

# Granadilla seed extract as antimicrobial and bioactive compounds source: mathematical modelling of extraction conditions

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## RESEARCH ARTICLE

### Abstract

Extracts of *Passiflora ligularis* Juss. (granadilla) residues (peel, seed and mixed peel, seed) were initially evaluated for antimicrobial potential against *Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Serratia marcescens*, *Escherichia coli* and *Salmonella enteritidis*. Notably, 80% acetone granadilla seed extract showed inhibitory activity against major bacteria (five) compared to the other extracts. Next, the extraction conditions of this extract were optimised, varying the solid/solvent ratio (0.5:10-6.0:10), incubation time (10-60 min) and temperature (30-60 °C), to obtain maximum antimicrobial activity, total phenolic content (TPC) and total flavonoid content (TFC). A central composite rotatable design (2<sup>3</sup> factorial arrangement with six axial points and three central points) was used. The temperature and solid/solvent ratio were most significant parameters to obtain extracts with antibacterial potential and significant bioactive compounds content (TPC and TFC). The response surfaces indicated that extracts obtained with increased solid/solvent molar ratio and temperature possess maximum inhibitory potential against most of the tested bacteria. However, extracts obtained with decreased molar ratio and increased temperature will have maximum TPC and TFC, respectively. Collectively, the data demonstrate that granadilla residue extracts have excellent potential as a source of natural antimicrobial and bioactive compounds, under optimised extraction conditions.

**Keywords:** optimisation, exotic fruit, fruit residue, polyphenols, response surface

## 1. Introduction

*Passiflora ligularis* Juss., commonly called sweet granadilla, belongs to the Passifloraceae family, which comprises more than 500 species arranged in 18 genera. *P. ligularis* is highlighted, not only because it is the largest genus of the family but also due to its economic importance (Pereira *et al.*, 2015). This exotic fruit can be found in the cool highlands of Indonesia, New Guinea, Jamaica, Sri Lanka, India and several other tropical regions of the world. The granadilla fruit pulp consists of a considerable amount of nutrients, like proteins, carbohydrates, amino acids, vitamin C and crude fibre, and the peel primarily possesses (1→4)-linked galacturonic acid, along with sugars, such as xylose, glucose, galactose, galactosamine and fructose. The acetone

extract of *P. ligularis* pulp has shown effective antioxidant, antidiabetic and antimicrobial activities (Saravanan and Parimelazhagan, 2014), and the ethanolic extract of the plant material has exhibited relevant antiproliferative activity against human hepatocellular carcinoma cells (Carraz *et al.*, 2015). Navarro *et al.* (2014) also verified that hydroalcoholic extracts of leaves and flowers of granadilla showed higher antioxidant potential than the aqueous one, which was more efficient as antimicrobial against *Escherichia coli* and *Staphylococcus aureus*. Ethyl acetate extracts of granadilla plant material showed strongest antimicrobial activity against gram-positive bacteria and ethanol extracts higher antioxidant potential (Vasic *et al.*, 2012). No report about granadilla residues was found.

Considering that by-products of exotic tropical fruits can sometimes exhibit higher amounts of bioactive compounds than the edible parts, many studies have focused on exploring recovery technologies and new applications for these kinds of wastes (Mirabella *et al.*, 2018). The solvent extraction technique is most commonly used for the preparation of extracts from plant materials, due to its efficiency, ease of use and wide applicability. However, the yield of solvent extraction depends on several factors, including the type of solvents with varying polarities, solid/solvent ratio, extraction time and temperature (Dai and Mumper, 2010). The response surface methodology (RSM) is a statistical tool that enables the evaluation of the effects of many factors and their interactions on response variables. Its principal advantage is the reduced number of experimental runs required to generate sufficient information for a statistically reliable result (Chen *et al.*, 2004). For instance, Sood and Gupta (2015) investigated the effect of solid/solvent ratio, incubation time and temperature on the bioactive content of pomegranate (*Punica granatum* L.) peels using RSM. However, to the authors best knowledge, this approach has not yet been attempted on granadilla residues extracts. Therefore, this research evaluated the effect of extraction conditions on the antimicrobial activity, total phenolic content (TPC) and total flavonoid content (TFC) of *P. ligularis* Juss. (granadilla) residue extracts using RSM.

## 2. Materials and methods

### Bacteria

*Pseudomonas aeruginosa* (INCQS 00025/ATCC 1544), *S. aureus* (INCQS 00014/ATCC 13150), *Bacillus cereus* (INCQS 00003/ATCC 11778), *Bacillus subtilis* (INCQS 00002/ATCC 19659), *Enterococcus faecalis* (INCQS 00531/ATCC 51299), *Serratia marcescens* (INCQS 00131/ATCC 14756), *E. coli* (INCQS 00032/ATCC 11229) and *Salmonella enteritidis* (INCQS 00258/ATCC 13076) were provided by the National Institute of Health and Quality Control/Oswaldo Cruz Foundation (Manguinhos, Rio de Janeiro, Brazil). The strains were stored frozen (-80 °C) in brain heart infusion broth with 20% glycerol. All culture media were purchased from Oxoid (São Paulo, Brazil).

### Obtention of granadilla residues

Fresh granadilla fruit (mature stage) was acquired from the local market of Aracaju, Sergipe. The fruit was sanitised with 200 mg/kg sodium hypochloride solution for 15 min. The peels and seeds were manually separated and dried at 50 °C for 24 h and then crushed in a blender to obtain a uniform powder.

### Preparation of extracts of granadilla residues

The extracts of the residues (peels, seeds and a mix of peels and seeds) were prepared with distilled water and acetone, methanol and ethanol solutions, respectively, at 40, 50, 60, 70 and 80% (v/v) (Sujata *et al.*, 2011). Erlenmeyer flasks, containing 2 g residue and 10 ml solvent, were maintained under orbital shaking at 200 rpm, 30 °C for 60 min. Then, the extracts were filtered through qualitative filter paper, and the antibacterial activity of the filtrate was determined by disk diffusion test. All the experiments were performed in triplicate.

### Antibacterial activity of granadilla extracts

The antibacterial activity of granadilla extracts was evaluated by the disk diffusion method (in triplicate) against eight foodborne bacteria (CLSI, 2015). Petri plates containing Mueller-Hinton agar were inoculated with  $1.5 \times 10^8$  cfu/ml of each bacterial strain, adjusted to McFarland standard 0.5. Sterile filter paper disks (6 mm) containing 10 µl of the extracts were placed on the agar surface with sterile forceps (four disks per plate) and incubated at 37 °C for 24 h. The antibiotics, gentamicin (30 µg/disk) and chloramphenicol (30 µg/disk), were used as positive controls. The diameters of the inhibition zone (DIZ) were measured with a calliper and expressed in millimetres (disk diameter included). The sensitivity to extracts was classified as not sensitive (diameters <8 mm), sensitive (diameters 9-14 mm); very sensitive (diameters 15-19 mm) and extremely sensitive (diameters >20 mm) (Djenane *et al.*, 2011).

### Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs) were determined for those with the higher antibacterial activity (according to the disk diffusion tests) among the extracts, using the broth microdilution (CLSI, 2015). Cultures of each bacterial strain were obtained from 24 h broth cultures and adjusted to 0.5 McFarland standard turbidity ( $1 \times 10^8$  cfu/ml). Bacterial suspensions were then diluted to  $1 \times 10^6$  cfu/ml in Mueller-Hinton broth. Extracts were prepared in a concentration range of 0.78-400 µl/ml. Next, 100 µl of each concentration was placed in a 96-well microplate inoculated with 100 µl of each bacterial strain. The microplate was incubated aerobically at 37 °C for 24 h. The MIC was defined as the lowest concentration of extract required to prevent visible bacterial growth. The solvent and bacterial suspension were used as negative and positive controls, respectively. The MBC was determined by sub-culturing 100 µl aliquots from MIC wells with no visible growth onto Mueller-Hinton agar plates and incubating at 37 °C for 24 h. The lowest concentration that yielded no visible colonies was considered the MBC.

## Experimental design

For the residue extract that showed the highest antibacterial potential, the effect of extraction conditions (temperature from 30 to 60 °C, solid/solvent ratio from 0.5:10 to 6.0:10 and incubation time from 10 to 60 min) on their bioactivity (antimicrobial activity and bioactive content) was investigated using RSM.

A central composite rotatable design, including a  $2^3$  factorial arrangement, with six axial points ( $\pm\alpha=(2^k)^{1/4}$ , where  $k$ =number of independent variables) and three central points (to evaluate the repeatability of the analysis and determine the pure error of the model) was performed, to obtain a second-order model, according to Equation 1:

$$y = \beta_0 + \sum_j \beta_j x_j + \sum_{i < j} \beta_{ij} x_i x_j + \sum_j \beta_{jj} x_j^2 + \varepsilon \quad (1)$$

where  $y$  is the predicted response (DIZ, TPC or TFC),  $\beta_0$  is the global mean,  $\beta_j$  is the linear coefficient,  $\beta_{ij}$  is the coefficient of interaction,  $\beta_{jj}$  is the quadratic coefficient,  $\varepsilon$  is the error of the model, and  $x_i$  and  $x_j$  are the coded values of the independent variables.

The experimental data were analysed using Statistica software, version 8.0 (Statsoft, Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) tables were generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms were judged statistically, at the  $P < 0.05$  significance level. The quality of fit of the model equation was expressed by the coefficient of determination ( $R^2$ ), and its statistical significance was determined using the F test. For validation of the statistical results, the observed values (DIZ, TPC, TFC) were compared with the predicted values. Additional experiments were subsequently performed to validate the statistical experimental design.

## Estimation of total phenolic content

The TPC of the extracts was determined by the Folin-Ciocalteu colourimetric method, with modifications (Shetty *et al.*, 1995). Extracts of the residues (1 ml) were mixed with 1 ml of 95% ethanol, 5 ml distilled water and 0.5 ml Folin-Ciocalteu reagent (1 N). After homogenisation, 1 ml of 5% (w/v) sodium carbonate solution was added. The samples were vortexed and then rested for 60 min. Next, the spectrophotometric absorbance at 725 nm was measured. The control was prepared by replacing the sample with 95% ethanol. All determinations were made in triplicate, and the values were calculated from the gallic acid calibration curve (0-500 mg/l). Results were expressed as g of gallic acid equivalents (GAE) per 100 g of residue (GAE/100 g of residue).

## Estimation of total flavonoid content

The TFC of each extract was investigated using the aluminium chloride colourimetry method, with modifications (Dewanto *et al.*, 2002). The extract (2 ml) was mixed with 2 ml of 2% (w/v) aluminium chloride solution and vortexed. The mixture was kept at room temperature for 30 min. Then, the absorbance of the mixture was measured at 415 nm, using a UV-Vis spectrophotometer. The flavonoid content was expressed as mg quercetin equivalents (QE) per 100 g of residue (mg QE/100 g of residue).

## Identification of bioactive compounds using high-performance liquid chromatography system

The granadilla extracts, that showed higher antibacterial potential and TPC (from results shown in Table 2 and 5), were analysed using a Prominence ultra fast liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of two pumps (LC-20D), a degasser (DGU-20 A3), injector (SIL-20A HT), a photodiode array detector (SPD-M20A) and a data controller (CBM-20A). The data were processed using Lab Solutions software. The column used was Ascents Express F5 (150×2.1 mm, 2.7 µm particle; Sigma Aldrich, São Paulo, Brazil) operating at a flow rate of 0.2 ml/min and a temperature of 40 °C. The mobile phases were ultrapure water + 0.1% formic acid (Phase A) and acetonitrile + 0.1% formic acid (Phase B). The injection volume was 5 µl. The gradient elution was as follows: 0-1 min, 15% B; 1-7 min, 25% B; 7-9 min, 25% B; 9-13 min, 30% B; 13-16 min, 30%; 16-21 min, 40%; 21-23 min, 40% B; 23-25 min, 45% B; 25-28 min, 50% B; 28-33 min, 60% B; 33-37 min, 75% B; 37-38%, 15% B (Andrade *et al.*, 2017 with modifications). The identification of individual compounds was performed on the basis of their retention times comparing with retention times of each phenolic standard.

## Statistical analysis

The results were expressed as the average ± standard deviation. The data were compared by Tukey's range test at the  $P < 0.05$  significance level, using Assisat 7.7 beta software developed by Dr Francisco A.S. Silva of Federal University of Campina Grande, Brazil.

## 3. Results and discussion

### Screening of granadilla residue extract with antibacterial potential

The sensitivity of foodborne bacteria to the various granadilla residue extracts is provided in Table 1. Among the tested microorganisms, the acetone (40 to 80%) seed extracts were more effective to inhibit *B. cereus*, *B. subtilis*, *E. faecalis*, *P. aeruginosa* and *S. aureus*, with no significant

differences among them ( $P>0.05$ ). *E. coli* was most sensitive to 80% acetone and 80% methanol seed extracts, without significant difference between them ( $P>0.05$ ). The 80% methanol seed extract was effective against all bacteria, except *B. subtilis* and *S. aureus*. Methanol has been widely used as extractor solvent due to its adequate polarity. The methanolic pomegranate extracts have also been effective against *Listeria monocytogenes*, *S. aureus*, *E. coli* and *Yersinia enterocolitica* (Al-Zoreky, 2009; Ismail *et al.*, 2012). No bacteria were sensitive to granadilla peel extracts, except *S. marcescens*, which was sensitive to these extracts in 70 and 80% ethanol. *B. cereus*, *S. marcescens* and *S. aureus* were not sensitive to the granadilla mixed peel and seed extracts. The mixed peel and seed extracts in 70 and 80% acetone, 70 and 80% ethanol and 80% methanol, respectively, showed no significant differences in inhibition of *B. subtilis*. Conversely, *E. coli*, *E. faecalis* and *P. aeruginosa* were inhibited with extracts of mixed peel and seed in 70 and 80% acetone or 70 and 80% methanol, with no significant difference ( $P>0.05$ ) between them. *S. enteritidis*, *S. marcescens* and *S. aureus* were not sensitive to the mixed peel and seed extracts, and no bacterium was sensitive to distilled water extracts.

Granadilla seed extract in 80% acetone was more effective at inhibiting the tested bacteria than the other extracts. Plant byproducts such as peels, seeds, pulps and others are natural sources of valuable components as polyphenols, tannins, and flavonoids (Gyawali and Ibrahim, 2014). The phenolic compounds possess great structural variations and the presence of groups as hydroxyl (-OH) are thought to cause the antimicrobial effect (Lai and Roy, 2004). These groups can interact with the cell membrane of bacteria to disrupt membrane structures and cause the leakage of cellular components (Xue *et al.*, 2013). Then, probably the higher inhibitory effect of acetone granadilla seed extracts be due the higher quantity of antimicrobial phenolics compounds when compared with the others extracts. Saravanan and Parimelazhagan (2014) tested the antimicrobial activity of granadilla pulp extract and also found the acetone extract exhibited a potent inhibitory effect against, particularly, *Salmonella paratyphi* (28 mm), *Salmonella typhi* A (31 mm), *S. typhi* B (32 mm) and *Klebsiella pneumonia* (32 mm).

It is often difficult to compare studies with plant extracts, so Alves *et al.* (2000) suggested a classification scheme, where extracts with halos <9 mm are classified as inactive, from 9-12 mm, partially active, from 13-18 mm active, and >18 mm as very active. In this context, the granadilla residue extracts were considered partially active against the tested bacteria. Particularly, the seed extract in 80% acetone presented MIC and MBC values of 0.39 and 200 µl/ml, respectively, for all bacteria. Based on Aligiannis *et al.* (2001), who classified plant material antimicrobial activity as strong (MIC<0.5 µl/ml), moderate (MIC 0.6-1.5 µl/ml), and weak (MIC>1.6 µl/ml), the seed extract in 80% acetone possessed a strong antimicrobial activity.

### Predictive models for antibacterial activity of 80% acetone granadilla seed extract

The extraction conditions used to obtain 80% acetone granadilla seed extracts were varied, and the resultant antibacterial activities of the extracts are given in Table 2. The majority of the bacteria were sensitive-to-very sensitive to all extracts (DIZ values of 9.0 to 16.7 mm). The impact of the extraction conditions on the antimicrobial potential of the extracts was evaluated through RSM. It was possible to obtain mathematical models of inhibition for all bacteria, except *P. aeruginosa*, which had a low  $R^2$  value of 0.62 by the regression analysis. A relatively high  $R^2$  (close to 1) is desirable, as it indicates the authenticity of the equation representing the system, under the given experimental domain.

Among the parameters, temperature (quadratic parameter) was the more significant for *B. cereus* and *S. aureus* inhibition ( $P<0.05$ ; Table 3). In contrast, the solid/solvent ratio was more significant regarding the inhibition of *E. coli*, *E. faecalis*, *S. marcescens* and *S. enteritidis* ( $P<0.05$ ; Table 3). Hence, both temperature and solid/solvent ratio had a positive effect against these bacteria, i.e. higher values imply a higher antibacterial activity. For *B. subtilis*, the interaction of temperature and extraction time was the most significant for inhibition. However, the negative effect indicated that higher values of combined temperature and extraction time could result in extracts with low antimicrobial activity against *B. subtilis*.

The statistical significance of the model was verified by F test values (ANOVA) (Table 4), which were approximately 6, 7, 12, 27, 11, 13 and 6 times higher than the F tabulated values, for *B. cereus*, *B. subtilis*, *S. aureus*, *E. faecalis*, *E. coli*, *S. marcescens* and *S. enteritidis*, respectively. The  $R^2$  values ranged from 0.80-0.93, indicating a high degree of correlation between the experimental values and the fitted values. Consequently, the models were considered sufficiently accurate for predicting the inhibition of the bacteria, as a function of the significant parameters. The predictive models for the inhibition of each bacteria are shown in Equation 2-8:

*B. cereus*:

$$\text{DIZ (mm)} = 10.6 + 1.8X_1^2 + 1.0X_2 - 1.7X_1X_3 \quad (2)$$

*B. subtilis*:

$$\text{DIZ (mm)} = 9.8 + 4.0X_1^2 - 5.6X_1X_3 \quad (3)$$

*S. aureus*:

$$\text{DIZ (mm)} = 9.3 + 2.4X_1 + 2.6X_1^2 + 2.1X_2^2 - 1.1X_3^2 \quad (4)$$

**Table 1. Antimicrobial activity (in function of diameters of inhibition zone) of seeds, peels and mixture of seeds and peels extracts obtained in distilled water and 40-80% acetone, ethanol and methanol against foodborne bacteria.<sup>1,2</sup>**

	Acetone					Ethanol					Methanol																																																																																																																																																																																																																																																																																																																																																																																																																																				
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Seeds																A	10.5±0.5 <sup>abc</sup>	11.0±0.0 <sup>ab</sup>	11.0±0.0 <sup>ab</sup>	11.0±0.0 <sup>ab</sup>	12.0±0.0 <sup>a</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	8.5±0.5 <sup>c</sup>	8.5±0.5 <sup>c</sup>	9.0±0.0 <sup>bc</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	9.5±0.5 <sup>bc</sup>	10.0±1.0 <sup>abc</sup>	9.5±0.5 <sup>bc</sup>	B	10.5±0.5 <sup>abc</sup>	10.5±0.5 <sup>abc</sup>	10.0±1.0 <sup>abc</sup>	11.0±1.0 <sup>ab</sup>	12.0±1.0 <sup>a</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	8.5±0.5 <sup>c</sup>	9.0±0.0 <sup>bc</sup>	6.0±0.0 <sup>d</sup>	C	10.0±0.0 <sup>bc</sup>	11.0±0.0 <sup>ab</sup>	11.0±1.0 <sup>ab</sup>	11.0±0.0 <sup>ab</sup>	12.0±0.0 <sup>a</sup>	6.0±0.0 <sup>e</sup>	7.0±0.0 <sup>de</sup>	8.0±0.0 <sup>d</sup>	8.0±0.0 <sup>d</sup>	7.5±0.5 <sup>de</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	8.5±0.5 <sup>cd</sup>	10.0±0.0 <sup>bc</sup>	D	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	9.5±0.5 <sup>b</sup>	11.0±0.0 <sup>a</sup>	6.0±0.0 <sup>d</sup>	8.0±0.0 <sup>c</sup>	7.5±0.5 <sup>c</sup>	7.5±0.5 <sup>c</sup>	7.5±0.5 <sup>c</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	7.5±0.5 <sup>c</sup>	8.0±0.0 <sup>c</sup>	11.0±0.0 <sup>a</sup>	E	8.5±0.5 <sup>b</sup>	9.0±1.0 <sup>b</sup>	9.0±1.0 <sup>b</sup>	10.5±0.5 <sup>ab</sup>	11.5±0.5 <sup>a</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	8.5±0.5 <sup>bc</sup>	10.0±0.0 <sup>ab</sup>	F	9.0±0.0 <sup>a</sup>	9.5±0.5 <sup>a</sup>	9.5±0.5 <sup>a</sup>	9.0±0.0 <sup>a</sup>	10.5±0.5 <sup>a</sup>	6.0±0.0 <sup>b</sup>	9.0±1.0 <sup>a</sup>	8.5±0.5 <sup>a</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	9.5±0.5 <sup>a</sup>	10.0±0.0 <sup>a</sup>	G	6.0±0.0 <sup>d</sup>	9.5±0.5 <sup>bc</sup>	10.0±0.0 <sup>ab</sup>	11.0±0.0 <sup>a</sup>	11.0±0.0 <sup>a</sup>	7.5±0.5 <sup>e</sup>	8.0±0.0 <sup>de</sup>	8.5±0.5 <sup>de</sup>	8.0±0.0 <sup>de</sup>	8.0±0.0 <sup>de</sup>	6.0±0.0 <sup>f</sup>	6.0±0.0 <sup>f</sup>	8.5±0.5 <sup>de</sup>	9.0±0.5 <sup>bcd</sup>	9.0±0.5 <sup>bcd</sup>	H	10.0±0.0 <sup>bc</sup>	11.0±0.0 <sup>ab</sup>	11.0±1.0 <sup>ab</sup>	12.0±0.0 <sup>a</sup>	12.0±0.0 <sup>a</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	8.0±0.0 <sup>d</sup>	8.5±0.5 <sup>cd</sup>	6.0±0.0 <sup>e</sup>	Peels																A	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	B	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	C	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	D	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	E	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	F	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	G	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	H	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	Peels and seeds																A	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	B	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	C	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	D	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	E	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	F	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	G	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	H	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>
A	10.5±0.5 <sup>abc</sup>	11.0±0.0 <sup>ab</sup>	11.0±0.0 <sup>ab</sup>	11.0±0.0 <sup>ab</sup>	12.0±0.0 <sup>a</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	8.5±0.5 <sup>c</sup>	8.5±0.5 <sup>c</sup>	9.0±0.0 <sup>bc</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	9.5±0.5 <sup>bc</sup>	10.0±1.0 <sup>abc</sup>	9.5±0.5 <sup>bc</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
B	10.5±0.5 <sup>abc</sup>	10.5±0.5 <sup>abc</sup>	10.0±1.0 <sup>abc</sup>	11.0±1.0 <sup>ab</sup>	12.0±1.0 <sup>a</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	8.5±0.5 <sup>c</sup>	9.0±0.0 <sup>bc</sup>	6.0±0.0 <sup>d</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
C	10.0±0.0 <sup>bc</sup>	11.0±0.0 <sup>ab</sup>	11.0±1.0 <sup>ab</sup>	11.0±0.0 <sup>ab</sup>	12.0±0.0 <sup>a</sup>	6.0±0.0 <sup>e</sup>	7.0±0.0 <sup>de</sup>	8.0±0.0 <sup>d</sup>	8.0±0.0 <sup>d</sup>	7.5±0.5 <sup>de</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	8.5±0.5 <sup>cd</sup>	10.0±0.0 <sup>bc</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
D	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	9.5±0.5 <sup>b</sup>	11.0±0.0 <sup>a</sup>	6.0±0.0 <sup>d</sup>	8.0±0.0 <sup>c</sup>	7.5±0.5 <sup>c</sup>	7.5±0.5 <sup>c</sup>	7.5±0.5 <sup>c</sup>	6.0±0.0 <sup>d</sup>	6.0±0.0 <sup>d</sup>	7.5±0.5 <sup>c</sup>	8.0±0.0 <sup>c</sup>	11.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
E	8.5±0.5 <sup>b</sup>	9.0±1.0 <sup>b</sup>	9.0±1.0 <sup>b</sup>	10.5±0.5 <sup>ab</sup>	11.5±0.5 <sup>a</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	6.0±0.0 <sup>c</sup>	8.5±0.5 <sup>bc</sup>	10.0±0.0 <sup>ab</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
F	9.0±0.0 <sup>a</sup>	9.5±0.5 <sup>a</sup>	9.5±0.5 <sup>a</sup>	9.0±0.0 <sup>a</sup>	10.5±0.5 <sup>a</sup>	6.0±0.0 <sup>b</sup>	9.0±1.0 <sup>a</sup>	8.5±0.5 <sup>a</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	6.0±0.0 <sup>b</sup>	9.5±0.5 <sup>a</sup>	10.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
G	6.0±0.0 <sup>d</sup>	9.5±0.5 <sup>bc</sup>	10.0±0.0 <sup>ab</sup>	11.0±0.0 <sup>a</sup>	11.0±0.0 <sup>a</sup>	7.5±0.5 <sup>e</sup>	8.0±0.0 <sup>de</sup>	8.5±0.5 <sup>de</sup>	8.0±0.0 <sup>de</sup>	8.0±0.0 <sup>de</sup>	6.0±0.0 <sup>f</sup>	6.0±0.0 <sup>f</sup>	8.5±0.5 <sup>de</sup>	9.0±0.5 <sup>bcd</sup>	9.0±0.5 <sup>bcd</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
H	10.0±0.0 <sup>bc</sup>	11.0±0.0 <sup>ab</sup>	11.0±1.0 <sup>ab</sup>	12.0±0.0 <sup>a</sup>	12.0±0.0 <sup>a</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	6.0±0.0 <sup>e</sup>	8.0±0.0 <sup>d</sup>	8.5±0.5 <sup>cd</sup>	6.0±0.0 <sup>e</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
Peels																A	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	B	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	C	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	D	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	E	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	F	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	G	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	H	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	Peels and seeds																A	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	B	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	C	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	D	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	E	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	F	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	G	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	H	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																
A	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
B	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
C	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
D	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
E	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
F	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
G	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
H	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
Peels and seeds																A	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	B	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	C	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	D	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	E	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	F	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	G	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	H	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																
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D	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
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G	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																
H	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>	6.0±0.0 <sup>a</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																

<sup>1</sup> A: *Bacillus cereus*; B: *Bacillus subtilis*; C: *Enterococcus faecalis*; D: *Escherichia coli*; E: *Pseudomonas aeruginosa*; F: *Salmonella enteritidis*; G: *Serratia marcescens*; H: *Staphylococcus aureus*.

<sup>2</sup> For each treatment, different letters in the same line indicate significant differences ( $P<0.05$ ) between the mean values according to Tukey's test.

Table 2. Inhibition zone diameters against foodborne bacteria for 80% acetone granadilla seed extract obtained under various extraction conditions (experiments 1-17).

Exp.	Temperature (°C)	Solid/solvent ratio	Time (min)	Inhibition zone diameters (mm) (mean ± standard deviation)										
				Gram positive bacteria					Gram negative bacteria					
				<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Serratia marcescens</i>	<i>Salmonella enteritidis</i>			
1	-1 (35)	-1 (15)	-1 (20)	10.0±1.0	8.3±0.5	8.7±0.5	8.3±0.5	9.3±0.5	9.3±0.5	9.3±0.5	8.3±0.5	9.3±0.5	8.3±0.5	
2	+1 (50)	-1 (15)	-1 (20)	12.7±0.5	15.3±0.5	12.0±0.8	11.3±0.5	14.7±0.9	11.7±0.5	11.3±0.9	13.0±0.8	11.3±0.9	13.0±0.8	
3	-1 (35)	+1 (50)	-1 (20)	11.3±0.5	10.3±0.5	9.3±0.9	11.0±0.8	12.3±0.5	11.3±0.5	13.0±1.0	11.0±0.8	11.3±0.5	11.0±0.8	
4	+1 (50)	+1 (50)	-1 (20)	13.7±0.5	16.3±0.5	13.7±0.5	13.7±0.5	15.7±0.5	14.3±0.5	12.3±0.5	15.7±0.5	12.3±0.5	15.7±0.5	
5	-1 (35)	-1 (15)	+1 (50)	11.3±0.5	15.7±0.5	9.3±0.5	12.0±1.0	14.3±0.5	11.3±0.9	11.3±0.9	11.7±0.5	11.3±0.9	11.7±0.5	
6	+1 (50)	+1 (15)	+1 (50)	12.3±0.5	12.0±0.8	11.3±0.5	10.7±0.9	14.7±0.5	11.7±0.5	12.0±0.8	12.7±0.9	12.0±0.8	12.7±0.9	
7	-1 (35)	+1 (50)	+1 (50)	13.0±0.8	15.7±0.5	11.7±0.5	13.3±0.9	14.0±0.8	13.0±0.8	12.0±1.0	14.3±0.5	12.0±1.0	14.3±0.5	
8	+1 (50)	+1 (50)	+1 (50)	10.7±0.5	10.0±0.8	13.0±1.0	13.3±0.5	11.7±0.5	14.0±0.8	14.3±0.5	12.7±0.5	14.3±0.5	12.7±0.5	
9	-1.68 (30)	0 (33)	0 (35)	12.7±0.5	16.7±0.9	11.3±0.5	11.0±0.8	14.0±1.0	12.3±0.5	11.3±0.9	14.3±0.5	11.3±0.9	14.3±0.5	
10	+1.68 (55)	0 (33)	0 (35)	12.7±0.5	11.7±0.5	14.0±0.8	13.7±0.5	12.3±0.9	13.7±0.9	14.0±0.8	13.3±0.5	14.0±0.8	13.3±0.5	
11	0 (43)	-1.68 (5)	0 (35)	8.3±0.5	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	
12	0 (43)	+1.68 (60)	0 (35)	10.7±0.5	10.7±0.5	10.7±0.5	12.0±0.8	13.0±0.8	11.3±0.5	11.7±0.5	11.3±0.5	11.7±0.5	11.3±0.5	
13	0 (43)	0 (33)	-1.68 (10)	10.3±0.9	9.3±0.9	9.0±1.0	8.7±0.9	10.0±0.8	10.3±0.9	11.3±0.5	11.0±1.0	11.3±0.5	11.0±1.0	
14	0 (43)	0 (33)	+1.68 (60)	11.7±0.5	10.7±0.5	11.7±0.9	11.7±0.9	11.7±0.5	11.3±0.5	13.0±0.8	12.0±2.0	13.0±0.8	12.0±2.0	
15	0 (43)	0 (33)	0 (35)	10.3±0.5	10.3±0.5	9.0±0.8	10.7±0.5	10.7±0.9	11.0±1.0	11.3±0.9	12.0±2.0	11.3±0.9	12.0±2.0	
16	0 (43)	0 (33)	0 (35)	11.0±0.8	10.3±0.5	9.3±0.5	9.0±0.8	11.0±1.0	10.3±0.5	12.3±0.5	11.0±1.0	12.3±0.5	11.0±1.0	
17	0 (43)	0 (33)	0 (35)	10.7±0.5	9.3±0.9	9.7±0.0	10.7±0.5	11.3±0.5	10.7±0.9	12.3±0.5	11.0±1.0	12.3±0.5	11.0±1.0	

**Table 3. Significant parameters on antibacterial activity, total phenolic content and total flavonoid content of 80% acetone granadilla seed extracts estimated from the results of the central composite rotatable design.<sup>1</sup>**

		Effect	Standard error	t	P-value
<i>Bacillus cereus</i> *	Mean	10.6	0.2	52.0	0.00036
	X <sub>1</sub> (Q)	1.8	0.2	8.5	0.01366
	X <sub>2</sub> (L)	1.0	0.2	5.2	0.03518
	X <sub>1</sub> versus X <sub>3</sub>	-1.7	0.3	-6.7	0.02128
<i>Bacillus subtilis</i> *	Mean	9.8	0.3	29.5	0.00114
	X <sub>1</sub> (Q)	4.0	0.3	11.4	0.00766
	X <sub>1</sub> versus X <sub>3</sub>	-5.6	0.4	-13.7	0.00527
<i>Staphylococcus aureus</i> *	Mean	9.3	0.2	46.0	0.00048
	X <sub>1</sub> (L)	2.4	0.2	12.5	0.00632
	X <sub>1</sub> (Q)	2.6	0.2	12.4	0.00647
	X <sub>2</sub> (L)	2.1	0.2	11.0	0.00825
	X <sub>3</sub> (Q)	1.1	0.2	5.1	0.03643
<i>Enterococcus faecalis</i> *	Mean	10.1	0.6	17.6	0.00314
	X <sub>2</sub> (L)	2.8	0.5	5.3	0.03326
<i>Escherichia coli</i> *	Mean	10.6	0.2	52.3	0.00037
	X <sub>1</sub> (L)	1.3	0.2	7.0	0.01950
	X <sub>1</sub> (Q)	2.2	0.2	10.5	0.00886
	X <sub>2</sub> (L)	2.6	0.2	13.5	0.00545
<i>Serratia marcescens</i> *	Mean	12.0	0.3	35.8	0.00078
	X <sub>2</sub> (L)	2.6	0.3	8.2	0.01440
	X <sub>2</sub> (Q)	-1.9	0.3	-5.4	0.03298
<i>Salmonella enteritidis</i> *	Mean	11.3	0.4	30.0	0.00110
	X <sub>1</sub> (Q)	2.2	0.4	5.7	0.02925
	X <sub>2</sub> (L)	2.5	0.4	7.0	0.01962
	X <sub>1</sub> versus X <sub>3</sub>	-2.5	0.5	-5.4	0.03223
Total phenolic content*	Mean	424.6	14.4	29.5	0.00115
	X <sub>1</sub> (Q)	-99.6	15.0	-6.7	0.02168
	X <sub>2</sub> (L)	-890.2	13.5	-65.7	0.00023
	X <sub>2</sub> (Q)	663.2	15.0	44.5	0.00050
Total flavonoid content*	Mean	244.8	6.6	37.2	0.00072
	X <sub>1</sub> (L)	79.0	6.2	12.8	0.00606
	X <sub>3</sub> (L)	33.0	6.2	5.4	0.03303
	X <sub>3</sub> (Q)	32.0	7.0	4.8	0.04149
	X <sub>1</sub> versus X <sub>2</sub>	-89.0	8.0	-11.0	0.00809

<sup>1</sup> \* = significant factors ( $P < 0.05$ ); L = linear parameters; Q = quadratic parameters; X<sub>1</sub> = temperature; X<sub>2</sub> = solid/solvent ratio; X<sub>3</sub> = time.

*E. faecalis*:

$$\text{DIZ (mm)} = 10.1 + 2.8X_2 \quad (5)$$

*E. coli*:

$$\text{DIZ (mm)} = 10.6 + 1.3X_1 + 2.2X_1^2 + 2.6X_2 \quad (6)$$

*S. marcescens*:

$$\text{DIZ (mm)} = 12.0 + 2.6X_1 - 1.9X_2^2 \quad (7)$$

and *S. enteritidis*:

$$\text{DIZ (mm)} = 11.3 + 2.2X_1^2 + 2.5X_2 - 2.5X_1X_3 \quad (8)$$

where, DIZ is the diameter of inhibition zone; X<sub>1</sub> is the temperature, X<sub>2</sub> is the solid/solvent ratio, and X<sub>3</sub> is the incubation time.

The response surfaces (Figure 1 and 2) showed that maximum inhibition of *B. cereus*, *E. coli*, *E. faecalis* and *S. enteritidis* is possible with extracts obtained with increased

**Table 4. Analysis of variance for antibacterial activity, total phenolic content and total flavonoid content of 80% acetone granadilla seed extracts.**

	Source of variation	Sum of squares	Degrees of freedom	Mean squares	$F_{\text{test}}$
<i>Bacillus cereus</i> $R^2=0.84$ $F_{(0.95;3;13)}=3.41$	Regression	25.6	3	8.5	21.3
	Residue	4.8	13	0.4	
	Lack of fit	4.6			
	Pure error	0.2			
	Total	30.4			
<i>Bacillus subtilis</i> $R^2=0.80$ $F_{(0.95;2;14)}=3.74$	Regression	125.5	2	62.7	27.0
	Residue	32.0	14	2.3	
	Lack of fit	31.2			
	Pure error	0.7			
	Total	157.5			
<i>Staphylococcus aureus</i> $R^2=0.93$ $F_{(0.95;4;12)}=3.26$	Regression	63.0	4	15.8	39.5
	Residue	5.0	12	0.4	
	Lack of fit	4.7			
	Pure error	0.3			
	Total	68.0			
<i>Enterococcus faecalis</i> $R^2=0.88$ $F_{(0.95;1;15)}=4.54$	Regression	61.0	1	61.0	122.0
	Residue	8.0	15	0.5	
	Lack of fit	6.0			
	Pure error	2.0			
	Total	69.0			
<i>Escherichia coli</i> $R^2=0.90$ $F_{(0.95;3;13)}=3.41$	Regression	54.0	3	18.0	36.0
	Residue	6.7	13	0.5	
	Lack of fit	6.5			
	Pure error	0.2			
	Total	60.7			
<i>Serratia marcescens</i> $R^2=0.88$ $F_{(0.95;2;14)}=3.74$	Regression	50.0	2	25.0	50.0
	Residue	6.7	14	0.5	
	Lack of fit	6.0			
	Pure error	0.7			
	Total	56.0			
<i>Salmonella enteritidis</i> $R^2=0.83$ $F_{(0.95;3;13)}=3.41$	Regression	68.2	3	22.7	21.2
	Residue	13.9	13	1.0	
	Lack of fit	13.1			
	Pure error	0.8			
	Total	82.1			
Total phenolic content $R^2=0.84$ $F_{(0.95;4;12)}=3.26$	Regression	4,260,692	4	1,065,173	15.7
	Residue	813,741	12	67,811.8	
	Lack of fit	812,491			
	Pure error	1,250			
	Total	5,074,433			
Total flavonoid content $R^2=0.70$ $F_{(0.95;4;12)}=3.26$	Regression	46,700	4	11,675	6.0
	Residue	23,140	12	1,928	
	Lack of fit	22,880			
	Pure error	260			
	Total	69,840			

the solid/solvent ratio and temperature. Extracts generated

with increased temperature and time of extraction could

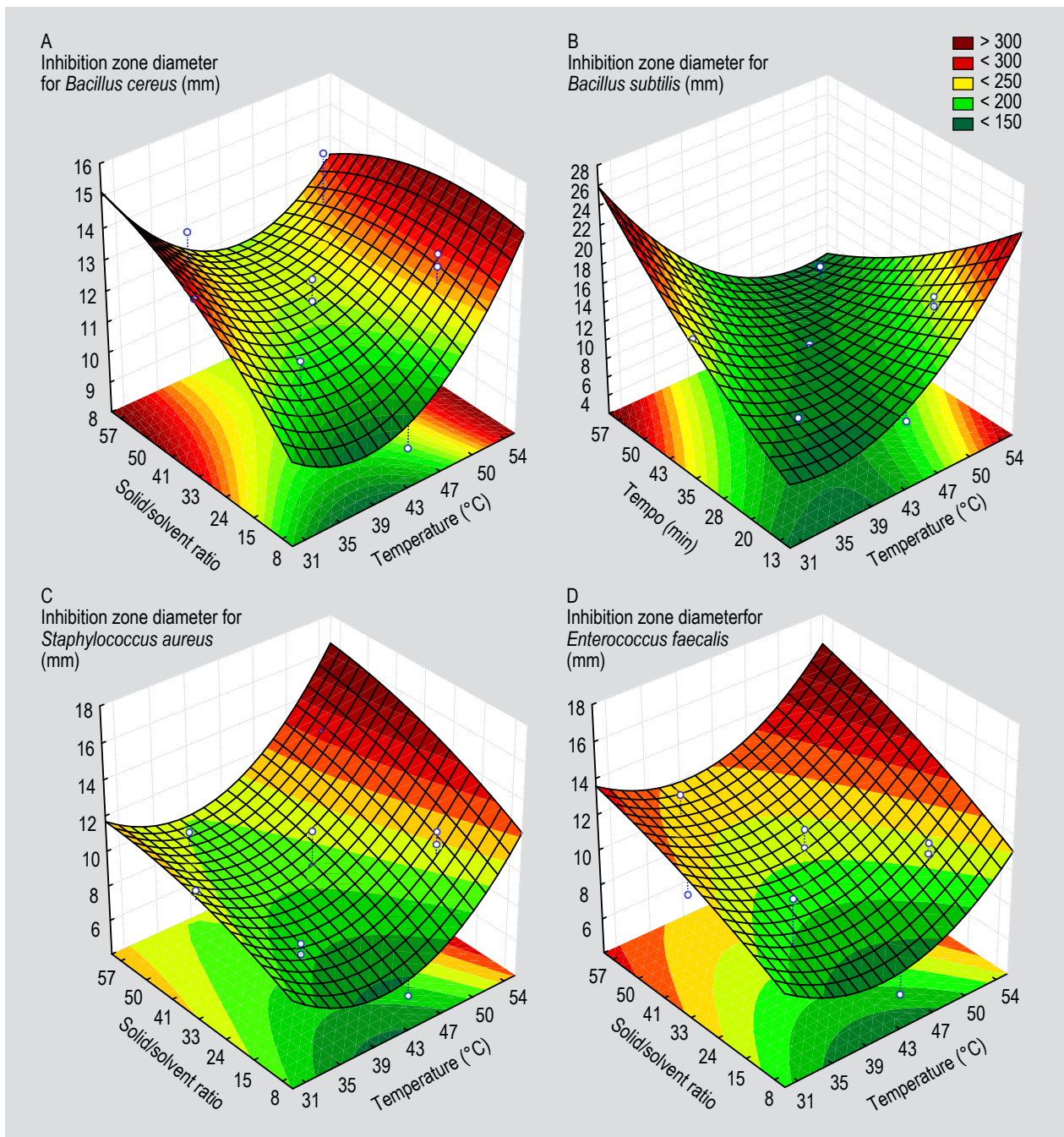
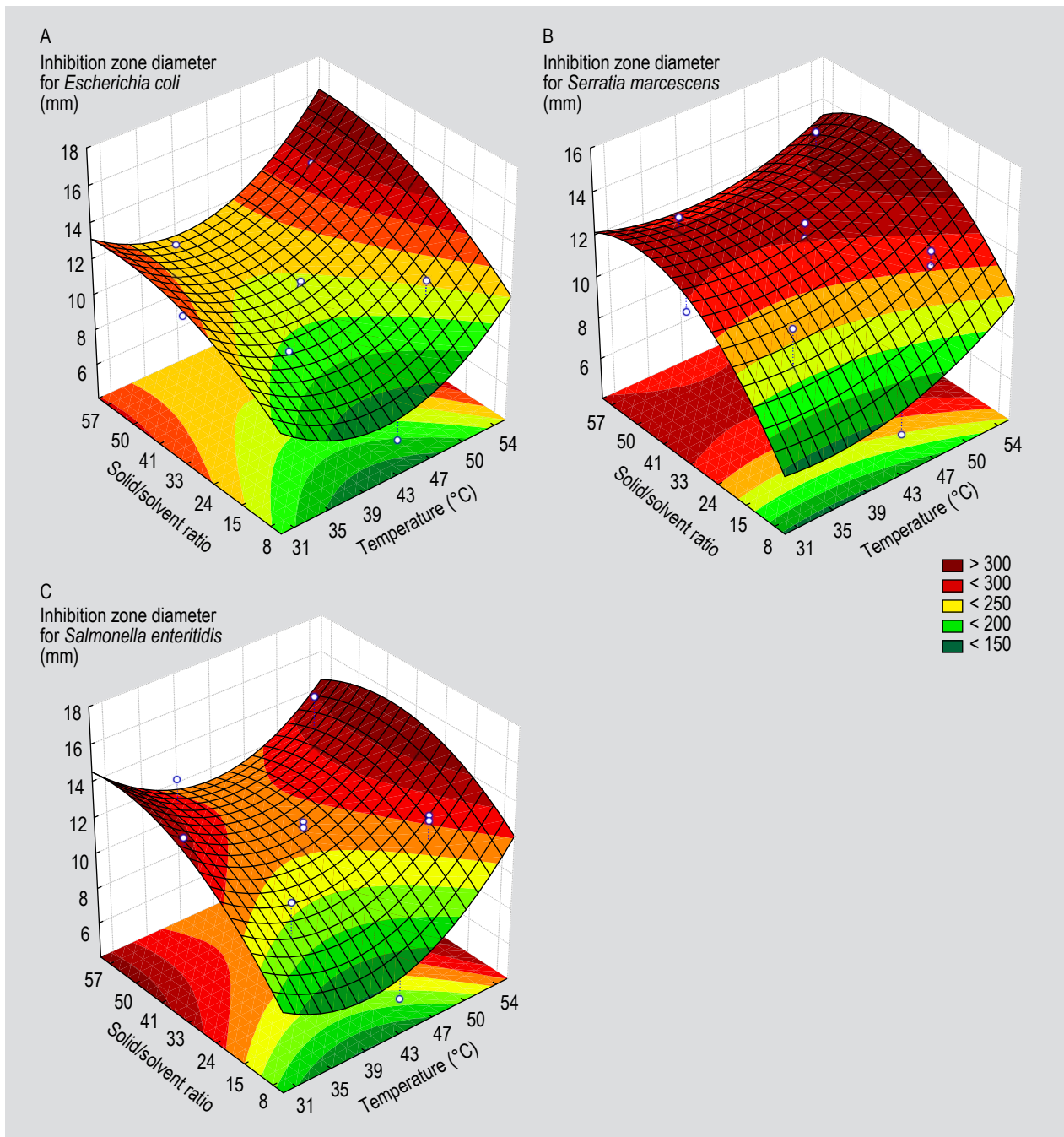


Figure 1. Response surfaces for diameters of inhibition zone obtained with 80% acetone granadilla seed extracts against: (A) *Bacillus cereus*, (B) *Bacillus subtilis*, (C) *Staphylococcus aureus* and (D) *Enterococcus faecalis*.

enhance the inhibition of *B. subtilis*. Extracts with maximum potential to inhibit *S. marcescens* can be obtained with intermediate values of solid/solvent ratio combined with increased temperature. Extracts obtained with increased temperatures are more adequate for *S. aureus* inhibition.

#### Predictive models for TPC and TFC in 80% acetone granadilla seed extract

The influence of the extraction conditions (temperature, solid/solvent ratio, extraction time) on the TPC and TFC of 80% acetone granadilla seed extracts was also evaluated (Table 5). The solid/solvent ratio (linear) was the most significant parameter ( $P < 0.1$ ) to obtaining extracts with superior TPC and TFC (Table 3). The extraction time was significant for extraction of both types of bioactive



**Figure 2.** Response surfaces for diameters of inhibition zone obtained with 80% acetone granadilla seed extracts against: (A) *Escherichia coli*, (B) *Serratia marcescens* and (C) *Salmonella enteritidis*.

compounds. However, the temperature only influenced the TFC extraction. These parameters showed a positive effect, i.e. higher values result in greater amounts of extracted compounds.

The F test values (ANOVA) were approximately 5.0 and 2.0 times greater than the F tabulated values, and the  $R^2$  values were 0.84 and 0.70, respectively, for TPC and TFC (Table 4).

Next, the predictive models were generated as a function of the significant parameters, according to Equation 9 and 10:

$$\text{TPC} = 424.6 + 99.6X_1^2 - 890.2X_2 + 663.2X_2^2 \quad (9)$$

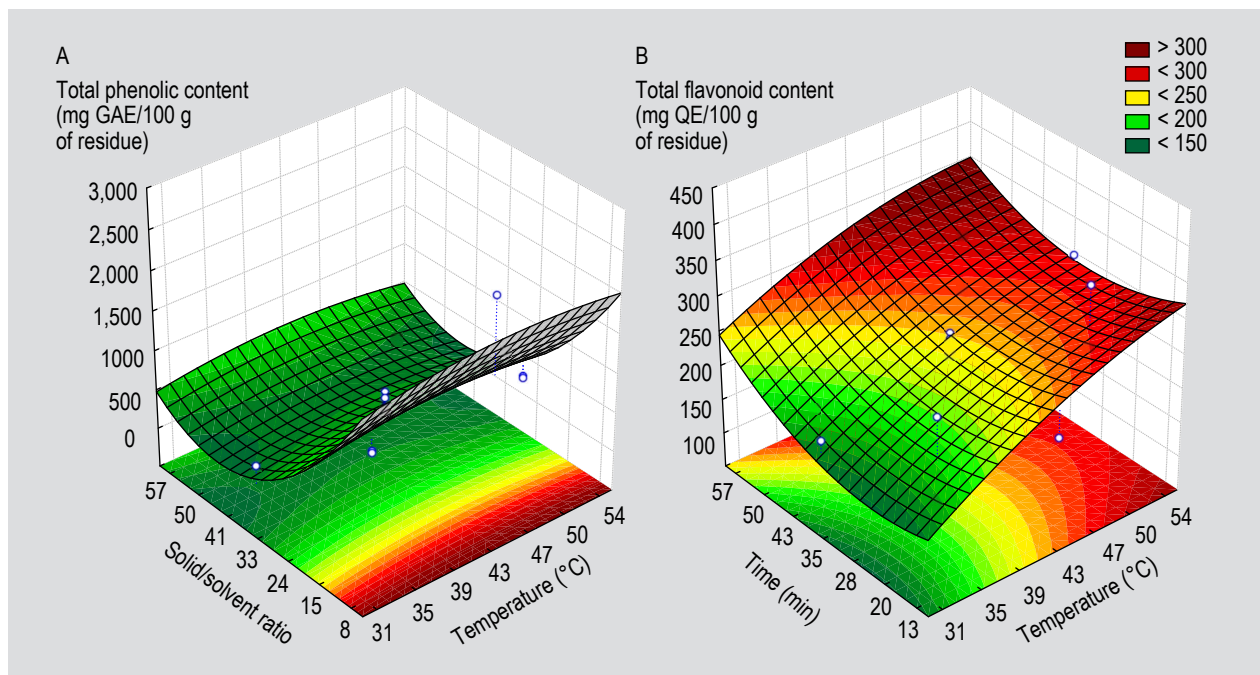
$$\text{TFC} = 244.8 + 79.0X_1 + 33.0X_3 - 32.0X_3^2 - 89.0X_1X_2 \quad (10)$$

where TPC is total phenolic content, TFC is total flavonoid content,  $X_1$  is the temperature,  $X_2$  is the solid/solvent ratio, and  $X_3$  is the extraction time.

**Table 5.** Total phenolic content and total flavonoid content for 80% acetone granadilla seed extracts obtained by varying the extraction conditions (experiments 1-17).<sup>1</sup>

Exp.	Temperature (°C)	Solid/solvent ratio	Time (min)	Total phenolic content mg GAE/100 g of residue	Total flavonoid content mg QE/100 g of residue
1	-1 (35)	-1 (15)	-1 (20)	858 <sup>cd</sup> ±7	130±5 <sup>k</sup>
2	+1 (50)	-1 (15)	-1 (20)	914 <sup>b</sup> ±12	340±0 <sup>b</sup>
3	-1 (35)	+1 (50)	-1 (20)	330 <sup>kl</sup> ±4	254±5 <sup>de</sup>
4	+1 (50)	+1 (50)	-1 (20)	362 <sup>jk</sup> ±3	253±0 <sup>de</sup>
5	-1 (35)	-1 (15)	+1 (50)	837 <sup>e</sup> ±8	143±1 <sup>k</sup>
6	+1 (50)	-1 (15)	+1 (50)	891 <sup>bc</sup> ±14	300±0 <sup>c</sup>
7	-1 (35)	+1 (50)	+1 (50)	307 <sup>l</sup> ±3	216±0 <sup>i</sup>
8	+1 (50)	+1 (50)	+1 (50)	334 <sup>kl</sup> ±1	222±1 <sup>gh</sup>
9	-1.68 (30)	0 (33)	0 (35)	443 <sup>h</sup> ±4	193±0 <sup>j</sup>
10	+1.68 (55)	0 (33)	0 (35)	423 <sup>h</sup> ±7	296±2 <sup>c</sup>
11	0 (43)	-1.68 (5)	0 (35)	2672 <sup>a</sup> ±31	264±0 <sup>d</sup>
12	0 (43)	+1.68 (60)	0 (35)	347 <sup>jk</sup> ±4	243±0 <sup>ef</sup>
13	0 (43)	0 (33)	-1.68 (10)	622 <sup>f</sup> ±0	214±0 <sup>i</sup>
14	0 (43)	0 (33)	+1.68 (60)	541 <sup>g</sup> ±6	410±11 <sup>a</sup>
15	0 (43)	0 (33)	0 (35)	407 <sup>hi</sup> ±5	254±2 <sup>de</sup>
16	0 (43)	0 (33)	0 (35)	382 <sup>ij</sup> ±3	232±5 <sup>gh</sup>
17	0 (43)	0 (33)	0 (35)	432 <sup>h</sup> ±2	240±7 <sup>efg</sup>

<sup>1</sup> For each treatment, different letters in the same column indicate significant differences ( $P<0.05$ ) between the mean values according to Tukey's test.



**Figure 3.** Response surfaces for (A) total phenolic content and (B) total flavonoid content in 80% acetone granadilla seed extracts.

The response surfaces (Figure 3) showed that maximum TPC and TFC can be obtained with decreased solid/solvent ratio and increased temperature values, respectively.

#### Validation of experimental design

In order to validate the adequacy of polynomial equations (Equation 2-10), experimental runs were carried out under different combinations of process parameters. The

**Table 6. Observed and predicted results (diameter of inhibition zone, total phenolic and total flavonoid content) obtained from the validation experiments.<sup>1</sup>**

Parameters				Responses					
X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Bacterium	DIZ		TPC		TFC	
				Observed	Predicted	Observed	Predicted	Observed	Predicted
55	60	35	<i>Bacillus cereus</i>	17.0	17.0	-	-	-	-
			<i>Staphylococcus aureus</i>	6.0	26.0	-	-	-	-
			<i>Escherichia coli</i>	21.0	23.0	-	-	-	-
			<i>Enterococcus faecalis</i>	19.0	14.8	-	-	-	-
			<i>Salmonella enteritidis</i>	6.0	21.7	-	-	-	-
55	33	60	<i>Bacillus subtilis</i>	6.0	6.0	-	-	575.3	523.3
55	50	35	<i>Serratia marcescens</i>	6.0	6.0	-	-	-	-
43	5	35	-	-	-	2,700.0	3,792.0	-	-

<sup>1</sup> DIZ = diameter of inhibition zone (mm); TFC = total flavonoid content; TPC = total phenolic content; X<sub>1</sub> = temperature (°C); X<sub>2</sub> = solid/solvent ratio; X<sub>3</sub> = time (min).

parameters values were selected within the maximum region of response surfaces. The observed (experimental) values were in agreement with the predicted values, with exception of DIZ values for *S. aureus* and *S. enteritidis* (Table 6). Therefore, the models proved to be suitable for predicting the potential of inhibition of granadilla extracts against the bacteria (*B. cereus*, *E. coli*, *E. faecalis*, *B. subtilis* and *S. marcescens*) and the bioactive compounds content (total phenolic and total flavonoid).

#### Identification of bioactive compounds

The granadilla extracts obtained from conditions 4 (temperature of 50 °C, solid/solvent ratio 5:10, 20 min of extraction) and 11 (temperature of 43 °C, solid/solvent ratio 0.5:10, 35 min of extraction) showed high DIZs values and TPC, respectively. A total of 12 compounds were identified in the extracts (Figure 4A and 4B), being the highest intensity (peak area) obtained in the extract 4. The epigallocatechin and caffeic acid phenethyl ester (CAPE) were the majoritary compounds found in the extract 4 and epigallocatechin in the extract 11. The CAPE is the ester of caffeic acid and has shown inhibitory activity against *Helicobacter pylori*, a bacterium associated to gastric diseases (Cui *et al.*, 2013). The epigallocatechin is a polyphenolic product of the secondary metabolism of plants and has showed potential for inhibition of gram-negative bacteria when exposed to light emitting diode (LED light) (Nakamura *et al.*, 2015).

#### 4. Conclusions

This work reported the effect of extraction conditions on the antimicrobial activity, TPC and TFC of granadilla seed extracts. Among the evaluated extracts, those extracted with 80% acetone provided superior potential inhibitory activity against the majority of the tested bacteria. The temperature and solid/solvent molar ratio were the most significant parameters for obtaining extracts with maximum antimicrobial activity, TPC and TFC. The predictive models developed enable obtaining extracts with high antibacterial potential and bioactive content. In particular, the extracts 4 and 11 contained majoritary compounds (epigallocatechin, caffeic acid phenethyl ester) of great medical and pharmaceutical importance. These extracts showed potential to be used as natural antimicrobials or natural source of bioactive compounds.

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#### Conflict of interest

The authors declare that they have no conflicts of interest.

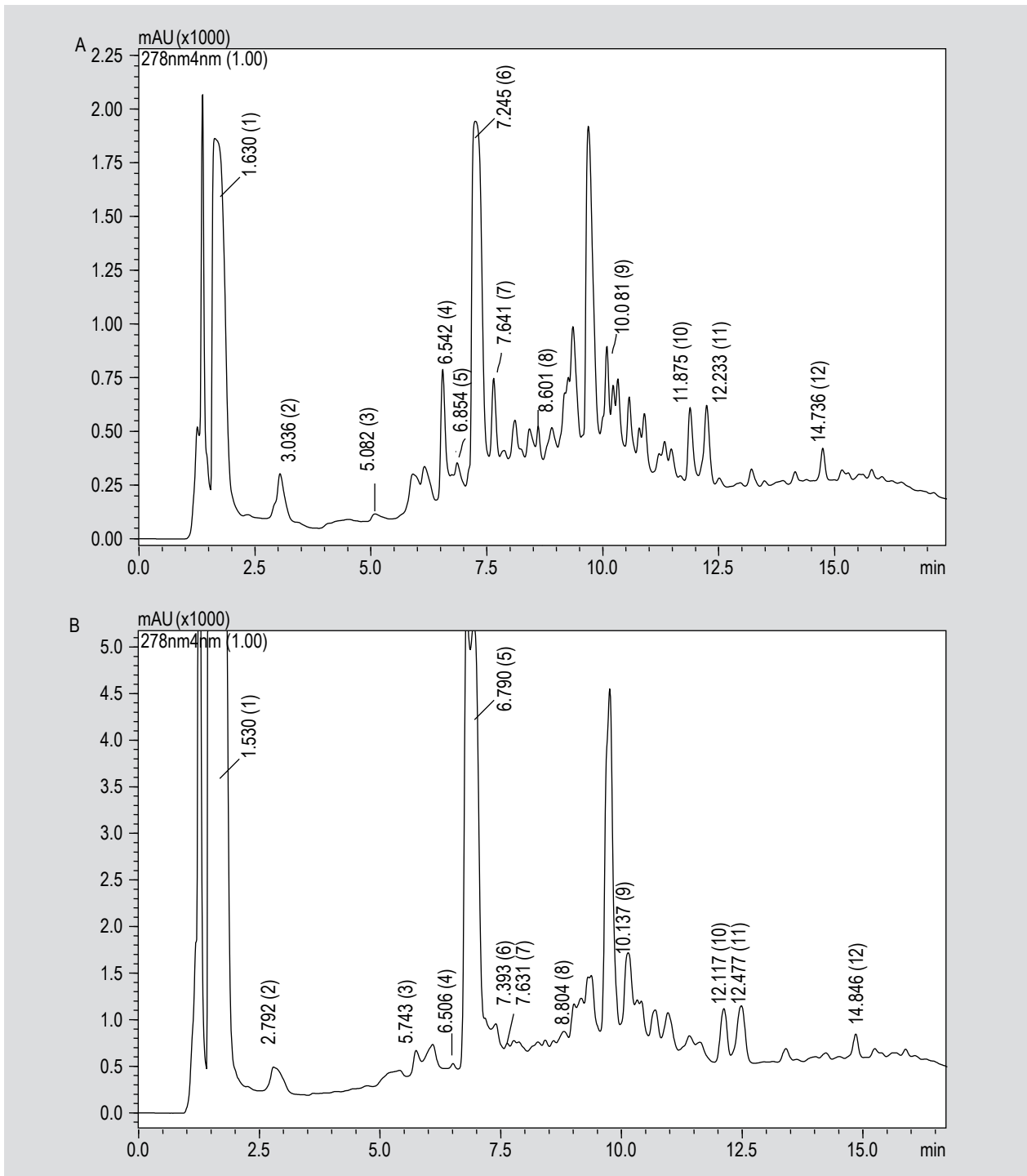


Figure 4. Chromatograms of granadilla extracts obtained from (A) extraction condition 4 (temperature of 50 °C, solid/solvent ratio 5:10, 20 min) and (B) extraction condition 11 (temperature of 43 °C, solid/solvent ratio 0.5:10, 35 min). (1) = epigallocatechin; (2) = epigallocatechin gallate; (3) = epicatechin gallate; (4) = kaempferol-3-glucoside; (5) = propyl gallate; (6) = caffeic acid pheneth; (7) = hesperedin; (8) = coumarin; (9) = trans-cinnamic acid; (10) = quercetin; (11) = luteolin; (12) = naringenin.

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