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Optimization of Ultrasound-Assisted Extraction of Bioactive Compounds from Peel Jambolan and Application of the Extract in Soy Protein Films

Medeiros, K. A. S.[1] , Nogueira, T. Y. K.[2], Lago-Vanzela, E. S.[2], Moritz, C. M. F.[1], Amado, L. R.[2], Silva, K. S.[1], Picolloto, A.M.[1]

[1] Department of Technology, UEM – Maringa State University, Angelo Moreira da Fonseca Avenue, 1800, 87506-370 – Umuarama, PR, Brazil

[2] Department of Food Engineering and Technology, São Paulo State University (UNESP), Institute of Biosciences, Humanities and Exact Sciences, Cristovão Colombo Street 2265, 15054-000 - São José do Rio Preto, SP, Brazil

ABSTRACT: This study aimed to optimize extraction of bioactive compounds from jambolan peels using ultrasound-assisted extraction and to develop active film from soy protein isolate and jambolan peel extract. For this purpose, the effect of time process and solvent concentration on total phenolic compounds, antioxidant and bacterial activity of the extract were evaluated. From the ideal conditions of these parameters, films were prepared and the effect of the extract addition on the mechanical properties, bacterial activity and antioxidant capacity of the active films were evaluated. The results showed that high ethanol concentration reduced the extraction of the bioactive compounds with antioxidant activity from jambolan peel, however, the interaction between extraction time and ethanol concentration presented positive effect on the extraction of these compounds. The extract with bactericidal action against the *Staphylococcus aureus* and *Escherichia coli* was not obtained by optimized conditions. Extract addition reduced the tensile strength of the SPI film, but increased its elasticity and antioxidant capacity. SPI + extract films affected the integrity of the bacterial cell membrane of the *Staphylococcus aureus* and *Escherichia coli*. SPI films with jambolan peel extract can be an alternative to limit undesirable oxidative processes and to inhibit the bacterial development in food packaged.

KEYWORDS: antioxidant; bioactive-compounds; jambolan; SPI-film; ultrasound-assisted.

INTRODUCTION

Jambolan (*Syzigium cumini L.*) is a plant belonging to the family Myrtaceae originates of India that bears fruit round or oblong or ellipsoid, with white pulp and dark purple peel (Seraglio *et al*., 2018). The jambolan fruit is rich in total phenolic compounds (TPC) as anthocyanins (based on delphinidin, petunidin and malvidin) and hydrolysable tannins, flavanonols, flavanols and flavon-3-ols and for this reason presents high potential for antioxidant activity (AA) and antibacterial properties (Ayyanar and Subash-Babu, 2012; Tavares e*t al*., 2016). For comparison, anthocyanin content of the jambolan peel is higher than obtained in jabuticaba peel and presents phenolic compounds (PC) higher than one found in açaí (Costa *et al*., 2013).

In USA and other European countries, the industrial pulp extraction is widely used in the production of health drinks, juices, squashes and jellies (Singh *et al*., 2015) which results in a significant amount of residues that can be used to extract bioactive compounds. In Brazil, jambolão is easily found in several states in domestic cultivars, but it is still under-exploited commercially. (Sabino *et al*., 2021). Phenolic compounds extracted from jambolan peels may be used in the country as safe, natural and low-cost additives in food products in order to reduce the use of synthetic antioxidant, to enhance nutritional quality and to protect the food against undesirable oxidative reactions. Moreover, its use as a natural antioxidant source may be an alternative to contribute to a sustainable food chain. Another alternative for preventing oxidation in packaged foods is incorporation of natural antioxidants in packaging material (Filipini; Romani; Martins, 2020). Antioxidants extracted from different fruits and fruit by-products incorporated into packaging can scavenging oxygen in the atmosphere, reduces oxygen passage through the material layer or migrate directly into solid food and prevent oxidation (Rangarai *et al*., 2021; Severo *et al*., 2021).

The extraction of PC from plant sources can be influenced by the bioactive compounds content in the plant, chosen method for extraction, the type and polarity of the solvent, the solid-solvent ratio, the time, temperature, pressure and the pH of the medium. Thus, it becomes a challenge to establish adequate and efficient extraction protocols for each type of plant matrix that results in total

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recovery of the compounds of interest without altering their qualitative and quantitative composition. (Alara; Abdurahman; Ukaegku, 2021).

Solid-liquid extraction at atmospheric pressure is a conventional method commonly used because it is simple, easy to operate and adaptable. However, its disadvantages include the need for large quantities of organic reagents, long extraction times, low efficiency and negative environmental impact. The reduction of the use of harsh organic solvents and the use of extraction techniques more environmentally friendly has been proposed by many researchers as a way to get natural extract employing the concept of green extraction (De la Guardia and Armenta, 2011). Ultrasonic extraction (US) is a simple, easy to operate, efficient, economical method and classified as green extraction technique. The efficiency of ultrasound extraction is influenced by temperature and time process, and by the properties of the sample and solvent used (Cong-Cong *et al.*, 2017). Some research has shown that the ultrasound extraction method for extracting PC from plant matrix is more efficient than the conventional method and provides bioactive compounds purer (Caldas *et al*., 2018; Deng *et al*., 2017). It is necessary to evaluate the influence of different mixtures of solvents, as well as concentrations and their nature (polarity) on the quality of the extraction process using the ultrasound technique in each type of plant matrix. (Martínez-Ramos *et al*., Phuong *et al*., 2020). Ethanol and water are the most common food grade solvents used for the extraction from vegetal sources due to safety from toxicological point of view (Tauchen *el al*., 2015). Ethanol-water mixtures are more environmentally preferable than pure alcohol (Mustafa and Turner, 2011). Binary solvents can to improve the extraction efficiency when comparing with simple solvent since one solvent could increase the solubility of polyphenols while other could improve the analyte desorption (Jovanović *et al*., 2017). A tool that has been used to assist in optimizing the parameters of the phenolic compounds extraction process is response surface methodology (RSM) (Alara *et al*., 2017; Charkraborty; Uppaluri; Das, 2020).

The aim of the present study was to optimize extraction of antioxidant compounds from jambolan peels using ultrasound technique; to investigate the combined effects of time process and solvent concentration on phenolic, antioxidant compounds content extracted and bacterial activity; to develop biodegradable antioxidant active packaging from soy protein isolate and jambolan peel extract; and to determine the effect of the extract addition on the mechanical properties, bacterial activity and antioxidant capacity of the packaging.

2. MATERIALS AND METHODS

2.1 Sampling and preparation of jambolan

Sample (2 kg) of the ripe fruit of jambolan [*S. Cumini (L)*, 2019 crop] was collected in the city of São José do Rio Preto (São Paulo, Brazil), located at 20º47'08"s and 49° 21'36" E and 544 m above sea level (World geodetic System, 1984). The species was identified by Dr. Regina Sampaio in a copy of voucher (number 32,214) was deposited in the SJRP herbarium at IBILCE/UNESP, in São José do Rio Preto, São Paulo, Brazil. The fruits were sanitized in distilled water with sodium hypochlorite for 15 minutes, then were carefully peeled in order not to grind the peel or to collect part of the pulp. After removal, the peels were dried in a fixed bed dryer equipped with a centrifugal fan with a 2 CV engine (CV3600, Ibram, São Paulo, SP, Brazil), during 12 hours at 40 ºC.

2.2 Extraction procedure

The extraction procedure was performed in three replicates using ultrasonic equipment (ECO-SONICS, Q3.8, 40 kHz, 88 Watts). The dry jambolan peels were mixed with a hydroethanolic solution acidified at pH 3 with phosphoric acid, in the proportion of 1:10 (solute:solvent), at 30 °C. The time of extraction by ultrasound (A) and the amount of ethanol used in the extraction process (B) were considered independent variables, according to experimental design presented in Table I. After extraction time, samples were centrifuged at 7,500 rpm, at 4 ºC for 15 minutes (HERMLE, Z 326 K – Germany) and the supernatant was concentrated on a vacuum rotary evaporator (TECNAL-TE-211) at 40 °C. The extract obtained was stored at −18 °C until further use.

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Table I - Concentration of total phenolics, anthocyanin and antioxidant activity of jambolan peel extract obtained by ultrasound.

	Variables		Responses				
	A:	B:	TPC	Anthocyanin	DPPH	FRAP	ABTS°⁺
Trials	Extraction time (min)	Ethanol (% w/w)	(mg GAE/100g)	(mg mv-3,5-dglc/100g)	(µM Trolox/100g)	(µM Trolox/100g)	(µM Trolox/100g)
1	20 (-1)	20 (-1)	696.92 ± 3.00	2404.48 ± 4.58	6846.08 ± 46.45	10891.34 ± 110.77	8275.91 ± 237.69
2	60 (+1)	20 (-1)	864.15 ± 3.58	1474.39 ± 3.71	3778.17 ± 95.26	7563.81 ± 44.90	6354.49 ± 154.14
3	20 (-1)	80 (+1)	788.59 ± 2.40	1357.91 ± 1.87	2995.49 ± 0,00	4842.61 ± 13.58	8789.45 ± 485.55
4	60 (+1)	80 (+1)	1307.32 ± 4.48	2723.65 ± 3.71	7346.87 ± 187.93	12765.73 ± 154.51	12862.74 ± 578.71
5	40 (0)	08 (-1.41)	994.79 ± 4.79	1233.13 ± 1.86	3820.10 ± 0,00	8206.74 ± 18.02	8208.57 ± 144.99
6	40 (0)	92 (+1.41)	982.35 ± 1.20	673.67 ± 4.67	3098.95 ± 0,00	6379.18 ± 56.52	4838.16 ± 36.38
7	12 (-1.41)	50 (0)	1100.00 ± 1.20	1616.78 ± 3.72	4255.17 ± 195.64	8655.42 ± 89.99	7520.69 ± 138.78
8	68 (+1.41)	50 (0)	1048.94 ± 5.99	1647.72 ± 1.86	4604.67 ± 15.08	9533.02 ± 99.18	8268.46 ±116.08
9	40 (0)	50 (0)	1269.16 ± 3.59	1440.34 ± 1.86	4900.64 ± 30.21	9664.15 ± 22.51	7794.45 ± 48.30
10	40 (0)	50 (0)	1272.46 ± 3.60	1444.69 ± 3.74	4962.81 ± 30.33	9662.22 ± 11.30	7825.11 ± 48.49
11	40 (0)	50 (0)	1272.41 ± 2.40	1444.16 ± 3.73	4930.54 ± 30.26	9664.96 ± 0.00	7807.96 ± 145.16

Abbreviations: TPC: total phenolic compounds, expressed as mg gallic acid equivalent (GAE)/g; mv-3,5-dglc: malvidina-3,5-diglicosídeo.

2.3 Sampling and preparation of jambolan

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2.5 *Optimization of the jambolan peel extract*

Extraction parameters were optimized applying a statistical planning of rotational composite design (DCCR) allied to Response Surface Methodology (MSR) using the Software Statistica 7.0 (Realese 7, StatSoft, INC, Tulsa, USA), with 4 factorial points (+1 and -1), 4 axial points (+1.41 and -1.41) and 3 central points (0), totalizing 11 experiments. The independent variables were ultrasound extraction time (A) and ethanol concentration (B). The dependent variables (responses) evaluated were the TPC, total anthocyanins content (ATC) and antioxidant activity (AA) by DPPH, ABTS and FRAP assays. The experiments were processed in a random order. The following general form of model was used to fit the observed data in DCCR.

$$Y_i = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} A.B \quad (1)$$

where Y_i is the response, β_0 is the intercept, A and B are the independent variable; $\beta_1, \beta_2, \beta_{11}, \beta_{22}, \beta_{12}$ are coefficients of the model (Meyer, 1971; Montgomery, 1991).

2.6 Determination of total phenolic compounds (TPC), total anthocyanins content (ATC) and antioxidant activity (AA) of the jambolan peel extract.

Determination of TPC in the extracts was performed using the Spectrophotometric method of Folin-Ciocalteu (Ough and Amerine, 1988). The TPC were quantified using Folin-Ciocalteu reagent and calibration curve with gallic acid (GAE), both from Dinâmica (Indaiatuba, São Paulo, Brazil), and the results expressed in mg of GAE.100g^{-1} of dry jambolan peel.

The ATC analysis was performed by the differential pH method (Ribéreau-Gayon, *et al*., 2006). The standard curve was determined using malvidine-3,5-glucoside as the standard. The results were expressed in mg of malvidin-3,5-glucoside (mv-3,5-glc).100g^{-1} of jambolan dry peels.

For the determination of AA of extracts, three methods were performed: the method of 2.2-defenil-1-picril-Hidrazil (DPPH), the ferric antioxidant power reducer (FRAP), both described by Brand-Williams *et al*. (1995) and Benzie & Strain (1996), and the method of capturing the 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) + radical (Surveswaran *et al*., 2007). Measurements of the DPPH, FRAP and radical ABTS methods were based on 6-hydroxy-2,5,7,8-Tetramethylchromano-2-carboxylic acid (TROLOX) calibration curves and the results expressed in µM of Trolox.100g^{-1} of dry jambolan peels. DPPH, 2,4,6-tris(2-pyridil)-s-triazine (TPZ), used in the FRAP methodology, ABTS and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) where acquires from Sigma-Aldrich (St. Louis, CO, USA).

2.7. Antibacterial activity

The tested bacteria came from the Collection of Reference Microorganisms in Health Surveillance - CMRVS, FIOCRUZ-INCQS, Rio de Janeiro, RJ, Brazil, with *Staphylococcus aureus* INCQS 00381 (ATCC 29213) and *Escherichia coli* INCQS 00033 (ATCC 25922).

2.8 Disc diffusion method

To determine the existence of antimicrobial activity in the obtained extracts, the disc-diffusion method (CLSI, 2003) with adaptations was used, as a preliminary scan to establish which extracts would be used for the application of the broth microdilution method. The bacteria were activated in Broth Brain Heart Infusion (BHI) and standardized on the 0.5 scale of MacFarland (equivalent to 1.0 × 10^8 UFC.mL^{-1}) using a spectrophotometer (Kasuaki, IL-227) with a wavelength of 625 nm. Mueller Hinton Agar (MHA) plates were seeded with the inoculum using a plastic stem swab. After spread, they were placed in 6 mm diameter paper discs and embedded with 15 µL of each extract. A control disc was added to the plates containing 15 µL of saline. The plates were incubated at 37 ºC for 24 hours and then the inhibition halos were read. Six replicates were performed for each bacterium tested. The data were compared using the Mann-Whitney method, at 5% significance.

2.9 Minimum Inhibitory Concentration (MIC)

The microdilution method (CLSI, 2009) was adapted to test extract which showed significant results of antimicrobial activity in the disk diffusion method. Microplates were used to prepare the serial concentrations, being tested the concentrations of 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9 and 0.97 mg.mL^{-1} of extract. The inoculants already standardized on the 0.5 scale of MacFarland were diluted in Broth Mueller Hinton (MH) to obtain the final concentration in each well of 5.0 × 10^5 UFC.mL^{-1}. The microplates were incubated in an oven at 37 ºC for 24 hours and after 50 µL of the 0.01% resazurin indicator was added to each well of the microplate for reading. For each bacterium, four replicates were performed and the Minimum Inhibitory Concentration

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(MIC) was considered the lowest concentration with bacterial inhibition in at least three replicates. Before adding the resazurin indicator, each microplate well was replated using stamping replicators in Petri dishes containing MHA. The plates were incubated for 24 hours at 37 °C. The non-development of bacterial colony at the peak of each well indicated bactericidal activity in 24 hours. Minimum Bactericidal Concentration (MBC) was considered the lowest concentration with bacterial inhibition in at least three replicates. The peaking of the microplate wells in MHA was also performed with only 2, 5 and 8 hours of incubation of the microplate, in order to determine the necessary contact time of the bacteria with the extract (concentrations greater than or equal to MBC) to occur bacterial death.

2.10. Film preparation

Film solutions were prepared adding 5% Soy protein isolate - SPI (Maxsoy® fibras & ingredients, Brazil), 2% glycerol (Dinâmica, Brazil) and distilled water. The pH was adjusted to 11 with NaOH solution (40% w/w) and then it was heated at 65 ºC for 10 minutes in thermostatic bath for protein solubilization. After heating, the solution was stirred on a mechanical stirrer for 2 hours for complete homogenization, and then heated at 70 ºC for 20 minutes. Next, solutions were cooled and the extract with higher antioxidant content (according to experimental design) was added. The solution was poured onto tray and dried on a convective oven (Marconi, model MA 035) for 16 hours at 35 ± 2 °C. Dried films without extract (SPI) and with extract (SPI+JE) were equilibrated in desiccator contained magnesium nitrate- 6-hydrate saturated solution (53% relative humidity) at 25 °C for one week before carry out the analyses.

2.11. Characterization of films without and with extract addition

The thickness of the films was measured at 10 different points using a digital micrometer (Mitutoyo, Japan).

Mechanical properties of the films were measured according to the guidelines of the ASTM D882-12 (ASTM D882-12, 2012) standard method using a texture analyzer (TA.XT Plus, Stable Micro Systems, Surrey, UK) at 25 °C. Tensile strength, elongation-at-break and Young's modulus were obtained using the software EXPONENT, version 5.1.1.0.

The TPC, DPPH, ABTS and FRAP analyzes of the extracts of the films were carried out on the following day of the extraction according to item 2.4.2. For this purpose, the extraction of the film with and without extract was carried out in a proportion of 1:10 (w/v) of film and extracting solution. The ethanol water ratio and ultrasound time used were the same employed to obtain the extract added in film. The cut film was mixed with extracting solution and crushed using a Turratec equipment (Tecnal, TE-102 model—Piracicaba, Brazil) for 5 minutes, then taken to the ultrasonic bath (LojaNetLab SSBu – 3,8 L, 100 W). The solution was centrifuged (MTD III PLUS) at 3000 rpm and the collected supernatant was standardized in volumetric flasks and packed in refrigerated flasks.

The TPC results were expressed in mg of GAE.100g^{-1} of film. The measurements of the DPPH, FRAP and radical ABTS° + methods were based on TROLOX calibration curves and the results expressed in MM of Trolox.100g^{-1} equivalents per of film.

In order to evaluate the action of volatilization of bioactive compounds on the bacteria *Staphylococcus aureus* and *Escherichia coli*, the microatmosphere diffusion method was used in the control and in the films at pH 3.0 (T3) and at pH 11.0 (T11). For this, the method described by Dannenberg *et al*. (2019) with adaptations was adopted. For each bacterium tested, an aliquot of 0.1 mL of approximately 10^3 CFU.mL^{-1} cell suspension was spread on the surface of 90 mm diameter Petri dishes containing Plate Count Agar (PCA). Disks of films T3 and T11 with 6 mm in diameter were placed on the inner surface of the lid of the seeded Petri dishes, in the inverted position. For the control treatment, plates were sown without film in the plate cover. The plates were incubated at 37 ºC for 24 hours. For each treatment, duplicates were performed and plates with counts between 25 and 250 colonies were considered.

The cell integrity of the bacteria was analyzed by determining the release of cell constituents into the supernatant, with adaptations to the method proposed by Diao *et al*. (2014). The activated bacterial cultures were washed and covered with 6 mm disc of films T3 and T11 and 0.5 mL of phosphate buffer solution (pH 7.4). Quadruplicates of the treatments were incubated at 37 ºC for 24 hours and an aliquot of 10 µL was diluted 1:20 in phosphate buffer solution (pH 7.4) and then read on a UV/Vis spectrophotometer (Nanodrop OneC, Thermo Scientific) with wavelength 260 nm.

2.12. Statistical analyses

The data expressed in mean ± standard deviation was statistically analyzed by an analysis of variance (ANOVA) and Tukey's test at a 5% significance level, using Statistic 7.0 software version 7.0 (StatSoft, Inc, Tulsa, USA). Principal Component Analysis

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(PCA) was performed in Past software to verify the relation between antioxidant activity, TPC and total anthocyanins content (ATC).

3. RESULTS AND DISCUSSION

3.1. Total phenolic compounds (TPC), total anthocyanins content (ATC) and antioxidant activity of the jambolan peel extract obtained by ultrasound-assisted extraction

In Table 1, it can be seen that the extracts obtained with the lowest and highest concentration of ethanol (% w/w), trials 5 and 6, respectively, with 40 minutes US showed very close values of TPC which may indicate that the PC extracted in these two conditions have similar interactions and polarities (Caldas *et al*., 2018).

Comparing the results, it is possible to observe that the lower values of AA were obtained by the DPPH method, which indicates that the mechanism of the antioxidant activity of the extracts is principally based on single electron transfer (ABTS value) and ferric-reducing ability (FRAP value) (Yen and Chen, 1995).

In order to evaluate the relation between TPC and ATC extracted from jambolan peels with the AA of the bioactive compounds present in extract, multivariate analysis was employed (Figure 1). The PC1 (principal component) (70.40%) and PC2 (19.84%) together explained 90.24% of the results variation. Antioxidant activity and ATC showed high correlation with PC1 (R value above 84%) while TPC showed higher correlation with PC2 (R = 89,88%). A Pearson's correlation analysis shows a positive and significant ($p \leq 0.05$) relationship between the increasing of ATC and DPPH value (R = 0.90), FRAP value (R = 0.79) and ABTS value (R = 0.81), indicating that the high antioxidant activity of the extracts at pH 3 is more related to ATC than to others PC. This analysis explains why experiment 1 has the second highest antioxidant activity value measured by the DPPH and FRAP method, but has the lowest content of PC among all samples.

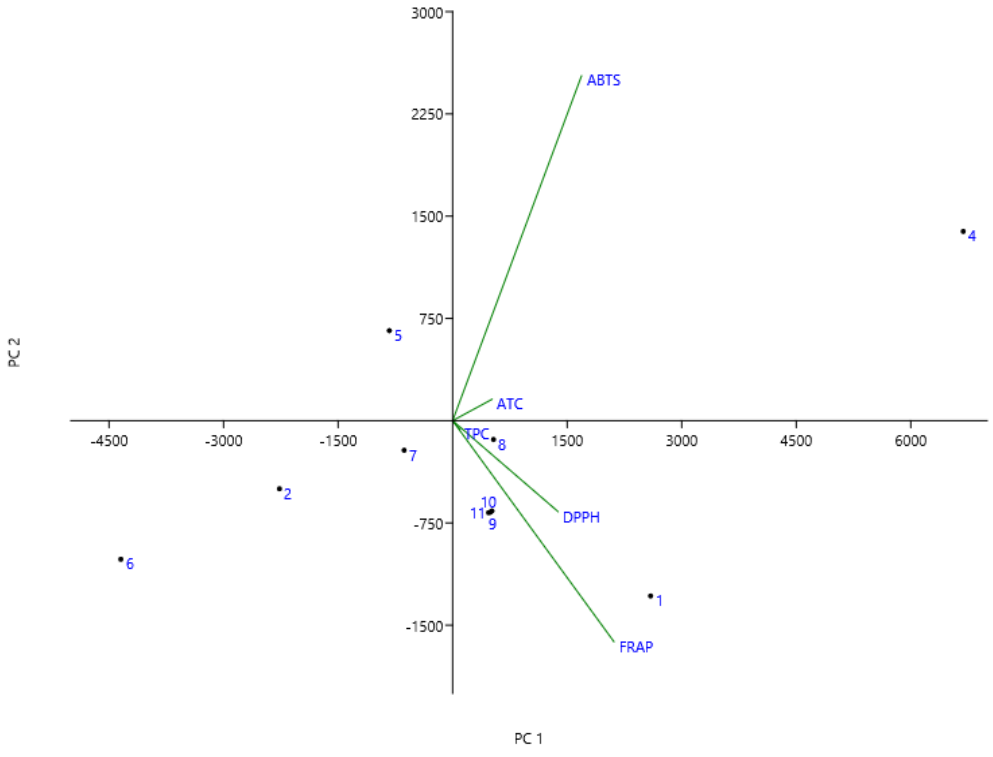

Figure 1: Effect of TPC and ATC on antioxidant activity of the jambolan peel extract.

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The effect of each independent variable on the response was assessed by using a Pareto diagram (Fig. 2). The vertical line represents the minimum value of each effect to be considered statistically significant, with 95% of the confidence interval. The horizontal column lengths indicate the degrees of significance for each effect. All linear and quadratic factors and its interaction had significant critical effects on TPC, ATC, DPPH, FRAP and ABTS values. Effects of ethanol concentration have a negative correlation with the responses analyzed, indicating that the increase in the magnitude of this independent variable can reduce significantly the antioxidant activity, PC and ATC of the jambolan peel extract. Nevertheless, it is possible to observe in Fig 2 that the interaction between extraction time and ethanol concentration was the most significant factor for the increase of the AA (FRAP, DPPH and ABTS value) and ATC in the extracts. The interaction between these variables also presented positive effect on TPC value, however, this factor had lower influence in comparison to quadratic effects of time and ethanol concentration which, in turn, influenced in negative way the TPC of the extracts.

Although ethanol reduces solvent polarity (Curren and King, 2001), it was possible to observe that high ethanol concentrations may reduce the extraction of the PC (Figure 2).

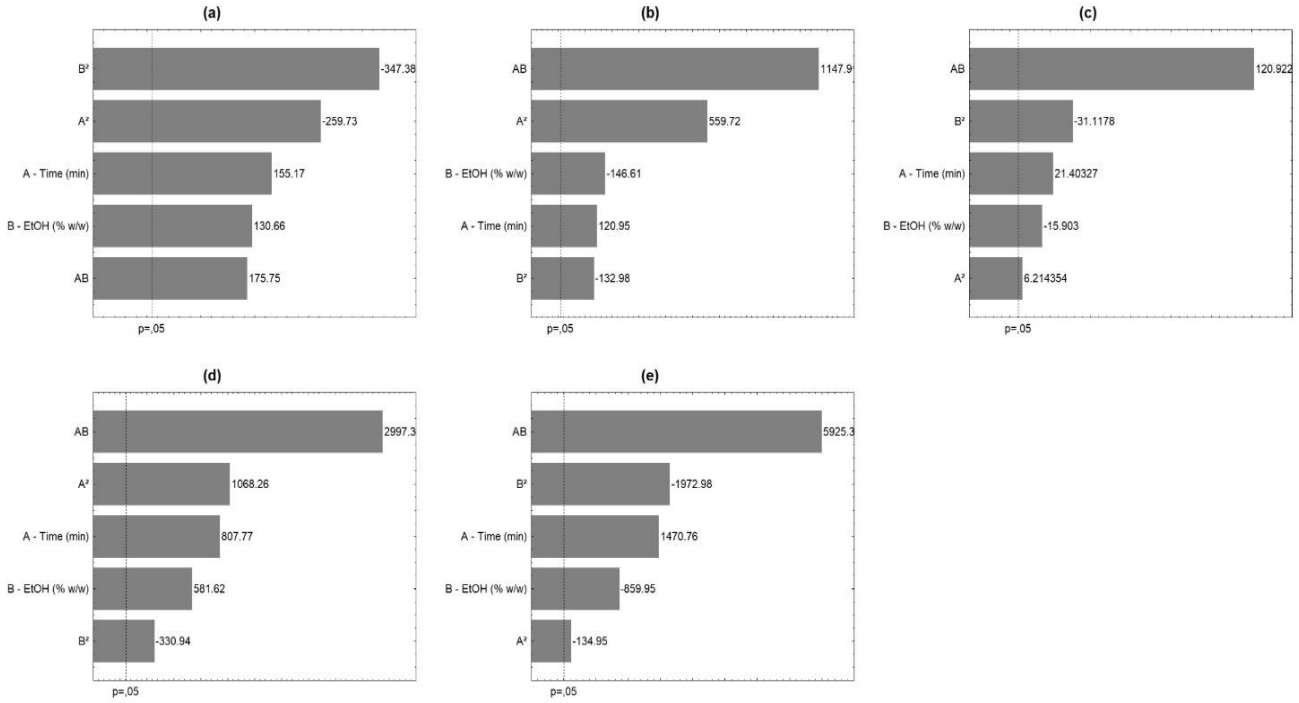

Figure 2: Pareto diagram of the effect of time (A – min) and ethanol concentration (B – EtOH - %) on the TCP – a; ATC – b; FRAP – c; DPPH – d and ABTS – of the jambolan peel extract.

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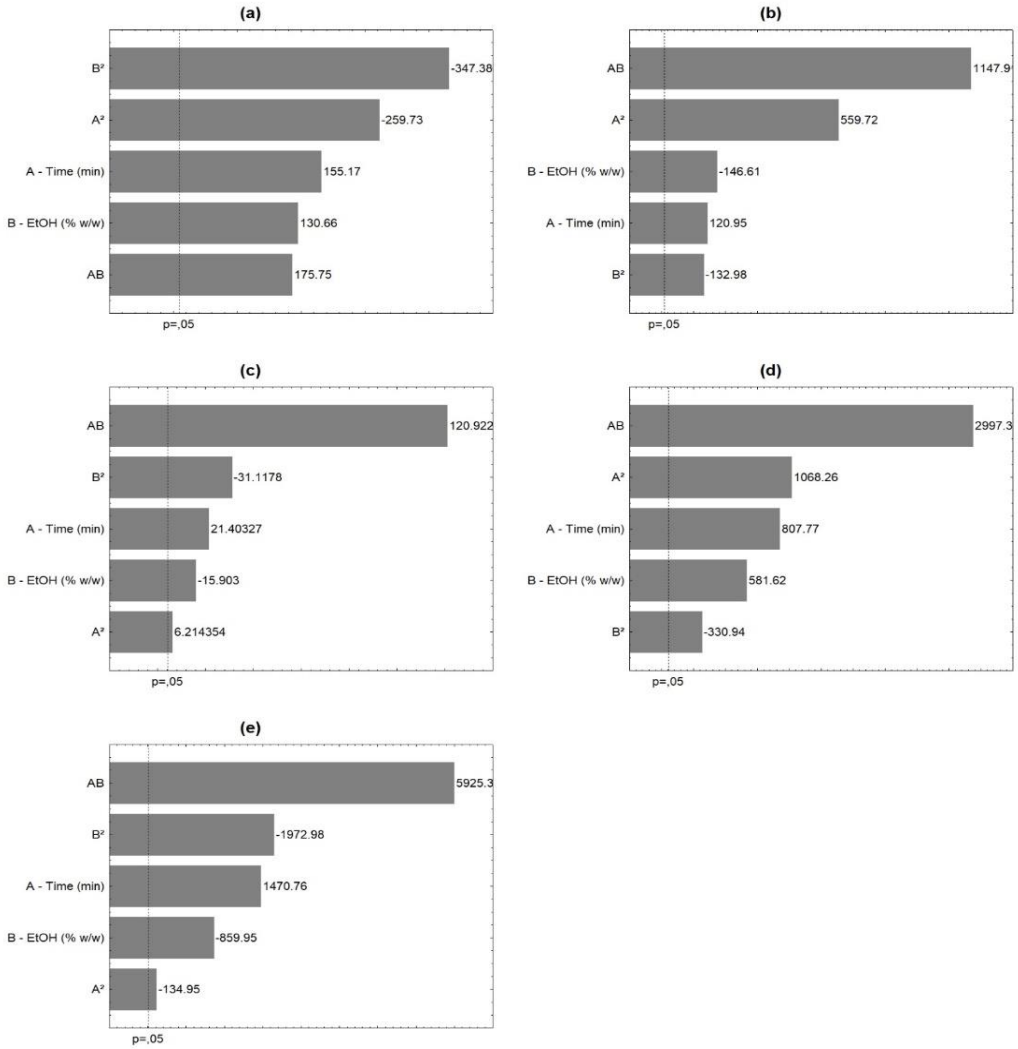

Figure 2: Pareto diagram of the effect of time (A – min) and ethanol concentration (B – EtOH - %) on the TCP – a; ATC – b; FRAP – c; DPPH – d and ABTS – of the jambolan peel extract.

Table II shows analysis of variance (ANOVA) of the obtained data generated which displays F- test to assess the goodness of fit. The calculate F was higher than the tabulated one for TPC, FRAP and DPPH, indicating that the model is predictive and statistically significant ($p < 0.05$) for the obtained experimental results. The value of the determination coefficient (R^2) was acceptable for TPC ($R^2 = 0.70$), but it was remarkable for FRAP and DPPH indicating that 93% of the variability in the antioxidant activity analyzed by FRAP and 82% of the variability analyzed by DPPH could be explained by the fitted model. The mathematic model obtained to FRAP and DPPH presented good accuracy to experimental data (low AAD value), however, the model obtained to TPC shown low accuracy. The influence of the ethanol concentration and extraction time on total ATC and the ability of antioxidant from jambolan peel extracts to reduce the radical cation ABTS+ to ABTS could not be explained by the model due to calculate F lower than tabulated F.

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Table II – ANOVA for responses surface quadratic model.

Source	Df	Sum of Square				
		TPC (mg GAE/100g)	Anthocyanin (mg mv-3,5-dglc/100g)	DPPH (µM Trolox/100g)	FRAP (µM Trolox/100g)	ABTS°⁺ (µM Trolox/100g)
A-Time (min)	1	47672.90***	28965.24***	442834.49**	4283033.60***	1291922.27***
B-EtOH (% w/w)	1	33804.24***	42556.01***	24478.71*	1464234.87***	669805.07***
AB	1	30887.61***	1317703.28***	14134948.21***	31644314.70***	8984155.38***
A^2	1	92693.69***	430477.39***	37331.30**	25025.17***	1568034.00***
B^2	1	16513.72***	24299.39***	936053.52**	5348706.94***	150485.36**
Residual	5	133799.33	1104075.48	341927.92	3189978.12	24361653.73
Variance analysis of quadratic square						
F-Tabulated		5.05	5.05	5.05	5.05	5.05
F-Calculated		8.32	5.01	13.85	40.22	1.56
R-Squared		0.70311	0.63819	0.82382	0.93096	0.34998
AAD (%)		40.12	---	1.41	4.97	---

*Significant at ($p < 0.05$)
** Significant at ($p < 0.01$)
***Significant at ($p < 0.001$)

In cases which the model was predictive, the 3D response surface and 2-D contour plots were plotted (Fig. 3 and 4).

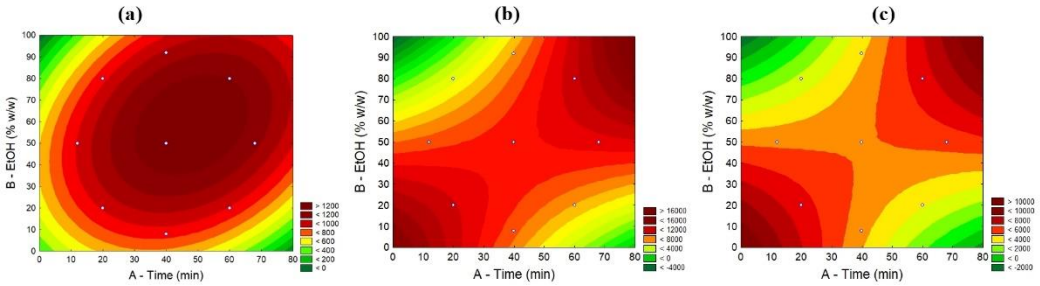

Figure 3: 2D surface plots of TPC (a) and antioxidant activity (FRAP – b; DPPH – c) of jambolan peel extract as affected by time (A – min) and ethanol concentration (B – EtOH - %).

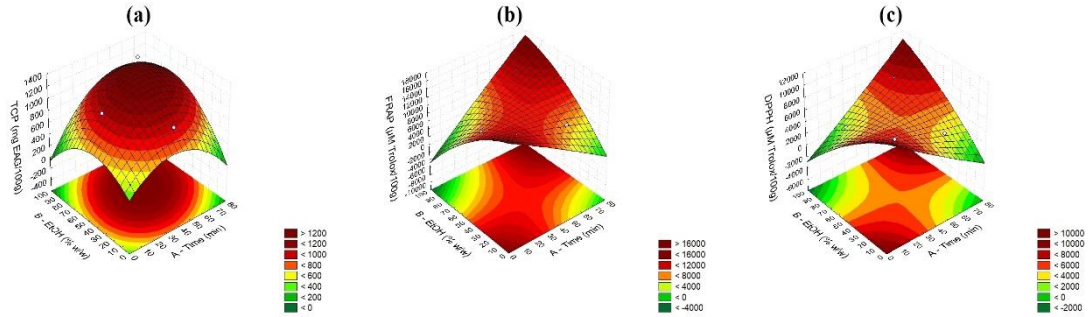

Figure 4: 3D surface plots of TPC (a) and antioxidant activity (FRAP – b; DPPH – c) of jambolan peel extract as affected by time (A – min) and ethanol concentration (B – EtOH - %).

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Based on the significant terms, the regression equation for the US efficiency was obtained Eq 2, 3 and 4.

$TPC = 299.76 + 22.53A - 0.32A^2 + 15.62B - 0.19B^2 + 0.15AB$ (2)

$DPPH = 10183.41 - 161.31A + 0.21A^2 - 85.32B - 0.46B^2 + 3.13AB$ (3)

$FRAP = 15263.67 - 184.12A - 0.17A^2 - 92.23B - 1.10B^2 + 4.69AB$ (4)

Extraction condition used to obtain extract 4 (80% (w/w) ethanol and 60 minutes of US) was the best condition for extracting the bioactive compounds from the dry jambolan peel (Table 1), since it presented the highest TPC values, ATC and AA. The condition used to obtain extract 4 resulted in TPC and ATC values 88 and 75% higher, respectively, when compared to the process condition less efficient (Trial 1, TPC = 696.92 ± 3.00 mg GAE / 100g and Trial 6, ATC = 673.67 ± 4.67 mv-3.5-dglc).

3.2. Antibacterial activity *of the jambolan peel extract*

In the disc-diffusion test only extract obtained by trial 6 developed a significant inhibition halo for both *Staphylococcus aureus* and *Escherichia coli* (median equal to 10mm).

In the microdilution test for extract n. 6 a MIC of 7.8 $mg.mL^{-1}$ against *Staphylococcus aureus* and 15.6 $mg.mL^{-1}$ against *Escherichia coli* was determined. The values of inhibition halo and MIC found for extract n. 6 were 0.5, 2.0 and 2.0 for *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA) and *Escherichia coli*, respectively.

According to Table III, with only two hours of contact with jambolan extract n. 6 it was sufficient to cause the mortality of all cells of the two bacteria in the concentration of 62.5 $mg \cdot mL^{-1}$. After 24 hours of contact, it was found that the lowest concentration in which there was no bacterial growth was 31.2 $mg \cdot mL^{-1}$, then this concentration was considered as the MBC in both tested bacteria. It is possible to observe in Table 1 that the extraction conditions used to obtain extract 6 resulted in the extraction of PC with low AA, however, the extracted phenols had high bactericidal action against the microorganisms tested (Table III).

Table II – Minimum Bactericidal Concentrations (MBC) of jambolan extract n.6 for the bacteria tested in each incubation time interval.

Bacterials	**Time in hour of MBC (**$mg.mL^{-1}$)			
	2	**5**	**8**	**24**
Staphylococcus aureus (ATCC 29213)	62.5	62.5	62.5	31.2
Escherichia coli (ATCC 25922)	62.5	62.5	31.2	31.2

3.3. Effect of jambolan peel extract addition on properties of the films

According to Table 1 and Pearson's correlation analysis (Figure 1) the higher AA of the jambolan peel extracts (JE) at pH 3 is related to the ATC. The anthocyanin is more stable in solutions at acidic pH than basic one and this stability is related to the changing chemical form of the anthocyanin with the variation pH of the solution (Sui *et al*., 2014). However, Faria *et al.* (2011) studied the influence of pH in extraction of bioactive compounds of pulp and peel of jambolan and observed that obtained ethanolic extract at basic pH presented higher free radical scavenging capacity than at acid pH. According to authors, the colourless (hemiacetals/chalcones) and quinonoidal base forms of anthocyanins from pulp and peel jambolan, obtained at basic pH, presented higher reducing capacity than flavylium cation species, obtained at acid pH.

Aiming to study the influence of extract rich in flavylium cation species (acid pH) and extract rich in colourless (hemiacetals/chalcones) and quinonoidal base forms of anthocyanins (basic pH), SPI films with JE addition were manufactured at pH 3 and 11. Therefore, extract n. 4 was used to manufacture SPI + JE, due to its higher content of TCP, DPPH, FRAP and ABTS $^{\circ+}$ (Table I). In preliminary tests, it was identified that the addition of 4% of extract in the solution was the maximum quantity necessary to form continuous films at pH 11 (Figure 5). At pH 3, however, it was not possible to obtain homogeneous films without cracking, adding any quantity of the extract. Thus, the production of SPI films with the addition of JE at pH 3.0 would not be commercially viable. For this reason, films with and without JE addition at pH 11 were manufactured and characterized by mechanical properties, polyphenol content and AA.

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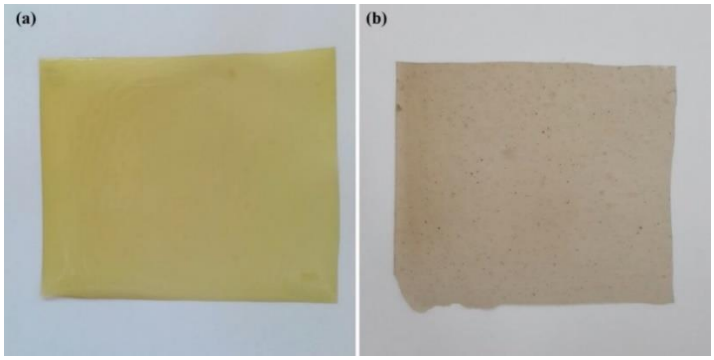

Figure 5 – Images of the produced SPI film without (a) and with (b) JE addition, both at pH 11.

Despite the low viability of producing SPI + JE films at pH 3.0, the application of this solution in acidic pH as a coating on fresh meats could have potential if it were found that films at this pH were efficient to inhibit the bacterial activity of meat products. Considering this hypothesis, films with and without JE addition at pH 3 and 11 were manufactured and characterized by antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Table V). For this purpose, extract n. 6 was used to manufacture SPI + JE, as it developed an inhibition halo for both microorganisms tested, as explained in items 3.2

Visual aspect of the films manufactured is presented in Figure 5. Tensile strength (TS), elongation-at-break (EB), young module (YM), TPC and antioxidant activity values of the SPI films with or without jambolan peel extract (JE) addition are present in Table

Table IV. Chemical physical properties of the SPI films with and without jambolan peel extract (JE).

Formulação	TS (MPa)	EB (%)	YM (MPa)	TCP (mg GAE/100g film)	DPPH (µM Trolox/100g film)	FRAP (µM Trolox/100g film)	ABTS (µM Trolox/100g film)
SPI	3.90 ± 0.39^{a}	33.20 ± 3.52^{b}	51.79 ± 10.22^{a}	62.62 ± 0.50^{a}	394.60 ± 8.04^{a}	0.01 ± 0.00^{a}	784.33 ± 7.15^{a}
SPI + 8g JE	2.69 ± 0.24^{b}	131.36 ± 39.68^{a}	28.81 ± 7.79^{b}	379.40 ± 6.10^{b}	1540.29 ± 4.46^{b}	0.22 ± 0.001^{b}	2860.77 ± 15.21^{b}

Mean ± SD.
Means with the same letter, in the same column, did not differ significantly at $p < 0.05$ according to the Tukey test.

Table IV. Chemical physical properties of the SPI films with and without jambolan peel extract (JE).

Properties	SPI	SPI+ 8g JE
TS (MPa)	3.90 ± 0.39^{a}	2.69 ± 0.24^{b}
EB (%)	33.20 ± 3.52^{b}	131.36 ± 39.68^{a}
YM (MPa)	51.79 ± 10.22^{a}	28.81 ± 7.79^{b}
TCP (mg GAE/100g film)	62.62 ± 0.50^{a}	379.40 ± 6.10^{b}
DPPH (µM Trolox/100g film)	394.60 ± 8.04^{a}	1540.29 ± 4.46^{b}
FRAP (µM Trolox/100g film)	0.01 ± 0.00^{a}	0.22 ± 0.001^{b}
ABTS (µM Trolox/100g film)	784.33 ± 7.15^{a}	2860.77 ± 15.21^{b}

Mean ± SD. Means with the same letter, in the same line, did not differ significantly at $p < 0.05$ according to the Tukey test.

Tensile strength and young module of SPI + JE film were 31% and 44%, respectively, smaller than TS and YM of SPI film without extract addition, however EB was almost 4 times higher. Soy protein contains four globulin fractions in its composition, the main ones being glycinin (11S) and conglycinin (7S). The Glycinin represents almost 30% of total protein in soybeans (Janjarasskul

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and Krochta, 2010; Song *et al*., 2011). The tendency of glycinin to form disulfide bridges is greater than conglycinin, moreover the bridges formed between the 11S fraction are stronger than bridges formed by the 7S fraction (Song *et al*., 2011). Caffeic acid, present in large quantities in the jambolan peel (Tavares *et al*., 2016), has a great capacity to react with thiol groups of the glycinin and to reduce the formation of the disulfide bridges between the protein chains (Rawel *et al.*, 2002). This reduction increases the molecular mobility of the polymer chains resulting in greater flexibility of the film.

SPI film showed considerable values of AA and TPC, which can be attributed to the amino acids (phenylalanine, tyrosine, and tryptophan) and PC (isoflavones and chlorogenic, caffeic, and ferulic acids) naturally present in SPI (Amigo-Benavent *et al.* 2008, Ciannamea *et al*., 2016; Kang *et al*., 2013). JE addition increased, significantly, the content of total phenolic compounds (TPC – 6×) and the antioxidant activity (DPPH ≅ 4×; FRAP – 22×; ABTS$^{\circ+}$ ≅ 3,6×) of the SPI films.

The results of colony counts on the plates subjected to microatmosphere diffusion of the tested films are shown in Table 5.

Table V – Colony counts obtained in the microatmosphere diffusion assay for control films and SPI films + extract 6 at pH 3.0 (T3) and 11.0 (T11).

Treatments	**Colony count (UFC.mL^{-1})** *Staphylococcus aureus* (ATCC 29213)	*Escherichia coli* (ATCC 25922)
Control	1665 ± 261^{a}	3375 ± 714^{a}
T3	1490 ±84^{ab}	3185 ± 289^{a}
T11	868 ± 2^{b}	1107 ± 81^{b}

Different letters in the same column indicate a significant difference of 5%.

Considering the bacterial counts of the control treatment as total growth, it was possible to verify that T11 was more efficient in bacterial inhibition than T3, because the treatment T3 reduced 10.51% and 5.63% of the colonies of *Staphylococcus aureus* and *Escherichia coli*, respectively and T11 reduced 47.86% and 67.20% for the respective bacteria. Possibly the less folded structure of the protein at pH 11.0 (Nishinari *et al.*, 2014) provided greater volatilization of bioactive compounds when compared to the more globular structure of SPI at pH 3.0. The volatilized compounds came into contact with the bacteria fixed on the plate resulting in a reduction in the number of colonies of the bacteria.

The effect of films with extract n. 6 on the integrity of the bacterial cell membrane was investigated by the principle of the release of cell constituents due to changes in the integrity of the cell membrane. The greater the quantity of constituents released, detected by the absorbance of the solution, the greater the damage caused to the cell membrane, with consequent ruptures. Results are presented in Table 6.

Table VI - Effect of SPI films + extract 6 on pH 3.0 (T3) and 11.0 (T11) on the release of bacterial cell constituents.

Treatments	**Absorbance (λ = 260 nm)** ***Staphylococcus aureus*** **(ATCC 29213)**	***Escherichia coli*** **(ATCC 25922)**
Control	-0.0250 ± 0.057^{a}	0.0333 ± 0.0153^{a}
T3	0.3525 ± 0.1801^{a}	0.1950 ± 0.0896^{a}
T3S	1.0075 ± 0.0310^{b}	0.7125 ± 0.1797^{b}
T11	0.3100 ± 0.0183^{a}	0.2100 ± 0.0183^{a}
T11S	1.8300 ±0.4503^{c}	0.9325 ± 0.2943^{b}

Different letters in the same column indicate a significant difference of 5%.

When comparing the pH influence, it is noted that the film at pH 11 promoted greater damage to the cell membrane of *Staphylococcus aureus* than the film at pH 3. This result may be linked to two factors: one of them is that soy protein has greater solubility at pH 11.0 than at pH 3.0 (Amado *et al.*, 2019) which may have facilitated the dissolution of bioactive compounds in the

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solution; and the other is that, probably, bioactive compounds with greater inhibition of bacterial activity of *S. aureus* have greater action at basic pH than at acidic pH, as was reported by Faria *et al*. (2011) for antioxidant activity of jambolan peel extracts at basic pH. For *E. coli*, however, it is noted that the increase in the amount of constituents released by the bacteria in contact with the active film (higher absorbance value) was not significant with the increase in pH, although it is possible to observe a clear trend of increase in bacterial cell membrane damage at basic pH.

4. CONCLUSION

High ethanol concentrations may reduce the extraction of the antioxidant activity, PC content and anthocyanin content of the jambolan peel extract. Interaction between extraction time and ethanol concentration presented positive effect on antioxidant activity, TPC and monomeric anthocyanin content in the extracts. The extract obtained in the optimized condition could be a good option to be used as a dye and natural antioxidant in functional foods, drinks and active edible films. Extract with bactericidal action against the *Staphylococcus aureus* and *Escherichia coli* was not obtained by optimized conditions.

SPI + JE films were manufactured at pH 3 and 11, however, only films at pH 11 were homogeneous and without cracking.

Extract addition reduced the tensile strength of the SPI films at pH 11, but increase significantly the elasticity (4 times) and the antioxidant capacity (DPPH ≅ 4×; FRAP – 22×; $ABTS^{\circ+}$ ≅ 3,6×) of the packaging.

Jambolan peel extract addition in SPI films can be an alternative to produce active packaging that limit undesirable oxidative processes in food like lipid oxidation in meat and meat products. Moreover, the antimicrobial behavior of SPI films with jambolan peel extract addition at pH 11, demonstrated by the microatmosphere diffusion methods and the effect of these films on the integrity of the bacterial cell membrane when in contact with the cells of the *S. aureus* and *E. coli*, confirm the possibility of applying these films as protective food packaging in order to inhibit the bacterial development and prolong product life.

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