean products and/or ingredients with improved functional properties related to their health benefits.

KEYWORDS

anti-inflammatory potential, antioxidant potential, hydrothermal processing, sonication, soybean flavonoids

1 | INTRODUCTION

Soybeans are well known for its high protein content and is also rich in polyphenols and tocopherols (de Camargo, Biasoto, et al., 2019; Shahidi & de Camargo, 2016). Flavonoids, more specifically isoflavones, are the most prominent group of phenolic compounds in soybeans (Perez-Vizcaino & Duarte, 2010). Isoflavones may exist as aglycones or in the conjugated forms.

The higher biological activity of isoflavone aglycones can be explained by their low molecular weight, which facilitates their absorption in the lower gut. However, conjugated isoflavones are the major phenolics in unprocessed soybeans. In fact, the content of malonylglucoside isoflavones may range from 70% to 90%, considering the isoflavone profile, while the content of β-glucoside isoflavones varies from 10% to 26%. The contribution of isoflavone aglycones is in the range of 1 to 7% (Lima & Ida, 2014).

Hydrothermal treatment and ultrasound (US) exposure (de Lima, Kurozawa, & Ida, 2014; Falcão et al., 2018) can change the isoflavone profile of the feedstock before the production of soybean products. Soaking and US affect the activity of endogenous enzymes involved in the conversion of conjugated isoflavones to their respective aglycones (de Lima et al., 2014; Falcão et al., 2018; Silva et al., 2019).

Soybean consumption has been linked to potential health benefits. These include the prevention of cardiovascular diseases (CVD) (Messina, 2016; Omoni & Aluko, 2005; Ruscica et al., 2018; Siow & Mann, 2010; Sirtori, Pavanello, Calabresi, & Ruscica, 2017). Furthermore, inflammation and oxidative stress are common to both diseases. Due to their improved biological activity, investigations on the sources of phenolic compounds in the aglycone form have been addressed in several studies (Larkin, Price, & Astheimer, 2008).

Falcão et al. (2018) demonstrated that the use of US prior to the soaking of soybeans can increase its content of isoflavone aglycones as well as to decrease the soaking time. However, the effect of US on the total phenolic content (TPC) and changes in their isoflavone profile in terms of antioxidant and anti-inflammatory potential have not been addressed. Therefore, the aim of the current study was to optimize the TPC and the recovery of isoflavone aglycones using the Box–Behnken design as well as to evaluate the potential bioactivity of the samples obtained under optimized conditions. Considering the critical role of oxidative stress linked to the nuclear factor kappa B (NF-κB) activation, the ability of phenolic extracts of unprocessed and US-treated samples was evaluated for their radical scavenging activity (RSA) and their ability in reducing the NF-κB activation. In addition, the protein complex NF-κB is commonly found in the cytoplasm (Lingappan, 2018). Therefore, the resultant isoflavones need to cross the cell membranes to exert the intracellular bioactivity, which strengths the importance of our findings in a biological model.

2 | MATERIALS AND METHODS

2.1 | Materials

Soybean samples (BRS 257), crop year 2015/2016, were donated by SL Alimentos (Londrina, Paraná, Brazil). Malonylglucoside and acetylglucoside isoflavones were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Meanwhile, β-glucoside isoflavones, isoflavone aglycones, *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG), gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), and Trolox, RPMI (Roswell Park Memorial Institute) medium, fetal bovine serum, penicillin, streptomycin, MTT (3-methyl-[4-5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), *Escherichia coli* lipopolysaccharide (LPS), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Macrophage RAW264.7 cells were procured from the cell bank of Rio de Janeiro (ATCC, Rio de Janeiro, Brazil). The remaining chemicals and solvents were of analytical or chromatographic grade.

2.2 | Experimental design and US treatment

A Box–Behnken experimental design (Box & Behnken, 1960) was employed to screen the effects of US treatment prior to soaking in the TPC and total isoflavone aglycone of soybeans. Uncoded (X_1 = temperature, X_2 = exposure time, and X_3 = US intensity) and coded (x_1, x_2, and x_3) as well as independent variables are shown in Table 1. Different treatments, including four replications at the central point, were numbered from 1 to 16 [treatment 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, (central point, treatment 13, 14, 15, and 16)]. Soybean grains (50 g) in distilled water (1:1.5; m/v) were used in each assay. US pretreatment procedure and equipment have been described elsewhere (Falcão et al., 2018). The samples were heated in a MARCONI MA127 water bath (MARCONI, Piracicaba, Brazil) until reaching the desired temperature, as defined in the experimental plan (Table 1). To maintain the internal temperature, the samples with the soaking water were transferred to a beaker (TE-2005), which was coupled to a Tecnal thermostatic water bath (Tecnal, Piracicaba, Brazil). A Q700 ultrasound (Qsonica, Newtown, CT, USA) and a low-intensity probe sonicator were used. The frequency was 20 kHz and both the time of exposure (X_2) and intensities (X_3) employed were defined according to the experimental plan (Table 1). At the end of each treatment, soybean samples were cooled down using ice to reach 25°C. The soaking water was discarded and the samples were lyophilized using an Alpha 1–2 LD Plus freeze dryer (Martin Christ, Germany). Lyophilized grains were ground (MDR301, Cadence, Navegantes, Brazil) and the resultant flour (0.5 mm of granulometry) was stored at −22°C. The TPC and the total aglycone content (TAC) were the response functions.

The response functions were subjected to the regression and variance analysis (ANOVA) to evaluate the effect of the independent variables (linear and quadratic effects) as well as their interactions. Determination coefficients (R^2 and R^2 adjusted) and lack of fit (p) were calculated. Some nonsignificant effects were removed from the model after a first coefficient evaluation (β) following a new statistical analysis to obtain a better adjusted model by considering the experimental data (Granato, de Araújo Calado, & Jarvis, 2014). The better adjusted R^2 (α = .05) was used to explain the response function. The data were fitted to a second-order model represented by the equation below.

TABLE 1 Box–Behnken design with coded (x) and uncoded (X) independent variables used for the ultrasound pretreatment of soybeans and their response functions

	Assays conditions							
	Coded variables			Uncoded variables			Response functions	
Treatment	x_1	x_2	x_3	X_1	X_2	X_3	TAC (μmol/g)[a]	TPC (mg GAE/g)[a]
1	−1	−1	0	35	5	15	0.19	3.18
2	1	−1	0	55	5	15	0.28	2.99
3	−1	1	0	35	25	15	0.21	2.85
4	1	1	0	55	25	15	0.23	3.34
5	−1	0	−1	35	15	6	0.18	3.04
6	1	0	−1	55	15	6	0.18	3.03
7	−1	0	1	35	15	24	0.20	3.04
8	1	0	1	55	15	24	0.32	3.62
9	0	−1	−1	45	5	6	0.16	3.15
10	0	1	−1	45	25	6	0.19	2.78
11	0	−1	1	45	5	24	0.20	2.91
12	0	1	1	45	25	24	0.22	3.01
13	0	0	0	45	15	15	0.19	2.90
14	0	0	0	45	15	15	0.22	2.95
15	0	0	0	45	15	15	0.20	3.03
16	0	0	0	45	15	15	0.22	–

Note: x_1, x_2, and x_3 are coded independent variables for X_1 (temperature, °C), X_2 (exposure time, min), and X_3 (ultrasound intensity, W/cm^2), respectively.
[a]Data represent mean values (n = 3). TAC: Total aglycone content (daidzein + genistein) and TPC: Total phenolic content. Isoflavones are expressed as μmol of TAC/gram of sample on a dry weight and full fat basis. TPC is expressed as mg of gallic acid equivalents/gram of sample on a dry weight and full fat basis.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} x_i x_j \quad (1)$$

where Y = response function (TPC or TAC) and x_i and x_j are the coded independent variables (i and j vary from 1 to k); β_0 = intercept coefficient; β_i, β_{ii} and β_{ij} are the estimated linear, quadratic, and interaction coefficients of the model, respectively; and k = number of parameters of the model. In case of this study, k = 3. Adjusted models of TPC and TAC were used to obtain the surface response and desirability function graphics were generated using the software Statistica 10.0 (StatSoft, Tulsa, OK, USA).

2.3 | Analytical methods

2.3.1 | Extraction of phenolic compounds

Ground flours in hexane (1:10; m/v) were defatted under agitation (MA 140/CFT, Marconi, Piracicaba, SP, Brazil) at 305 rpm for 1 hr. The material was vacuum filtered and stored at −22°C. The extracts were obtained as described elsewhere (Handa, Couto, Vicensoti, Georgetti, & Ida, 2014). The solvent system was ethanol/acetone/water (1:1:1, v/v/v) (Falcão et al., 2018). These phenolic extracts were used in all assays described below.

2.3.2 | Estimation of total phenolics

The TPC was estimated according to the method described by de Camargo, Regitano-d'Arce, Gallo, and Shahidi (2015). In brief, the extracts (0.50 ml), phenol reagent (0.50 ml), and deionized water (4.0 ml) were mixed thoroughly in test tubes. A saturated solution of sodium carbonate (0.5 ml) was added after 3 min. The reaction was carried out in the dark for 2 hr. The absorbance was read at 760 nm using a Biochrom Libra S22 spectrophotometer (Biochrom, Cambridge, England). The results were expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) of sample on a dry weight and full fat basis.

2.3.3 | Isoflavone profile of ultra-performance liquid chromatography

The separation and quantification of isoflavones were carried out according to the method described by Falcão et al. (2018) using an ultra-high performance liquid chromatography (ACQUITY UPLC® System, Waters, USA) equipped using a reversed-phase BEH C18 column (2.1 mm × 50 mm, 1.7 μm, Waters). The samples were filtered (Millex filter-H, 0.22 μm) and aliquots of 1.4 μl were automatically injected (ACQUITY UPLC® System, Waters, Milford, MA, USA). The

binary mobile phase containing 0.4% formic acid (A) and acetonitrile (B) was applied. The flow rate was adjusted to 0.3 ml/min and the column temperature was kept at 27°C. The elution gradient used was as follows: 0 min, 95% A; 8 min, 20% A; 9.5 min, 95% A, with a total run time of 12 min. For UPLC analysis, the identification of the compounds was based on the retention times and UV spectra at 260 nm. The TAC was calculated as the sum of isoflavones aglycones daidzein and genistein. The concentration was expressed as μmol of isoflavone per gram of sample (μmol/g) on a dry weight and full fat basis.

2.3.4 | Radical scavenging activity

The RSA toward the DPPH radical was according to Melo et al. (2015), as modified for the microplate determination to evaluate the effect of US on the antioxidant potential of soybeans. Likewise, the RSA toward the ABTS radical cation was evaluated as that for DPPH (Melo et al., 2015) to confirm the effect of low-intensity US pretreatment prior to soaking. The results were expressed as μmol Trolox equivalents per gram (μmol TE/g) of sample on a dry weight and full fat basis.

2.3.5 | Anti-inflammatory potential

Prior to the anti-inflammatory potential assays, the phenolic extracts were dried in a speed vacuum concentrator for 12 hr at 1.200 rpm using an RC10.22 vacuum concentrator (RC10.22, Jouan, Winchester, VA). The concentrated samples obtained were used for viability assay (MTT) and NF-κB activation assay, as described below.

Cell culture

RAW 264.7 macrophages transfected with the NF-κB-pLUC gene (Applied Biological Materials Inc., Vancouver, BC, Canada) were cultured in a RPMI medium supplemented with 10% of fetal bovine serum (FBS), 100 U/ml of penicillin and, 100 μg/ml of streptomycin. Cells were cultured in a CO_2 incubator (Sanyo, Tokyo, Japan) at 37°C with 5% humidity.

Viability assay (MTT)

Cell viability was determined in the presence of phenolics obtained from US-treated soybeans in triplicate by the MTT method. Briefly, cells (10^5 cells/well) were adhered to 96-well plates for 24 hr with supplemented RPMI. After adhesion, the supernatant was discarded and the dried samples from phenolic extracts were added at 1, 10, and 100 μg/ml diluted in unsupplemented RPMI. Treatment was carried out for 24 hr and the supernatant was then discarded and in its place diluted 0.01% MTT was added in unsupplemented RPMI for 3 hr. Finally, the supernatant was discarded and 200 μl of 100% DMSO was added. The lysate absorbance was measured at 550 nm (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA) and viability was determined by comparing the absorbance of the control untreated group with the treated groups (Franchin et al., 2016).

NF-κB activation assay

Cells were adhered to 24-well plates at 3×10^5 cells/well density for 24 hr with supplemented RPMI. After adhesion, the supernatant was discarded and phenolic extracts from control and pretreatment with US were added to the wells at 100 μg/ml. The dried samples from treatments were diluted in unsupplemented RPMI. After 30 min of treatment, *E. coli* LPS was added to each well to initiate an inflammatory stimulus (except in the control group). The final concentration of LPS was 10 ng/ml and stimulated cells were incubated for 4 hr. Afterward, the supernatant was then discarded and cells were lysed using a 50 μl aliquot of Tris-NaCl-Tween buffer on ice for 20 min. Finally, a 20 μl aliquot of the lysate was added in a 96-well white opaque plate with 25 μl of the luciferin reagent (Promega, Madison, WI, USA). Luminescence was then measured (FlexStation 3, Molecular Devices, Sunnyvale, CA, EUA) (Franchin et al., 2016).

2.3.6 | Statistical analysis

Experimental and predicted values for the response functions of TAC and TPC were compared using the student's *t*-test ($p < .05$) to validate the models. The concentration of individual isoflavones as well as the RSA and NF-κB activation were evaluated by the one-way analysis of variance (ANOVA), followed by the Tukey's test ($p < .05$).

3 | RESULTS AND DISCUSSION

3.1 | Multi-response optimization of the effects of soybean US pretreatment in relation to the TAC and TPC

Response surface methodology was employed to optimize the parameters used during the soybean US pretreatment in order to increase the recovery of TAC and TPC. Regression coefficients (β) and analysis of variance (ANOVA) of TAC and TPC are shown in Table 2. The coefficients related to temperature (β_1) and US intensity (β_3) as well as their interaction (β_1 and β_3) were positive with respect to TAC recovery. Some coefficients were not significant ($\beta_1{}^2$ and $\beta_3{}^2$); however, they were maintained considering their contribution to the model (higher adjustment, R^2). The linear (β_1), and quadratic coefficients ($\beta_1{}^2$) and the interaction ($\beta_1\beta_2$ and $\beta_1\beta_3$) were significant with respect to TPC. Likewise, nonsignificant coefficients (β_3 e $\beta_2\beta_3$) which also contributed to adjust the TPC model by increasing R^2 were therefore maintained in the model.

The lack of fit was not significant ($p > .05$) for both models and, according to the R^2, 85 and 84% of data fitted the model obtained for TAC and TPC, respectively. Thus, the models obtained ([TAC = 0.21 + 0.03 x_1^* + 0.02 x_1^2 + 0.03 x_3^* + 0.01 x_3^2 + 0.03 $x_1x_3^*$] and [TPC = 2.96 + 0.11x_1^* + 0.18x_1^{2*} + 0.07x_3 + 0.17$x_1x_2^*$ + 0.15$x_1x_3^*$ + 0.12x_2x_3]) can be used for predictive purposes. The highest TAC recovery (0.32 μmol/g) occurred when soybeans were subjected to the US at X_1 = 55°C and X_3 = 24 W/cm^2. In contrast, at a lower US

TABLE 2 Estimated coefficients and the analysis of variance for response functions of total aglycone contents (TAC) and total phenolic contents (TPC)

	Estimated coefficients (β)[a]		Significance of the coefficients[b]	
Parameters	**TAC**	**TPC**	**TAC**	**TPC**
Intercept				
β_0	0.207426	2.961381	0.000014	0.000067
Linear				
β_1	0.027149	0.108574	0.013097	0.040709
β_2	–	–	–	–
β_3	0.029429	0.072593	0.010464	0.084795
Quadratic				
β_1^2	0.022887	0.175045	0.050857	0.033921
β_2^2	–	–	–	–
β_3^2	−0.011976	–	0.196751	–
Interaction				
$\beta_1 \beta_2$	–	0.171071	–	0.033185
$\beta_1 \beta_3$	0.028876	0.148093	0.028240	0.043566
$\beta_2 \beta_3$	–	0.116720	–	0.067494
Lack of fit (p)[c]	NS	NS	0.34	0.23
R^{2c}			0.85	0.84
R^2 adjusted[c]			0.78	0.72
MS pure error			0.0002098	0.004087

Abbreviation: NS, no significant coefficients.
[a]Estimated coefficients for: TAC = daidzein +genistein. TAC is expressed as μmol of TAC/gram of sample on a dry weight and full fat basis. TPC is expressed as mg of gallic acid equivalents/gram of sample on a dry weight and full fat basis.
[b]Significant at 5%.
[c]Determination coefficients (R^2 and R^2 adjusted) and lack of fit (p) of the adjusted models. β_1, β_2, and β_3 (subscript numbers) stand for temperature (°C), exposure time (min), and ultrasound intensity (W/cm^2), respectively. (–) nonsignificant terms removed from the models.

intensity (X_3 = 6 W/cm^2), but at the same temperature, a lower TAC (0.21 μmol/g) was recovered, showing a reduction of 34% and supporting an intensity response of the US treatment (Figure 1a).

As for the TPC recovery (Figure 1b), the highest yield (3.75 mg GAE/g) was obtained when the test samples were subjected to the US treatment at X_1 = 55°C, X_2 = 25 min, and X_3 = 24 W/cm^2, which lends support to the results obtained for the TAC. Furthermore, a lower TPC recovery also occurred when a lower US intensity (X_3 = 6 W/cm^2) was employed. A concentration decrease of esterified phenolics with a concurrent increase in the fraction containing free phenolics may be due to the hydrolysis and removal of the sugar moiety (de Camargo et al., 2015), similar to that observed in the present study. Furthermore, Teh and Birch (2014) applied heat during the US-assisted extraction of phenolic compounds from hemp, flax, and canola seed cakes and observed a higher extraction yield.

According to Shahidi and Yeo (2016), the concentration of insoluble-bound phenolics in fruits, vegetables, and legume/seeds may reach 60%. These insoluble-bound compounds are linked to the cell wall of plant materials (e.g., pectin, cellulose, arabinoxylan, and structural proteins) via covalent bonds and, therefore, must be chemically or enzymatically hydrolyzed to play their role as bioactive compounds. The presence of isoflavones in the aglycone form has already been reported in some soybean cultivars (Zilić, Akıllıoglu, Serpen, Peric, & Gökmen, 2013). The conversion of soluble isoflavones into their corresponding aglycones is well established. Thus, both the literature (Zilić et al., 2013) and our results support the view that US-induced extraction of insoluble-bound isoflavone aglycones is contemplated.

The multi-response optimization of response variables was carried out using the overall desirability function. According to our results (Figure 1c), the highest recovery of TAC (0.30 μmol/g) was found at X_1 = 55°C, X_2 = 5 to 25 min, and X_3 = 19.5 to 24 W/cm^2. The treatment 8 (X_1 = 55°C, X_2 = 15 min, and X_3 = 24 W/cm^2) falls within these parameters (Table 1). The TAC recovery of the aforementioned assay was 0.32 μmol/g, which is not different from that of the estimated optimum condition (p = .73). Likewise, treatment 8 (TPC = 3.62 mg GAE/g) did not show any difference (p = .60) when compared to the TPC obtained under the optimum condition (3.75 mg GAE/g). Therefore, considering the overall desirability function and statistical analysis, our proposed model can be used for predictive purposes.

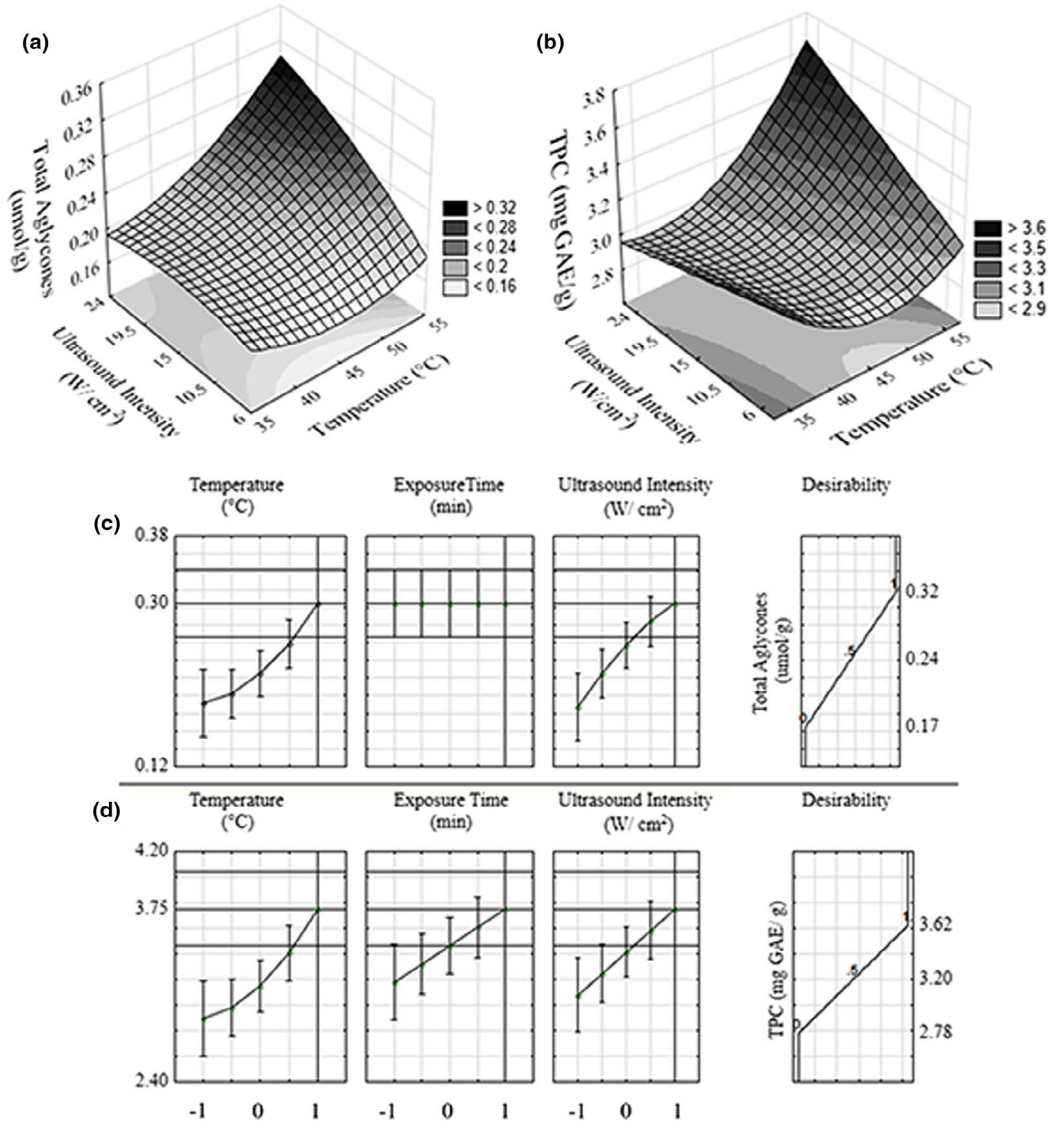

FIGURE 1 Response surface plotting as a function of temperature (X_1), and ultrasound intensity (X_3) from ultrasound soybeans pretreatment: (a) Total aglycone content (TAC) = daidzein + genistein, and (b) TPC is total phenolic content. (c) Profile for predicted values and desirability in the condition of maximum responses for TAC [μmol of TAC/gram of sample on a dry weight and full fat basis]; and (d) TPC [mg of gallic acid equivalents/gram of sample on a dry weight and full fat basis]

3.2 | Effects of soybean US pretreatment

The sample obtained during the treatment 8 (X_1 = 55°C, X_2 = 15 min, and X_3 = 24 W/cm^2) showed higher TAC and TPC. Accordingly, this sample was selected for further experiments. Likewise, a sample subjected to the US under the same conditions (55°C, 15 min) but at a lower US intensity, as a contrasting sample (6 W/cm^2) as well as a control (without any treatment) were investigated.

3.2.1 | Isoflavone profile

According to Shahidi, Varatharajan, Oh, and Peng (2019), isoflavones show the greatest bioavailability among the remaining flavonoids which has been explained by the relatively moderate lipophilicity of isoflavones as aglycones. The present study was designed to evaluate the effects of US pretreatment of soybeans with respect to its major phenolic compounds and potential outcomes in terms of bioactivity. Daidzein, glycitein, and daidzein and their respective conjugated forms are the major phenolic compounds in soybeans and their products. Malonylglucoside isoflavones made the major isoflavone contribution (64.43%), which was followed by β-glucoside isoflavones (29.13%) and aglycones (6.47%). Furthermore, acetyl-β-glucoside was not present. The results of this study are in line with those of de Lima et al. (2014).

The contents of malonylglucoside and β-glucoside isoflavones decreased upon the US treatment, while a significant concurrent increase in their respective aglycones was observed. At the higher US intensity (24 W/cm^2), the concentration of malonylglucoside and β-glucoside isoflavones decreased by 15% and 26%, respectively. In contrast, the concentration of genistein and daidzein increased by 95% and 16%, respectively. Furthermore, a US intensity-dependent

increase of total aglycones was observed in the present study. The literature data also suggest that the US may be used to decrease allergens in soybean products (Yang, Gao, Yang, & Chen, 2015). Therefore, the US treatment may provide a feedstock with a higher content of bioactive aglycones with a lower potency in allergenic reactions.

3.2.2 | Biological activity in vitro

Much has been discussed about food processing and its outcome in terms of nutritional quality of food and/or the effects in terms of food bioactives and relation to health. However, it is well known that specific chemical compounds respond differently to each process as well as the conditions employed. Structure-activity analyses carried out by Ma, Kinneer, Ye, and Chen (2003) suggested that the inhibition of the NF-κB function involves the redox cycling properties of the antioxidants, which was supported by other studies. According to Thakur, Pritchard, McMullen, Wang, and Nagy (2006), lipopolysaccharide increases the production of reactive oxygen species (ROS). Furthermore, ROS can regulate the activation of NF-κB (Nakajima & Kitamura, 2013). In addition, a recent study (de Camargo, Biasoto, et al., 2019) demonstrated that colorimetric methods were able to anticipate the reduction in the activation of NF-κB using LPS-activated RAW 264.7 macrophages. Accordingly, in this contribution, two antioxidant methods (ABTS and DPPH), based on chemical reactions, were employed as a screening method to test the hypothesis that isoflavones as aglycones from US pretreated samples could show improved bioactivity by reducing the activation of NF-κB.

Regardless of the method (ABTS or DPPH), the US pretreatment induced a positive effect by increasing the RSA of the test material by showing an intensity-dependent response (Table 3) and both methods exhibited a higher antioxidant potential than that of the control. In fact, the scavenging activity of samples treated with US at 24 W/cm^2 was 129 and 68% higher than that of untreated samples (control) toward ABTS radical cation and DPPH radical, respectively. Furthermore, even at a lower intensity (6 W/cm^2), the respective antiradical activity was 79% and 41% higher than that of the control.

The higher antiradical activity of samples subjected to the US at 24 W/cm^2 is supported by the higher TPC as well as by the higher TAC (Figure 1). This is in good agreement with the literature with respect to the chemistry of phenolic compounds. A recent study by Yoshiara et al. (2018) demonstrated that cotyledons, epicotyls, radicles, and hypocotyls from germinated soybeans also showed a higher antiradical activity, which was related to their higher aglycones contents (e.g., genistein). However, as far as we know, this has not been reported for soybeans subjected to the US.

Oxidation of LDL-cholesterol (LDL-c) has been used as a biomarker to anticipate the potential development of CVD (Amarowicz, 2016). A study by Xu, Yuan, and Chang (2007) reported a high correlation (r = .97, p < .01) between the scavenging of DPPH radicals and the ability of phenolic extracts from several legumes, including soybeans, in counteracting the oxidation of copper-induced human LDL-c oxidation in vitro. Furthermore, the correlation between

TABLE 3 Isoflavone contents (μmol/g) and antiradical activity (μmol TE/g) as affected by the ultrasound soybean pretreatment in maceration

	Treatment		
	Control	6 W/cm^2*	24 W/cm^2*
Isoflavone contents			
Daidzin	0.344 ± 0.045a	0.264 ± 0.004a	0.238 ± 0.002a
Glycitin	0.311 ± 0.006a	0.281 ± 0.011b	0.294 ± 0.002ab
Genistin	0.353 ± 0.004a	0.276 ± 0.006b	0.212 ± 0.002c
Total β-glucosides	1.008 ± 0.043a	0.821 ± 0.001b	0.744 ± 0.002b
Malonyldaidzin	0.683 ± 0.004a	0.638 ± 0.024ab	0.614 ± 0.008b
Malonylglycitin	0.434 ± 0.041a	0.371 ± 0.010a	0.405 ± 0.006a
Malonylgenistin	1.113 ± 0.002a	0.910 ± 0.004b	0.878 ± 0.016b
Total malonylglucosides	2.230 ± 0.043a	1.919 ± 0.038b	1.898 ± 0.018b
Daidzein	0.148 ± 0.003a	0.092 ± 0.002b	0.171 ± 0.012a
Genistein	0.075 ± 0.003b	0.085 ± 0.001b	0.146 ± 0.005a
Total aglycones	0.224 ± 0.001b	0.177 ± 0.003c	0.317 ± 0.016a
Total isoflavones	3.461 ± 0.087a	2.917 ± 0.039b	2.958 ± 0.032b
Antiradical activity			
DPPH	2,165 ± 286c	3,048 ± 167b	3,634 ± 244a
ABTS	120.0 ± 14.2c	214.8 ± 3.08b	275.3 ± 13.0a

Note: Data represent the mean values for each sample ± standard deviation (n = 4). Means followed by the same letters within a row are not significantly different (α = .05). The results are expressed on a dry weight and full fat basis *Ultrasound pretreatment at 55°C and 15 min. Glycitein was not detected in any samples.
Abbreviation: TE, Trolox equivalent.

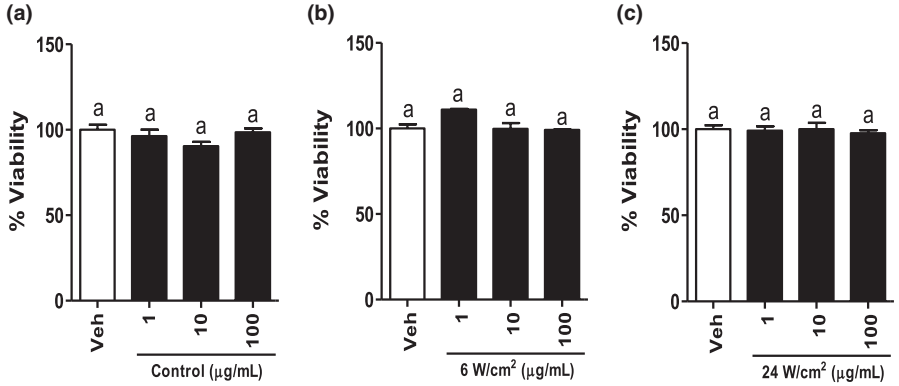

FIGURE 2 Cell viability by the MTT (3-methyl-[4-5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay of macrophages with the samples at different concentrations or vehicle (veh) for 24 hr (37°C, 5% of CO_2) for A = Control (soybeans without any treatment), B = Soybean pretreated with ultrasound at 6 W/cm^2, and C = Soybean pretreated with ultrasound at 24 W/cm^2. The data were expressed as the mean (n = 3) ± standard deviation. Different letters indicate a statistical difference (p < .05, Tukey's test)

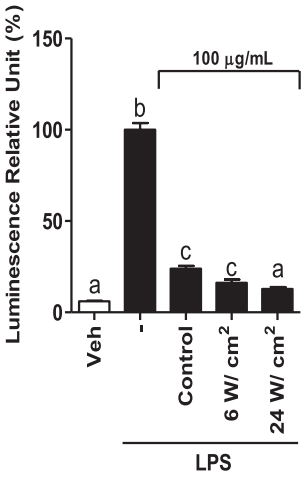

FIGURE 3 NF-κB (nuclear factor kappa B) activation assay in macrophages pretreated with the samples at the concentration of 100 μg/ml or vehicle (veh), 30 min prior to stimulation with lipopolysaccharide (LPS) (10 ng/ml). Control is soybeans without any treatment. Soybean was pretreated with ultrasound intensity at 6 W/cm^2. Soybean was pretreated with ultrasound intensity at 24 W/cm^2 Different letters indicate a statistical difference (p < .05, Tukey's test)

TPC and protection of LDL-c has also been demonstrated (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2014). In this study, US pretreated samples showed higher RSA toward DPPH and ABTS. Therefore, considering the existing literature (Xu et al., 2007), soybeans subjected to the US have a higher potential in protecting LDL-c and, as a consequence, higher potential in preventing CVD. Furthermore, genistein, which showed the highest increase in the present study, positively affects the activity of antioxidant enzymes (e.g., superoxide dismutase, catalase, and glutathione peroxidase) and decreases the production of malondialdehyde (MDA) in vivo (Lee, 2006; Valeri et al., 2012).

Inflammatory responses are common to several chronic ailments (Zhang & Tsao, 2016) such as type 2 diabetes, cancer, and CVD and the role of NF-κB in controlling many genes related to inflammation is well recognized. Accordingly, samples obtained from treatments 6 (US at 6 W/cm^2) and 8 (US at 24 W/cm^2) were evaluated for their ability in decreasing the activation of NF-κB, as a pivotal mediator of inflammatory responses. Initially, we determined the toxicity of the phenolic extracts to macrophages. The tested concentrations were nontoxic when compared to the untreated control group (Veh; Figure 2a–c), indicating no cytotoxic effects at concentrations equal or lower than 100 μg/ml (p > .05). Therefore, we have tested the higher nontoxic concentration (100 μg/ml) on the LPS-stimulated NF-κB activation assay. All treatments reduced the LPS-induced NF-κB activation (p < .05). The treatment with the phenolic extract obtained from treatment 6 reduced the NF-κB activation by 84% (Figure 3). Similarly, the sample obtained from treatment 8 rendered phenolic extracts that reduced the NF-κB activation by 86% (Figure 3). Interestingly, extracts obtained from untreated samples (control) showed to be significantly (p < .05) less effective than the extracts obtained from soybeans subjected to the US (treatment 8), since it reduced the NF-κB activation by 77% (Figure 3).

As mentioned in the manuscript "the concentration of genistein in treated soybean increased by up to 95%." The role of genistein as an anti-inflammatory compound was summarized by de Camargo, Favero, et al. (2019). The study of the mechanisms involved for the effect of genistein on the inflammatory responses in LPS-activated RAW 264.7 macrophages is beyond the mandate of the present study, as our main goal was to verify if US pretreated soybean samples would be more effective in inhibiting the activation of the NF-kB. Furthermore, the literature has already demonstrated that genistein decreases the activation of NF-κB and down-regulates the expression of pro-inflammatory mediators such as TNF-α, IL-6, IL-8, and IL-1β (Jeong et al., 2014; Ji et al., 2012; Venza, et al., 2018).

Macrophages are a heterogeneous group of immune cells that exert important immune functions in response to an insulting agent, such as bacterial infections. In general, these cells are scattered

all over the body as resident cells that act as sentinels, triggering a cascade of inflammatory mediators when facing a strange organism (Davies, Jenkins, Allen, & Taylor, 2013). Therefore, macrophages are one of the most important first-line defense under physiological conditions. However, the exacerbated number or activity of macrophages is closely related to the aggravation of several immune diseases, and the NF-kB activation is one of the most important routes in these cases (Wynn, Chawla, & Pollard, 2013). Hence, the inhibition of the NF-kB activation is a promising target to treat inflammatory diseases and natural molecules able to affect the NF-kB activation are interesting pharmacologic strategies to treat these diseases (Lazarini et al., 2016). Our results demonstrate that US (treatment 8) increases the anti-inflammatory potential of the soybean samples. Therefore, due to well documented action of isoflavone aglycones in modulating pro-inflammatory mediators such as TNF-α, IL-6, IL-8, and IL-1β (de Camargo, Favero, et al., 2019; Jeong et al., 2014; Ji et al., 2012; Venza et al., 2018), one can infer that US pretreated soybean samples may serve as a better source of anti-inflammatory molecules compared to the control and this may be attributed to their improved concentration of isoflavones as aglycones, especially genistein.

4 | CONCLUSION

The US pretreated soybeans showed increased TPC and improved concentration of isoflavones as aglycones. Phenolic extracts from treated samples also showed higher antioxidant and anti-inflammatory potential compared to those obtained from untreated samples. Oxidative stress and inflammatory responses are common to type 2 diabetes, CVD, and cancer. Therefore, US pretreated soybeans may provide a better option to counteract and/or prevent these diseases. However, due to the novelty of this study, further confirmation in vivo is deemed necessary. At this stage, it is possible to suggest that US pretreated soybeans have a good potential to be employed in the functional foods industry as a functional ingredient.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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