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Predictive modelling of foodborne bacteria inhibition by pomegranate (*Punica granatum* L.) peel extracts using response surface methodology

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RESEARCH ARTICLE

Abstract

In this work, predictive models based on response surface methodology (RSM) were developed, to describe the inhibition of foodborne bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Serratia marcescens*), using pomegranate (*Punica granatum* L.) peel extracts. The extraction conditions (solid/solvent ratio, 1.0:10-5.0:10), incubation time, 10-60 min and extraction temperature, 30-60 °C) were varied using a 2^3 factorial design with six axial points and three central points and different extracts were obtained. The antibacterial activity of the extracts was determined by the disk diffusion method and Gram-positive bacteria showed more sensitivity to them than Gram-negative bacteria. The RSM showed that the temperature and solid/solvent ratio, were the most significant parameters to obtain extracts with antimicrobial potential against *B. subtilis*, *S. aureus*, *E. faecalis*, *E. coli* and *S. marcescens*. The extraction conditions 4 (extraction temperature = 54 °C, solid/solvent ratio = 4.2:10, incubation time 20.0 min) and 12 (extraction temperature = 45 °C, solid/solvent ratio = 5.0:10, incubation time 35.0 min) resulted in extracts with the highest antibacterial activity against the tested bacteria with minimum inhibitory and minimum bactericide concentrations of 400 and >400 µl/ml, respectively. This is the first study about mathematical models for inhibition of foodborne bacteria using pomegranate peel extract. The results may help food industries to select optimal conditions for the preparation of pomegranate peel extracts able to inhibit foodborne bacteria.

Keywords: fruit residue, natural antimicrobial, pomegranate peel, response surface methodology

1. Introduction

Pomegranate (*Punica granatum* L.) (Punicaceae) is better known in some countries as the fruit of Eden (Al-Qur'an) due to its pleasant taste and excellent health benefits. The fruit is native to Afghanistan, Iran, China and the Indian sub-continent. Pomegranates have been cultivated for thousands of years in Pakistan, China and East India. From the west of Persia (modern day Iran), pomegranate cultivation stretched through the Mediterranean region, to the Turkish-European borders and American Southwest including California and Mexico (Celik *et al.*, 2009; Lansky and Newman, 2007).

The non-edible fractions of fruit and flora, such as peel, seeds, flowers, bark, buds and leaves, although typically considered as waste, often contain higher amounts of specific, nutritionally valuable and biologically active components, compared to the edible fruit. Both, the pomegranate fruit and its fruit extracts, have shown to possess preventive and attenuating activities against diseases, such as cancer, type 2 diabetes, atherosclerosis and cardiovascular diseases. Particularly, peels and peel extracts of pomegranate, which possess high molecular weight phenolics, ellagitannins, proanthocyanidins, complex polysaccharides, flavonoids and microelements, exhibit strong antimutagenic, antioxidant, antimicrobial and apoptotic properties (Akhtar *et al.*, 2015). Compounds, such as anthocyanins, catechins and other complex flavonoids, in addition to hydrolysable tannins, for instance, punicalin, pedunculagin, punicalagin, gallic and ellagic acid, are concentrated in pomegranate peel and juice, which account

for 92% of the antioxidant activity associated with the fruit (Zahin *et al.*, 2010). Besides their free radical scavenging properties, gallic acid, ellagic acid and punicalagin, also possess antibacterial activities against, *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* (Pai *et al.*, 2011).

In general solvent extraction is the most common technique used to prepare plant material extracts due to its efficiency, ease of use and wide applicability. However, the yield of solvent extraction depends on several factors, such as the type of solvents including their varying polarities, the sample/solvent ratio, extraction time and temperature, as well as the chemical composition and physical characteristics of the plant material (Dai and Mumper, 2010). Methanol has been widely used due to its adequate polarity. Methanolic extracts of pomegranate peel possess a higher antioxidant activity than that obtained with other solvents. Furthermore, methanolic pomegranate extracts have shown an inhibitory effect against *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Yersinia enterocolitica* (Al-Zoreky, 2009; Ismail *et al.*, 2012). The antibacterial activity of methanolic pomegranate extracts can be due to the presence of hydrolysable tannins (punicalins and punicalagins), ellagic acid, a component of ellagitannins, and gallic acid, a component of gallotannins, for example (Naz *et al.*, 2007; Reddy *et al.*, 2007).

Response surface methodology (RSM), originally described by Box and Wilson (1951), is a widely adopted tool for process optimisation (Nazni and Karunathara, 2011). This technique is effective for responses that affect many factors and their interactions. The central composite rotatable design (CCRD) was adopted to predict responses based on a few sets of experimental data in which, all factors were varied within a chosen range (Box and Hunter, 1957). RSM has been applied to optimise the extraction conditions of polysaccharides (Zhu *et al.*, 2015), as well as phenolics, flavonoids and antioxidant compounds (Kazemi *et al.*, 2016; Sood and Gupta, 2015; Tabaraki *et al.*, 2012; Wang *et al.*, 2013) from pomegranate residues. However, there are no reports about the use of RSM to predict the inhibition of bacteria using fruit extracts. Whereas the potential of pomegranate peel extracts as natural antimicrobials, this work aimed to investigate the effect of extraction conditions, using RSM, to obtain extracts with high antimicrobial activity against foodborne bacteria.

2. Materials and methods

Pomegranate peel flour

Fresh pomegranate fruits were collected directly from a tree cultivated at Aracaju city in Sergipe, Brazil. The fruits were sanitised with 200 mg/kg chloride solution for 15 min and manually peeled. The peels were dried at 50 °C for 24 h and then crushed in a blender.

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 in brain heart infusion broth with 20% glycerol in a -80 °C freezer. All culture media were purchased from Oxoid (São Paulo, Brazil).

Preparation of pomegranate peel extract

The extract was obtained with aqueous methanol (60%) using 1 g of residue and 10 ml of solvent. The mixture was kept under agitation using an orbital shaker at 250 rpm, at 30 °C for 60 min (Gullon *et al.*, 2016 with modifications). Then, the extract was filtered through qualitative filter paper to separate solid from liquid.

Response surface methodology

With the aim of maximise the antimicrobial activity of pomegranate peel extract against foodborne bacteria, the extraction conditions (temperature, solid/solvent ratio and extraction time) were varied and the effect of these parameters in inhibitory potential was analysed by response surface methodology. A CCRD including 2^3 factorial design with six axial points and three central points was performed to obtain a second-order model (Equation 1) and predict the antibacterial activity of pomegranate peel extracts against foodborne bacteria. The CCRD resulted in 17 experiments as shown in Table 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 (diameter of inhibition zone, DIZ), $\beta 0$ is the global mean, β_j is the linear coefficient, β_{ij} is the coefficient of interaction, β_{jj} is the quadratic coefficient, ε 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). The 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, according to the *P*-value at the 5%. 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

Table 1. Inhibition zone diameters (mean ± standard deviation) against foodborne bacteria for pomegranate peel extracts obtained under various extraction conditions (experiments 1-17).

				Gram-positive bacteria				Gram-negative bacteria		
Exp.	Temperature (°C)	Solid/solvent ratio	Time (min)	*Bacillus cereus*	*Bacillus subtilis*	*Staphylococcus aureus*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Serratia marcescens*
1	-1 (36.0)	-1 (1.8:10)	-1 (20.0)	10.5±0.7	6.0±0.0	10.0±0.0	14.5±0.7	6.0±0.0	9.5±0.7	8.0±0.0
2	+1 (54.0)	-1 (1.8:10)	-1 (20.0)	18.5±0.7	11.5±0.7	9.5±0.7	14.5±0.7	8.5±0.7	6.0±0.0	11.0±0.0
3	-1 (36.0)	+1 (4.2:10)	-1 (20.0)	15.5±0.7	10.0±0.0	11.5±0.7	15.5±0.7	6.0±0.0	11.0±0.0	12.0±0.0
4	+1 (54.0)	+1 (4.2:10)	-1 (20.0)	18.5±0.7	14.5±0.7	12.0±0.0	17.5±0.7	13.0±1.4	9.5±0.7	13.5±0.7
5	-1 (36.0)	-1 (1.8:10)	+1 (50.0)	14.5±0.7	9.0±0.0	6.5±0.7	14.5±0.7	6.0±0.0	9.5±0.7	6.0±0.0
6	+1 (54.0)	-1 (1.8:10)	+1 (50.0)	17.5±0.7	12.0±1.4	10.0±0.0	13.0±1.4	10.0±0.0	6.0±0.0	10.0±0.0
7	-1 (36.0)	+1 (4.2:10)	+1 (50.0)	15.5±0.7	11.5±0.7	12.0±1.4	15.5±0.7	6.0±0.0	13.0±0.0	11.0±0.0
8	+1 (54.0)	+1 (4.2:10)	+1 (50.0)	17.0±1.4	14.0±1.4	10.5±0.7	16.5±0.7	10.5±0.7	11.0±0.0	12.5±0.7
9	-1.68 (30.0)	0 (3.0:10)	0 (35.0)	15.5±0.7	13.0±1.4	9.5±0.7	15.0±0.0	10.0±0.0	9.0±0.0	11.0±0.0
10	+1.68 (60.0)	0 (3.0:10)	0 (35.0)	14.5±0.7	13.5±0.7	10.5±0.7	15.0±0.0	10.5±0.7	10.0±0.0	12.5±0.7
11	0 (45.0)	-1.68 (1.0:10)	0 (35.0)	16.0±0.0	6.0±0.0	7.5±0.7	15.0±0.0	8.0±0.0	8.0±0.0	6.0±0.0
12	0 (45.0)	+1.68 (5.0:10)	0 (35.0)	18.0±0.0	13.0±0.0	11.0±0.0	19.5±0.7	14.0±1.4	12.5±0.7	8.0±0.0
13	0 (45.0)	0 (3.0:10)	-1.68 (10.0)	16.0±0.0	11.5±0.7	9.0±1.4	19.5±0.7	11.5±0.7	13.0±0.0	9.0±0.0
14	0 (45.0)	0 (3.0:10)	+1.68 (60.0)	17.5±0.7	12.0±0.0	9.5±0.7	17.0±0.0	12.0±0.0	11.5±0.7	7.0±0.0
15	0 (45.0)	0 (3.0:10)	0 (35.0)	18.5±0.7	11.5±0.7	9.0±0.0	16.5±0.7	8.5±0.7	12.0±0.0	10.5±0.7
16	0 (45.0)	0 (3.0:10)	0 (35.0)	17.0±0.0	12.0±0.0	9.0±1.4	16.0±0.0	8.0±0.0	13.0±0.0	10.5±0.7
17	0 (45.0)	0 (3.0:10)	0 (35.0)	17.5±0.7	12.5±0.7	8.5±0.7	16.0±0.0	8.5±0.7	13.0±0.0	9.0±0.0

F-test. For validation of the statistical results, the observed DIZ (mm) values were compared with the predicted values.

Antibacterial activity of pomegranate peel extracts

The antibacterial activity of the extracts was evaluated by the disk diffusion method (in triplicate), as described by the Clinical and Laboratory Standards Institute (CLSI, 2015). Petri plates containing Mueller-Hinton agar, were inoculated with bacterial solutions containing 1.5×10^8 of each bacterium/ml, adjusted to 0.5 McFarland standard turbidity (1×10^8 cfu/ml). Sterile filter paper disks (6 mm), containing 10 µl of the extracts, were placed on the agar surface with sterile forceps (four disks on each 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 DIZ was measured with a calliper and expressed in millimetres (disk diameter included). The sensitivity to the extracts was classified according to the DIZ, as follows: not sensitive, for diameters <8 mm; sensitive, for diameters 9-14 mm; very sensitive, for diameters 15-19 mm, and extremely sensitive, for diameters >20 mm (Djenane *et al.*, 2011).

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for the extracts that showed high antibacterial activity in the experimental design, by the disk diffusion test. Before the experiments, the solvent was removed from the extracts by rotary evaporation (Heidolph G3, Schwabach, Germany) under a pressure of 90 mbar, at 40 °C. The MIC was performed using the broth microdilution method, according to the CLSI (2015). Various concentrations of extract diluted in the solvent were prepared, over the range 0.78-400 µl/ml. Cultures of each bacterial strain were obtained from 24 h broth cultures and adjusted to 0.5 McFarland standard turbidity (1×10^8 cfu/ml). The bacterial suspensions were then diluted to 1×10^6 cfu/ml in Mueller-Hinton broth. An aliquot (100 µl) of each extract dilution 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.

3. Results and discussion

Antibacterial activity of the extracts

The effects of the process variables of temperature, solid/solvent ratio and incubation time, on the antibacterial activity of the extracts, are shown in Table 1. The Gram-positive bacteria *B. cereus* and *E. faecalis*, were sensitive to very sensitive to all extraction conditions (Table 1), with DIZ between 10.5 and 18.5 mm and between 13.0 and 19.5 mm, respectively. The majority of the extracts obtained at 45 or 54 °C, were the most effective at inhibiting *B. cereus* (DIZ between 17.0 and 18.5 mm). Also, notable inhibition (19.5 mm) of *E. faecalis* was achieved with extracts obtained at 45 °C (conditions 12: 45 °C, 5:10 molar ratio, 35 min, and 13: 45 °C, 3:10 molar ratio, 10 min). *B. subtilis* and *S. aureus* were sensitive to the majority of the extracts (DIZ between 9.0 and 14.0 mm). However, highly efficacious inhibition of these bacteria was obtained with the extracts obtained under conditions 4 (54 °C, 4.2:10 molar ratio, 20 min) and 7 (36 °C, 4.2:10 molar ratio, 50 min), against *S. aureus.*

Compared to the Gram-positive bacteria, the Gram-negative bacteria were less sensitive to the extracts probably due the presence of an outer membrane surrounding their cell walls, which restricts the diffusion of hydrophobic compounds, contained in extracts, through its lipopolysaccharide covering (Burt, 2004). However, effective inhibition of *P. aeruginosa* (DIZ 14 mm), *E. coli* (DIZ 13 mm) and *S. marcescens* (DIZ 13.5 mm), was realised with extracts obtained under conditions 8 (45 °C, 5:10 molar ratio, 35 min), 7 (36 °C, 4.2:10 molar ratio, 50 min), 13 (45 °C, 3:10 molar ratio, 35 min), 15-17 (45 °C, 3:10 molar ratio, 35 min), and 4 (54 °C, 4.2:10 molar ratio, 20 min), respectively. In general, the antibacterial properties of the extracts were most potent, when high temperatures (45 and 54 °C) were used in the extraction process.

Predictive models of bacteria inhibition by pomegranate peel extracts

Table 2 and 3 show the effects of temperature, solid/solvent ratio and extraction time, on the antibacterial (*B. subtilis*, *S. aureus*, *E. faecalis*, *E. coli*, and *S. marcescens*) activity of the extracts, evaluated through statistical analysis of the experimental data (Table 1). For *B. cereus* and *P. aeruginosa*, it was not possible to obtain mathematical models of inhibition because regression analysis revealed low R^2 values of 0.7 and 0.5, respectively. A relatively high value of R^2 (close to 1.0), indicates that the model equation is capable of representing the system, under the given experimental domain.

Table 2. Main effects of pomegranate peel extracts on antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecalis*, estimated from the results of the central composite rotatable design.[1]

Bacteria		Effect	Standard error	t	*P*-value
B. subtilis	Mean	12.03	0.28	41.75	0.00057*
	X_1 (L)	2.40	0.27	8.85	0.01253*
	X_1 (Q)	0.70	0.30	2.36	0.14198
	X_2 (L)	3.41	0.27	12.60	0.00624*
	X_2 (Q)	-1.95	0.30	-6.55	0.02254*
	X_3 (L)	0.78	0.27	2.89	0.10170
	X_3 (Q)	-0.36	0.30	-1.20	0.35253
	X_1 vs X_2	-0.37	0.35	-1.06	0.40000
	X_1 vs X_3	-1.12	0.35	-3.18	0.08618
	X_2 vs X_3	-0.63	0.35	-1.76	0.21913
S. aureus	Mean	8.78	0.16	52.83	0.00356*
	X_1 (L)	0.54	0.15	3.45	0.07468
	X_1 (Q)	1.14	0.17	6.63	0.02196*
	X_2 (L)	2.33	0.15	14.90	0.00448*
	X_2 (Q)	0.61	0.17	3.55	0.07101
	X_3 (L)	-0.46	0.15	-2.96	0.09750
	X_3 (Q)	0.61	0.17	3.55	0.07101
	X_1 vs X_2	-1.00	0.20	-4.90	0.03923*
	X_1 vs X_3	0.50	0.20	2.45	0.13400
	X_2 vs X_3	0.50	0.20	2.45	0.13400
E. faecalis	Mean	16.28	0.17	97.88	0.00010*
	X_1 (L)	0.22	0.15	1.40	0.29478
	X_1 (Q)	-1.58	0.17	-9.18	0.01167*
	X_2 (L)	2.35	0.15	15.06	0.00438*
	X_2 (Q)	0.01	0.17	0.08	0.94053
	X_3 (L)	-0.98	0.15	-6.30	0.02440*
	X_3 (Q)	0.72	0.17	4.20	0.05229
	X_1 vs X_2	1.13	0.20	5.51	0.03138*
	X_1 vs X_3	-0.63	0.20	-3.06	0.09216
	X_2 vs X_3	0.13	0.20	0.61	0.60264

[1] *P<0.05; L = linear parameters; Q = quadratic parameters; X_1 = temperature; X_2 = solid/solvent ratio; X_3 = time.

The temperature (linear or quadratic parameter) and the molar ratio (linear parameter), were the more significant parameters, at a confidence level of 95% (P<0.05), to obtain extracts with antibacterial activity against all tested bacteria. These parameters had a positive effect against *B. subtilis*, *S. aureus* and *S. marcescens*, i.e. higher values imply a higher antibacterial activity. Conversely, for inhibition of *E. faecalis* and *E. coli*, the molar ratio and temperature had a positive and negative effect, respectively, therefore, extracts obtained

Table 3. Main effects of pomegranate peel extracts on antibacterial activity against *Escherichia coli* and *Serratia marcescens*, estimated from the results of the central composite rotatable design.[1]

Bacteria		Effect	Standard error	t	*P*-value
E. coli	Mean	12.74	0.33	38.30	0.00068*
	X_1 (L)	-1.30	0.31	-4.13	0.05379
	X_1 (Q)	-2.74	0.34	-7.94	0.01547*
	X_2 (L)	3.08	0.31	9.88	0.01010*
	X_2 (Q)	-2.20	0.34	-6.40	0.02354*
	X_3 (L)	0.14	0.31	0.46	0.69097
	X_3 (Q)	-0.79	0.34	-2.30	0.14947
	X_1 vs X_2	0.87	0.40	2.14	0.16532
	X_1 vs X_3	-0.13	0.40	-0.30	0.78839
	X_2 vs X_3	0.87	0.40	2.14	0.16532
S. marcescens	Mean	9.89	0.50	19.82	0.00253*
	X_1 (L)	1.83	0.47	3.91	0.05951
	X_1 (Q)	1.97	0.51	3.83	0.06199
	X_2 (L)	2.54	0.47	5.43	0.03232*
	X_2 (Q)	-1.38	0.51	-2.68	0.11496
	X_3 (L)	-1.22	0.47	-2.61	0.12052
	X_3 (Q)	-0.68	0.51	-1.31	0.31853
	X_1 vs X_2	-1.00	0.61	-1.63	0.24407
	X_1 vs X_3	0.25	0.61	0.40	0.72265
	X_2 vs X_3	0.25	0.61	0.40	0.72265

[1] *P<0.05; L = linear parameters; Q = quadratic parameters; X_1 = temperature; X_2 = solid/solvent ratio; X_3 = time.

Table 4. Analysis of variance (ANOVA) for the antibacterial activity of pomegranate peel extracts against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Serratia marcescens*.[1,2]

	Source of variation	SQ	DF	MS	F_{test}
B. subtilis	Regression	80.67	3	26.89	25.60
	Residue	13.59	13	1.05	–
	Lack of fit	13.09	5	–	–
	Pure error	0.50	2	–	–
	Total	94.26	16	–	–
S. aureus	Regression	27.26	3	9.08	15.13
	Residue	7.80	13	0.60	–
	Lack of fit	7.64	5	–	–
	Pure error	0.16	2	–	–
	Total	35.06	16	–	–
E. faecalis	Regression	37.21	4	12.40	15.90
	Residue	10.23	13	0.78	–
	Lack of fit	10.07	5	–	–
	Pure error	0.16	2	–	–
	Total	47.44	16	–	–
E. coli	Regression	68.80	3	22.93	18.50
	Residue	16.14	13	1.24	–
	Lack of fit	15.47	5	–	–
	Pure error	0.67	2	–	–
	Total	84.94	16	–	–
S. marcescens	Regression	66.03	1	66.03	59.00
	Residue	16.85	15	1.12	–
	Lack of fit	15.35	5	–	–
	Pure error	1.50	2	–	–
	Total	82.88	16	–	–

[1] SQ = sum of squares; DF = degrees of freedom; MS = mean squares.
[2] For *B. subtilis*: R^2=0.85; $F_{(0.95;3;13)}$=3.41; for *S. aureus*: R^2=0.80; $F_{(0.95;3;13)}$=3.41; for *E. faecalis*: R^2=0.80; $F_{(0.95;4;13)}$=3.18; for *E. coli*: R^2=0.81; $F_{(0.95;3;13)}$=3.41; for *S. marcescens*: R^2=0.80; $F_{(0.95;1;15)}$=4.54.

at lower temperatures and higher solid/solvent ratio were most effective against these microorganisms. The positive influence of the solid/solvent ratio, can be attributed to the higher amount of solvent, which causes more dissolution of bioactive compounds and, consequently, facilities the extraction (Mohamed and Chang, 2009).

The ANOVA (Table 4) indicated that the *F*-test values were approximately 7.5, 4.4, 5.0, 5.4 and 13.0 times higher than the *F*-tabulated values, for *B. subtilis*, *S. aureus*, *E. faecalis*, *E. coli* and *S. marcescens*, respectively. Also, the R^2 values ranged from 0.80-0.85, indicating a high degree of correlation between the experimental values and the fitted values. Thus, the models were valid to predict 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 2-6.

For *B. subtilis*:

$$DIZ(mm) = 12.03 + 2.40\,X_1 + 3.40\,X_2 - 1.96\,X_2^2 \quad (2)$$

For *S. aureus*:

$$DIZ(mm) = 8.78 + 1.14\,X_1^2 + 2.33\,X_2 - X_1X_2 \quad (3)$$

For *E. faecalis*:

$$DIZ(mm) = 16.28 - 1.58\,X_1^2 + 2.35\,X_2 - 0.98\,X_3 + 1.12\,X_1X_2 \quad (4)$$

For *E. coli*:

$$DIZ(mm) = 12.74 - 2.73\,X_1^2 + 3.08\,X_2 - 2.20\,X_2^2 \quad (5)$$

For *S. marcescens*:

$$DIZ(mm) = 10.00 + 2.54\,X_2 \quad (6)$$

where, DIZ is the diameter of inhibition zone; X_1 is the solid/solvent ratio, X_2 is the temperature, and X_3 is the time.

The generated response surfaces showed that the bacterial inhibition can be maximised, with extracts obtained under the following conditions: (1) *B. subtilis* (Figure 1A), solid/solvent ratio between 3.2:10 and 4.5:10, at >55 °C; (2) *S. aureus* (Figure 1B), solid/solvent ratio between 3.5:10 and 5.0:10, at 30 °C; (3) *E. faecalis* (Figure 1C) solid/solvent ratio between 4.5:10 and 5.0:10, between 42 and 60 °C, respectively; (4) *E. coli* (Figure 1D), solid/solvent

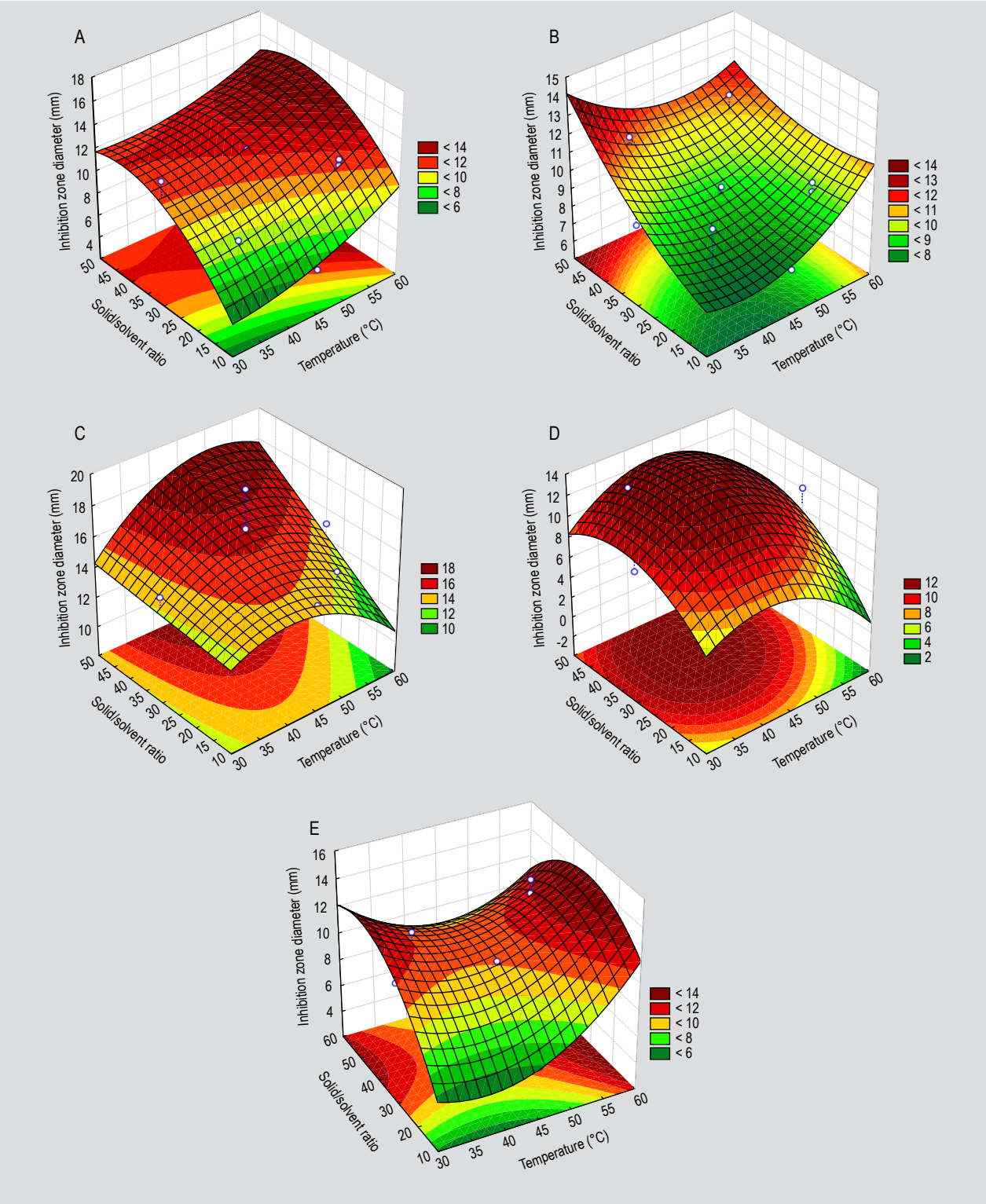

Figure 1. Response surface for diameters of inhibition zone obtained with pomegranate peel extract against: (A) *Bacillus subtilis*, (B) *Staphylococcus aureus*, (C) *Enterococcus faecalis*, (D) *Escherichia coli* and (E) *Serratia marcescens*.

ratio between 2.5:10 and 5.0:10, between 35 and 50 °C, respectively; and (5) *S. marcescens* (Figure 1E), solid/solvent ratio between 4.0:10 and 5.5:10, at about 25 °C. This study allowed to obtain the best conditions for the extraction of compounds of the pomegranate peel with high antimicrobial activity against *B. subtilis*, *S. aureus*, *E. faecalis* and *E. coli.*

The experimental and predictive values (obtained from Equation 2-6) were compared, to validate the generated mathematical models. The degree of replication, assuming a tolerable deviation from the calculated model of ≤15% (Gasparetti *et al.*, 2006), was considerably high, given the data showed deviation bands between 8 and 15%.

The antibacterial activity of pomegranate residues extracts has been determined by various researchers against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *E. coli*, *L. monocytogenes*, *Listeria innocua*, *Salmonella* sp., *Pseudomonas stutzeri*, *Clostridium difficile*, *Alicyclobacillus acidoterrestris* and *Vibrio parahaemolyticus* (Al-Zoreky, 2009; Devatkal *et al.*, 2013; Finegold *et al.*, 2014; Gullon *et al.*, 2016; Hayrapetyan *et al.*, 2012; Molva and Baysal, 2015; Wu *et al.*, 2016). However, there are no reports about developing predictive models of bacterial inhibition as a function of the conditions used to obtain pomegranate extracts.

Minimum inhibitory concentration and minimum bactericidal concentration of pomegranate peel extracts

The MICs and MBCs were determined for the extracts obtained under conditions 4 and 12 (as shown in Table 1), which, as above-mentioned, showed the highest antibacterial activity (highest DIZ) against the tested bacteria. For both extracts, the MIC and MBC values were 400 and >400 µl/ml, respectively, against all the bacteria tested. According to Aligiannis *et al.* (2001), the classification for plant material antimicrobial activity, is strong inhibition for MIC values <500 µg/ml; moderate inhibition for MIC values at 600-1,500 µg/ml, and weak inhibition for MIC values >1,600 µg/ml. Thus, the pomegranate peel extracts had strong inhibition against the tested bacteria, in this study.

The MIC values obtained in this study, were lower than those reported by some authors, such as Al-Zoreky (2009), who documented MIC values for 80% methanolic pomegranate peel extract ranging from 500-4,000 µg/ml, against *S. aureus*, *E. coli*, *S. enteritidi*s and *B. subtilis*. Gullon *et al.* (2016) obtained MIC and MBC values ranging from 40-50 and 50-60 mg/ml, respectively, against *S. aureus*, *E. coli*, *P. aeruginosa* and *Salmonella* spp. using methanolic pomegranate peel extract. Furthermore, Hayouni *et al.* (2011) obtained MIC values of 500 µg/ml, against *E. coli* and *P. aeruginosa*, and >2,000 µg/ml against *S. aureus*.

4. Conclusions

This work was the first study to describe predictive models for the inhibition of foodborne bacteria, such as *B. subtilis*, *S. aureus*, *E. faecalis*, *E. coli* and *S. marcescens*, using methanolic pomegranate peel extract. The RSM showed that temperature and solid/solvent ratio, were the more significant parameters to obtain extracts with high antimicrobial potential against the tested bacteria. This innovative work allowed obtaining mathematical models that predict the inhibition of these bacteria as a function of the extraction conditions (temperature, solid/solvent ratio and extraction time), to obtain methanolic pomegranate peel extracts. The optimised extracts can be used in future studies of food conservation.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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