RSC Advances

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Cite this: *RSC Adv.*, 2019, **9**, 39893

High pressure extraction of bioactive diterpenes from the macroalgae *Bifurcaria bifurcata*: an efficient and environmentally friendly approach†

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Received 20th August 2019
Accepted 22nd November 2019

DOI: 10.1039/c9ra06547d

rsc.li/rsc-advances

The brown macroalgae *Bifurcaria bifurcata* have gained special attention due to their ability to biosynthesize linear diterpenes (rarely found in other species). However, the conventional extraction methods normally used to extract these compounds involve organic solvents and often high temperatures, leading to the degradation of thermo-labile compounds. In this context, the main objective of this work was to study and optimize for the first time the extraction of diterpenes from *B. bifurcata* through an environmentally friendly methodology, namely, high pressure extraction (HPE) using ethanol : water. This was compared with conventional Soxhlet extraction, using dichloromethane. Box–Behnken design was employed to evaluate the linear, quadratic, and interaction effects of 3 independent variables (pressure (X_1), ethanol percentage (X_2), and time of extraction (X_3)) on response variables (extraction yield and diterpenes content (mg g^{-1} of extract and mg kg^{-1} of dry weight)) and the optimal extraction conditions (X_1: 600 MPa; X_2: 80%; X_3: 5 min) were estimated by response surface methodology (RSM). *B. bifurcata* extract obtained under HPE optimal conditions showed a diterpenes content (612.2 mg g^{-1} of extract) 12.2 fold higher than that obtained by conventional extraction (50.1 mg g^{-1} of extract). The HPE extract, obtained under optimal conditions, showed antioxidant and antibacterial (against *Staphylococcus aureus*) activities considerably higher than the Soxhlet extract, and also presented a promising synergic effect with antibiotics, improving the antibiotic efficacy against *S. aureus*. In conclusion, these results indicate that HPE is a promising methodology, compared to conventional methodologies to obtain linear diterpene rich extracts from *B. bifurcata* with great potential to be exploited in pharmaceutical or biomedical applications.

1. Introduction

Bioactive natural compounds from marine sources have gained increased interest.[1] *Bifurcaria bifurcata* is a brown macroalga that thrives, year-round, in the intertidal areas (the lower shore, rock-pools and mid-shore) along the coast of the Northern Atlantic, from Morocco (southern limit) to north-western Ireland (northern limit).[2–4] The chemical composition of this brown macroalga has gained particular attention, due to the abundance of a variety of acyclic diterpenes.[5–12] Compared to their cyclic counterparts, these linear diterpenes are relatively quite rare in nature,[2] yet quite interesting since a vast range of promising bioactivities have been recognized for them.[2,3,7,8,13–15] Recently, Santos *et al.*[5] demonstrated the antioxidant, anti-inflammatory and antibacterial activities of *B. bifurcata* lipophilic extracts, mainly composed of linear diterpenes. In addition, a promising synergism was observed when these extracts were used together with antibiotic families of major clinical importance.[5]

In general, this species could be a promising source of bioactive molecules useful for pharmaceutical, biomedical or even cosmetic industries.[3,5] Nonetheless, the commonly used conventional extraction methodologies involve the use of large amounts of organic solvents, often toxic to humans and harmful to environment, such as dichloromethane[5] or chloroform[16] which represents a major limitation to the industrial exploitation of *B. bifurcata* diterpenes rich extracts. In addition, these extraction methods have demonstrated poor selectivity as at the same time require long operation times frequently at high

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ra06547d

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temperatures, which can induce the degradation of thermolabile compounds.[13,14] Therefore, the evaluation and optimization of sustainable, economically viable and efficient methodologies to extract diterpenes from *B. bifurcata* macroalgae is an important challenge.

High pressure extraction (HPE) has been one of the emerging technologies that has been successfully exploited in the extraction of bioactive compounds from natural raw materials.[17,18] HPE provides a large differential pressure between the interior and exterior of the cells, which causes cell walls and membranes structural damages, increasing their permeability and thus enhancing compounds dissolution in the extraction media.[17] Additionally, HPE technology, usually performed at room temperature, presents a higher rate and efficiency of extraction than conventional methods, allowing to use safe solvents even presenting lower selectivity. Furthermore, this extraction methodology can be faster, with higher safety and energetically efficient.[17,19] Although a major limitation of HPE has been associated with the high capital and equipment costs, the perspective for an expansion of HPE implementation in the near future is expected to lead to a decrease in costs. Actually, HP technology has been adopted quickly as reflected by the increased number of units installed.[20,21]

Although some studies have endorsed the use of HPE to extract lipophilic components (namely fatty acids and terpenes) from natural sources,[18,19,22–26] no studies have been reported so far concerning the HPE of diterpenes. In addition, HPE has been only exploited in macroalgae to extract higher molecular weight components, namely sulfated polysaccharides from *Sargassum muticum*.[27]

In the present work the feasibility of HPE using ethanol : -water mixtures to extract diterpenes from *B. bifurcata* macroalga was studied for the first time. Besides, the process parameters of HPE were optimized by both Box–Behnken design and response surface methodology (RSM). As final output of this work, the higher efficiency of HPE to recover diterpenes from *B. bifurcata*, as well as the higher antioxidant and antibacterial activities and synergism with different antibiotics of the extract obtained, compared with those previously described are demonstrated.

2. Materials and methods

2.1 Sample preparation

The company ALGAplus, Produção e Comercialização de Algas e seus derivados, Lda. was responsible for the collection and preprocessing of algal samples. *B. bifurcata* (batch B1.2643.08F), was harvested in November 2013, at Aguda Beach (41°2′38″ N, 8°39′10″ W), region of Oporto, Portugal. Processing consisted in washing the biomass with running tap water and then with distilled water. Samples were preserved at −20 °C and then freeze-dried and milled at the laboratory of University of Aveiro.

2.2 Conventional extraction

For comparative purposes, three aliquots (5 g) of lyophilized macroalgae samples were Soxhlet extracted with dichloromethane (160 mL), for 9 h, following a procedure described before.[5] The solvent was evaporated to dryness and the extracts were weighed. The results are expressed in percent of dry weight (dw) material (w/w, %).

2.3 High pressure extraction

Each aliquot (5 g) of lyophilized macroalgae was dispersed in 50 mL of solvent (ethanol : water, as described in 2.3.1 and 2.3.2 sub-topics) and placed in a double packaged in low-permeability polyamide-polyethylene bags, which were heat-sealed under vacuum. The bags containing the biomass and solvent mixtures were subject to HPE under different time periods and pressures according to the established extraction conditions (Tables 2 and 3). HPE were performed in a hydrostatic press (Hyperbaric 55, Hyperbaric, Burgos, Spain), which has a pressure vessel of 200 mm inner diameter and 2.0 mm length with a maximum operating pressure of 600 MPa.

After HPE, the aqueous/ethanol extracts and the remaining residue were removed from the bags and filtered through a glass filter funnel (porosity 3). Ethanol was evaporated and the extracts were frozen at −80 °C until freeze-drying. Then, and similarly to the Soxhlet extract, the extraction yield (EY) was determined as weight percentage (w/w, %) of dried extract.

2.3.1 Preliminary experiments. Preliminary experiments were carried out (Table 2), in order to determine which independent variables would have an effect on HPE, as well as their range of values. The solid/liquid ratios used were 1 : 10 (5 g of dry macroalgae in 50 mL of aqueous/ethanol solution). The extraction time was 15 min.

2.3.2 Design of experiments. A Box–Behnken design was employed to determine the optimal extraction conditions for HPE of diterpenes from *B. bifurcata*. Based on the preliminary results, their main factors were chosen (pressure, ethanol percentage and time).

Table 3 presents the Box–Behnken design with three independent variables, designated as X_1, X_2, X_3, at three levels, coded +1, 0, −1 for high, intermediate and low values, respectively: namely extraction pressure (X_1: 0.1, 300 and 600 MPa), ethanol percentage (X_2: 40, 60 and 80%) and extraction time (X_3: 5, 17.5 and 30 min). A solid/liquid ratio of 1 : 10 was used. EY and the diterpenes content (DC) (expressed as milligram per gram of extract and milligram per kilogram of dw macroalgae) were used as response variables to evaluate the influence of the different levels of independent variables combined.

The response surface design consisted in 15 runs in randomized order, to minimize the effects of unexpected variability in the observed responses,[27] with three replicates in the centre point to estimate the pure error sum of squares (Table 3).

A full quadratic model was used to fit the data according to eqn (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} \beta_{ij} X_i X_j \qquad (1)$$

where Y is the predicted response, β_0, β_i, β_{ii} and β_{ij} are coefficients in the intercept, linear, quadratic and interaction terms, respectively, and X_i, X_j are the independent variables.

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Analysis of variance (ANOVA) for response surface quadratic model validation was performed, and the test for significance of each term to test for goodness of fit was conducted at $P < 0.05$. The design construction and analysis were achieved through Minitab 18 (Minitab Statistical Software, Pennsylvania State University, State College, PA) software.

2.4 Diterpenes analysis by gas chromatography-mass spectrometry (GC-MS)

Before GC-MS analysis, aliquots of each dried extract (nearly 20 mg each) and an accurate amount of internal standard (hexadecane, 0.8 mg) were dissolved in 1100 μL of dichloromethane. High pressure extracts were, prior to the injection, filtered through a syringe filter (0.2 μm Teflon filter).

The extracts were analysed by GC-MS following previously described methodologies[5,28,29] on a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) equipment, equipped with a DB-1 J&W (Agilent, Santa Clara, CA, United States) capillary column (30 m × 0.32 mm inner diameter, 0.25 μm film thickness). The chromatographic conditions were as follows: initial temperature, 80 °C for 5 min; temperature gradient, 52 °C min^{-1}; final temperature 285 °C for 8 min; injector temperature, 250 °C; transfer-line temperature, 290 °C; split ratio, 1 : 40.

The identification of compounds was carried out through the comparison of their mass spectra fragmentation profile with library (Wiley 275 and U.S National Institute of Science and Technology (NIST14)), their characteristic retention times obtained under the described experimental conditions[5] and by comparing their mass spectra fragmentation profiles with published data[9,10,28] or by injection of standards.

For semi-quantitative analysis and to determine the response factor for diterpenes, GC-MS was calibrated with phytol, relative to hexadecane. The respective response factor was calculated as an average of six GC-MS runs. Three aliquots of each extract were injected in duplicate, and the results correspond to the average of the concordant values obtained (less than 5% variation between injections of the same aliquot). The compound contents were expressed as milligram per gram of extract (mg g^{-1} of extract) and as milligram per kilogram of dw of macroalgae (mg kg^{-1} dw).

2.5 Scanning electron microscopy (SEM) analysis

SEM micrographs were obtained by a Hitachi SU-70 microscope operating at 4 kV. Dried samples (initial lyophilized macroalgae, and macroalgae after Soxhlet and high-pressure extractions) were placed in an aluminium support with double sided carbon tape and deposited with a carbon coating before SEM analysis.

2.6 *In chemico* and *in vitro* biological activities evaluation

2.6.1 Antioxidant activity evaluation. The antioxidant activity of *B. bifurcata* extracts was measured through the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) assay, which evaluates the hydrogen-donating or radical scavenging ability of extracts, following a procedure reported before.[5]

The dry extracts were previously dissolved in methanol (4 mg mL^{-1}). Sample aliquots (0.5 mL) were mixed with 0.125 mL of DPPH$^{\bullet}$ (0.8 mM in methanol) and 1.375 mL of methanol. The ranges of final concentrations were 50–1000 μg mL^{-1} for Soxhlet extract and 20–100 μg mL^{-1} for high pressure extracts. Mixtures were homogenized in vortex. After 30 min of incubation in the dark, at room temperature, the absorbance was read at 517 nm, against a blank, using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Duplicate measurements of each extract were carried out and each absorbance was compared to a control without extract.

The antioxidant activity was expressed as a percentage of DPPH radical reduction, using the following eqn (2):

$$\%\ \text{DPPH reduction} = \frac{(\text{Abs(control)} - \text{Abs (sample)})}{\text{Abs(control)}} \times 100 \quad (2)$$

The inhibitory concentration of the extract required to decrease the initial DPPH radical concentration by 50% (IC_{50}) was determined from the graph of DPPH reduction percentage in function of extracts concentration. The IC_{50} values were expressed in μg mL^{-1}.

2.6.2 Antibacterial activity evaluation. The antibacterial activity of *B. bifurcata* extracts was evaluated against Gram-positive bacteria *Staphylococcus aureus* ATCC® 43300, through minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determinations and, also, through a synergistic assay by conjugation of the extract with antibiotics. Antibacterial activity assays were performed according to Clinical and Laboratory Standard Institute (CLSI) guidelines[30] and following the procedure described in a previous work by Santos *et al.*,[5] with some modifications. Briefly, *S. aureus* cells in exponential phase were suspended in Mueller–Hinton Broth (MHB; Liofilchem, Italy) to obtain a concentration of 5×10^5 cfu mL^{-1} and *B. bifurcata* extracts, obtained from high pressure and Soxhlet extractions, were dissolved in dimethyl sulfoxide (DMSO; AppliChem, Germany) to a final stock concentration of 50 mg mL^{-1}. The method used was microbroth dilution, using a range of concentration from 8 to 2048 μg mL^{-1}, in a 96-well plate (TPP, Switzerland).[31] MIC was assessed qualitatively by adding Resazurin sodium salt 0.1 mg mL^{-1} (Sigma-Aldrich, USA) in each well, after 24 h of incubation at 37 °C, according to the protocol described by Riss *et al.*[32] with slight adaptations. Resazurin allows MIC visualization at naked eye where the originally blue colour does not turn to pink and fluorescent colour, product of viable cells metabolism. Experiments were performed three times and each one with triplicates ($n = 9$).

MBC, defined as the lowest concentration of the extract that results in killing 99.9% of bacterial cells, was determined by subculturing the corresponding MIC onto agar plates. In this assay, MIC and concentrations above were plated on Mueller–Hinton Agar (MHA; Liofilchem, Italy), using the spreading technique. The lowest concentration without visible growth corresponded with the MBC. Experiments were performed three times and each one with duplicates ($n = 6$).

The synergistic assay was performed following the protocol described above, according to Santos *et al.*[5] *B. bifurcata* high pressure extract was conjugated with the antibiotics rifampicin (Rif; Duchefa Biochemie, Alfagene), tetracycline (Tetra; Duchefa

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Biochemie, Alfagene), gentamicin (Gent; Duchefa Biochemie, Alfagene) and ampicillin (Amp; Duchefa Biochemie, Alfagene) in a concentration range from 2 to 256 μg mL^{-1}. Experiments were performed three times and each one with triplicates (n = 9). Factorial inhibitory concentration index (FICI) was calculated to classify interaction between *B. bifurcata* high pressure extract and antibiotics.[33] Each of the combinations was calculated according to the following eqn (3):

$$\mathrm{FICI} = \frac{\mathrm{MIC(antibiotic + extract)}}{\mathrm{MIC(antibiotic)}} \quad (3)$$

Results were interpreted as follows: FICI ≤ 0.5 synergistic (S), 0.5 < FICI < 1 partially synergistic (PS), FICI = 1 additive (ADD), 1 < FICI ≤ 4 indifferent (I) and FICI > 4 antagonistic (ANT).

3. Results and discussion

3.1 Lipophilic fraction obtained by soxhlet extraction

The lipophilic extract of wild *B. bifurcata* obtained by Soxhlet extraction presented an EY of 9.4 ± 0.1% (w/w) which was about three-fold higher than the Soxhlet EY obtained previously for *B. bifurcata* also from Portugal.[5] Additionally, the EY observed was considerably higher than those previously reported for other *Phaeophyta* species.[28,34] A detailed study of *B. bifurcata* Soxhlet extracts composition was performed by GC-MS analysis (Table 1), according to a previously established methodology.[5]

B. bifurcata has already been studied due to its variety of diterpenes.[2,6–12] However, only a single detailed study of its lipophilic fraction was performed by GC-MS analysis, in which other compounds were identified and quantified, namely sterols, fatty acids, long-chain aliphatic alcohols, monoglycerides, among others.[5]

Several diterpenes were identified in *B. bifurcata* Soxhlet extract, namely neophytadiene, phytol, *trans*-geranylgeraniol, 6,7,9,10,11,12,14,15-tetrahydrophytol, 6-hydroxy-13-oxo-7,7′,10,11-didehydrophytol, eleganolone, and 1-acetyl-10,13-dioxo-6,7,11,11′,14,15-tridehydrophytol. Some of these linear compounds were previously detected in *B. bifurcata* collected at different geographical points, such as France,[9,11,35] Morocco[10] and Spain.[36] Phytol and neophytadiene were also reported before as constituents of this macroalga from Portugal.[5] *trans*-Geranylgeraniol was already identified as *B. bifurcata* constituent from Morocco[8,37] and Brittany.[9,37]

The diterpenes in the studied extracts were identified by comparing the mass spectra fragmentation profile with libraries (Wiley 275 and U.S. National Institute of Science and Technology (NIST14)), their characteristic retention times obtained under the described experimental conditions[5] and literature data.[9,10,28]

Diterpenes accounted for 6395 mg kg^{-1} dw of *B. bifurcata*. Eleganolone and 6-hydroxy-13-oxo-7,7′,10,11-didehydrophytol were the major components of this family, accounting for 6180 mg kg^{-1} dw and 144 mg kg^{-1} dw, respectively. 1-Acetyl-10,13-dioxo-6,7,11,11′,14,15-tridehydrophytol and neophytadiene were also present in considerable amounts.

These linear diterpenes have been the focus of interest of several studies, since they are relatively rare in nature.[2,15] In addition, they have been associated with several biological activities, such as antioxidant, antimicrobial and anti-inflammatory properties.[5,38]

In order to obtain these extracts without the use of hazardous solvents and using a more sustainable approach we decided to study their extraction with ethanol : water under HPE.

3.2 Preliminary high-pressure extractions

Preliminary HPE experiments were performed considering two independent variables, namely the extraction pressure (600, 300 and 0.1 MPa) and the ethanol : water ratio (80 : 20 and 60 : 40). These experiments were carried out to choose the experimental design variables and their ranges of values, and thus the type of experimental design. As shown in Table 2, the differences observed in the EY and DC (mg g^{-1} of extract and mg kg^{-1} dw) values of the preliminary experiments indicated that these factors have possible significant effects on the results.

HPE extracts presented quite different EY values, accounting 6.9% (w/w) at 600 MPa and 80% ethanol and 11.9% (w/w) at 300 MPa and 60% ethanol. These values are also different from that obtained with Soxhlet extraction (9.4% (w/w)). The experiment 3 (Table 2), performed at atmospheric pressure and 80% ethanol, showed an EY of 8.4% (w/w), which is in the range of those obtained at higher pressure and lower than that obtained with Soxhlet extraction.

Table 1 Diterpenes identified in *B. bifurcata* Soxhlet extract expressed in mg g^{-1} of extract and in mg kg^{-1} dw

Compound	mg g^{-1} of extract[a]	mg kg^{-1} dw[a]	Rt (min)
Neophytadiene	0.13	15	26.7
Phytol	0.03	4	32.3
trans-Geranylgeraniol	0.08	10	33.9
6,7,9,10,11,12,14,15-Tetradehydrophytol	0.07	9	34.4
6-Hydroxy-13-oxo-7,7′,10,11-didehydrophytol	1.12	144	36.3
Eleganolone	48.44	6180	38.0
1-Acetyl-10,13-dioxo-6,7,11,11′,14,15-tridehydrophytol	0.27	33	40.6
Total	**50.14**	**6395**	

[a] Results correspond to the average value estimated from the injection of three aliquots analysed in duplicate (standard deviation < 5).

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Table 2 Preliminary experiments extraction yield (EY) and diterpenes content (DC)

	Independent variables		Response variables[a]		
Preliminary experiment no.	Pressure (MPa)	% Ethanol	EY (w/w, %)	DC (mg g^{-1} of extract)	DC (mg kg^{-1} dw)
1	600	80	6.9	413.0	31 557
2	300	60	11.9	333.5	43 527
3	0.1	80	8.4	4299	39 679

[a] Results correspond to the average value estimated from the injection of three aliquots analysed in duplicate (standard deviation < 5%).

All the preliminary extractions showed total amounts of diterpenes (31 557 and 43 527 mg kg^{-1} dw) higher than that obtained with Soxhlet extraction (6394 mg kg^{-1} dw). The experiments with 80% ethanol (1 and 3) showed the highest DC values, suggesting that the ethanol percentage could have a high effect on the diterpenes yield. Comparing the experiments at different pressures (1 and 3), with the same ethanol percentage, the DC at atmospheric pressure was slightly higher than that obtained at 600 MPa. However, the standard deviation associated to experiment 3 was considerably high and therefore the differences may not be statistically significant.

When a lower ethanol percentage (60%) was used with a pressure of 300 MPa, higher EY and lower DC were achieved, which could mean that under these HPE conditions, the extraction of other compounds, such as polysaccharides, can be favored in the detriment of diterpenes extraction.

With the preliminary experiments, it was verified that HPE could be more selective to the diterpenic compounds, than the conventional extraction methodology. Therefore, pressure and ethanol percentage were chosen as variables to optimize. In addition, and taking into account the high number of studies showing a high effect of extraction time on the EY of target compounds,[27,39,40] time was selected as the third variable for the experimental design. EY and DC (expressed as mg g^{-1} of extract and mg kg^{-1} dw) were selected as responses to optimize.

A pressure range between 0.1 and 600 MPa was selected, due to the highest DC observed for the preliminary experiments 1 (600 MPa) and 3 (0.1 MPa). Actually, 600 MPa correspond to the maximum value of pressure enabled by the equipment, so the full possible range was considered in order to enhance the maximum rupture of macroalga cell walls.[27]

In the same way, DC values were different at diverse mixture concentration. Therefore, the effect of this factor was evaluated in an extended range, namely between 40% and 80% of ethanol.

Finally, extraction time was selected to be 5–30 min, which has been in the range of most of the optimal extraction times reported in several studies.[27,39,40]

3.3 Analysis of the designed HPE experiments

HPE of diterpenes from *B. bifurcata* was optimized by response surface methodology using a Box–Behnken design. EY and DC expressed in mg g^{-1} of extract and in mg kg^{-1} dw of *B. bifurcata* extracts are shown in Table 3. The predicted values, within the limits of the experimental factors, are also listed in Table 3.

From a qualitative point of view and similarly to the observed in preliminary experiments, the lipophilic composition of HPE extracts was very similar to the Soxhlet extract.

3.3.1 Model fitting for experimental design. A Box–Behnken design was formulated to develop an empirical model for each measured response (namely, EY and DC, expressed as mg g^{-1} of extract and mg kg^{-1} dw) thereby evaluating the effect of the interaction of three independent variables (extraction pressure (X_1), ethanol percentage (X_2), and extraction time (X_3)) in the extraction of these bioactive compounds. The second order quadratic models were expressed as a function of the independent variables.

The results of 15 experiments including three replicates at the centre point were analysed, using the response surface methodology. Linear and quadratic effects of the three variables studied as well as their interactions were evaluated for regression coefficients.

3.3.2 Independent variables effect on extraction yield. Only the independent variables ethanol percentage (X_2) and time of extraction (X_3) had a significant effect ($P < 0.05$) on EY (Table 1S†). The EY of these 15 experiments ranged from 3.5 to 12.6% (w/w), and this last value was obtained under the following conditions: 600 MPa (X_1), 60% (X_2) and 30 min (X_3). Additionally, the percentage of ethanol was the most significant effect observed, presenting a F value of 19.96 (X_2), and extraction time was the second most significant factor exhibiting F value of 7.53 (X_3).

The main effects of independent variables on the measured responses can be observed through the interpretation of the 2D contour and 3D surface response plots (Fig. 1). The increase of extraction time and the decrease of ethanol percentage led to an increase of the extraction yield, which is also verified in the positive and negative values of the β-coefficient value (for coded variables) of the linear term (Table 1S†), respectively. This negative effect of the ethanol percentage could be related to the co-extraction of other components, namely polysaccharides, which are quite abundant in macroalgae, and their extraction may occur with high water contents on the extraction solvent mixture.

Extraction time also had a significant effect on the EY. As example, the yield increased from 3.5% (w/w) at 300 MPa, 80% ethanol and 5 min to 9.6% (w/w) at 300 MPa, 80% ethanol and 30 min, which is a variation of 64%. As HPE is known to be a faster methodology than other extraction methods,[19] the extraction time only has to be long enough to ensure the contact

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Table 3 Box–Behnken matrix and experimental and predicted values of the response variables for the HPE of *B. bifurcata*

	Coded levels of independent variables			Responses variables					
				EY (w/w, %)		DC[a] (mg g^{-1} of extract)		DC (mg kg^{-1} dw)	
Run no.	X_1 (pressure, MPa)	X_2 (% ethanol)	X_3 (time, min)	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	0 (300)	+1 (80)	−1 (5)	3.5	3.6	475.7	436.7	16 651	16 517
2	+1 (600)	+1 (80)	0 (17.5)	4.1	5.2	430.3	409.1	17 470	17 982
3	0 (300)	−1 (40)	−1 (5)	9.2	10.2	49.0	16.1	4484	4988
4	−1 (0.1)	0 (60)	−1 (5)	9.1	9.2	15.0	26.8	1370	1378
5	0 (300)	0 (60)	0 (17.5)	7.7	7.6	125.3	110.7	9620	8336
6	+1 (600)	0 (60)	+1 (30)	12.6	12.5	27.8	16.0	3496	3488
7	0 (300)	+1 (80)	+1 (30)	9.6	8.6	136.4	169.3	13 134	12 630
8	−1 (0.1)	−1 (40)	0 (17.5)	9.9	8.7	15.6	36.8	1540	1028
9	+1 (600)	0 (60)	−1 (5)	9.7	8.4	188.9	249.1	18 285	17 906
10	0 (300)	0 (60)	0 (17.5)	8.3	7.6	85.0	110.7	7048	8336
11	+1 (600)	−1 (40)	0 (17.5)	10.9	11.0	33.2	6.0	3608	3482
12	−1 (0.1)	0 (60)	+1 (30)	9.1	10.3	90.2	30.0	8213	8592
13	0 (300)	−1 (40)	+1 (30)	10.5	10.4	14.6	53.7	1541	1675
14	−1 (0.1)	+1 (80)	0 (17.5)	6.2	6.0	142.7	170.0	8888	9013
15	0 (300)	0 (60)	0 (17.5)	6.8	7.6	121.8	110.7	8340	8336

[a] Diterpenes content.

between the compounds and the solvent.[40] Notwithstanding, a higher amount of extractives is expected when increasing the extraction time.

No significant effect was observed for pressure ($P > 0.05$) (Fig. 1a and b). However, the maximum EY (12.6% (w/w)) was obtained at 600 MPa, corresponding to an increase of 28% compared to the yield obtained with the same conditions at atmospheric pressure (9.1% (w/w)).

According to the model, the EY was maximized at 600 MPa, 40% ethanol and an extraction time of 30 min, corresponding to a predicted value of 13.4% (w/w). A R^2 of 0.894 was obtained, which means that 10.6% of total variations are not explained by the model. However, the *P*-value for the lack-of-fit was 0.17, which shows that the model developed can represent well the results observed.[27,41]

3.3.3 Independent variables effect on diterpenes content (mg g^{-1} of extract). All linear effects (X_1, X_2 and X_3) showed to significantly affect ($P < 0.05$) the DC, expressed in mg g^{-1} of extract. Additionally, the interaction between ethanol percentage and extraction time (X_2X_3) shows also a significant effect ($P < 0.05$) (Table 2S†). All the quadratic effects showed to not affect significantly the DC.

Higher ethanol percentages and/or higher extraction pressures led to higher DC (mg g^{-1} of extract), as can be seen in

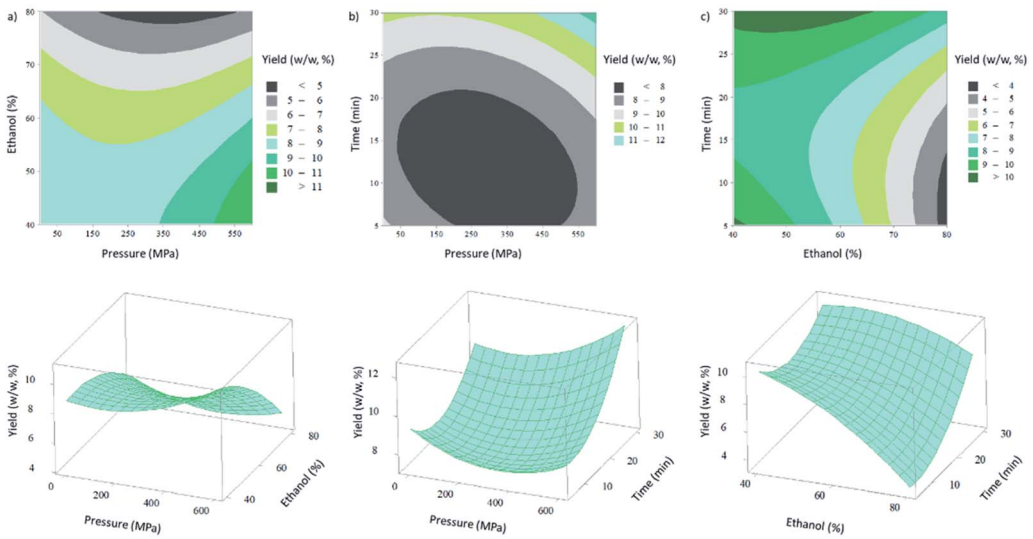

Fig. 1 Contour and response surface plots of extraction yield (w/w, %) as a function of (a) ethanol percentage (%) and pressure (MPa) (time = 17.5 min); (b) time (min) and pressure (MPa) (ethanol = 60%); (c) time (min) and ethanol percentage (%) (pressure = 300 MPa).

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contour and surface response plots (Fig. 2), through the positive β-coefficients values (for coded variables) of the linear terms (X_1 and X_2).

In general, an increase in extraction pressure resulted in an increase on DC. When the pressure changed from 0.1 MPa to 600 MPa, maintaining the remaining extraction conditions (X_2: 80% and X_3: 17.5 min), the DC increased from 142.7 to 430.3 mg g^{-1} of extract (~67%). This positive effect of pressure was expected, since at higher pressures the cell structures and membranes are destroyed, which increases mass transfer of solvents into raw materials, as well as of the soluble constituents into the solvents.[19] Concerning the effect of extraction time, the highest DC was obtained for the lower extraction time (5 min), which is also reflected in the negative value of the β-coefficient value (for coded variables) of the linear term. At 600 MPa and 60% of ethanol, the extractions carried out during 5 and 30 minutes, resulted in DC of 188.9 and 27.8 mg g^{-1} of extract, respectively. This means that the longer the extraction time, the lower the DC.

A hypothesis to explain the negative effect of extraction time is based on a higher abundance of co-extracted compounds, such as, polysaccharides, and a possible adsorption of diterpenes in the macromolecules. In fact, brown macroalgae are known for their high content in polysaccharides.[42,43] Before GC-MS analysis, several steps are performed, such as filtration, where losses can result in the reduction of co-extracted compounds. Upon elimination, the polysaccharides may consequently retain part of the diterpenes, which results in a decrease in this response.

The maximum of DC (475.7 mg g^{-1} of extract) was achieved at 300 MPa, the value of extraction pressure, with the lowest extraction time (5 min) and with the highest percentage of ethanol (80%). However, at 300 MPa an extraction time of 30 min and with a percentage of ethanol of 40%, the minimum amount of diterpenes per g of extract (14.6) was extracted. This means that the ethanol percentage and the extraction time were the most significant effects, which is in accordance to the F values of their linear effects (44.60 and 8.19, respectively) (Table 2S†).

According to the model, the maximum predicted DC (593.5 mg g^{-1} of extract) could be obtained with the following HPE conditions: extraction pressure (X_1), 600 MPa; ethanol percentage (X_2), 80%; and extraction time (X_3), 5 min.

The R^2 value (Table 2S†) of this model was 0.943, which represents a good correlation between the observed and predicted values, where more than 94% of responses variability are explained by the model. Furthermore, the P-value for the lack-of-fit non-significant (0.09) suggests once more that the developed model can represent the observed results.

3.3.4 Independent variables effect on diterpenes content (mg kg^{-1} dw). Regarding DC expressed as mg kg^{-1} dw, with the exception of ethanol : time interaction term (X_2X_3), all the linear and interaction effects showed to have significant effect (P-value < 0.05) (Table 3S†). The 2D contour and 3D surface response plots in Fig. 3 show that DC (mg kg^{-1} dw) increased with increased ethanol percentage and extraction pressure. In fact, the positive β-coefficients values (for coded variables) of the corresponding linear terms (Table 3S†) are in agreement with this effect. However, and similarly with the observed for the DC in a basis of extract, the effect of extraction time was opposite, obtaining higher diterpenes amount per kg of dw when the extraction has a short length. As expected, the linear effect of each variable in this response was quite similar to those of the previous response.

In this measured response, the effect of extraction pressure was more evident when compared with the other models. The two HPE performed with 60% of ethanol, for 5 min, at different values of pressure (0.1 and 600 MPa), resulted in a minimum (1370 mg kg^{-1} dw) and maximum (18 285 mg kg^{-1} dw) DC, respectively. Thus, the highest extraction pressure ensured a higher DC. In fact, when the compression level applied exceeds the deformation limit of the cells, can lead to formation

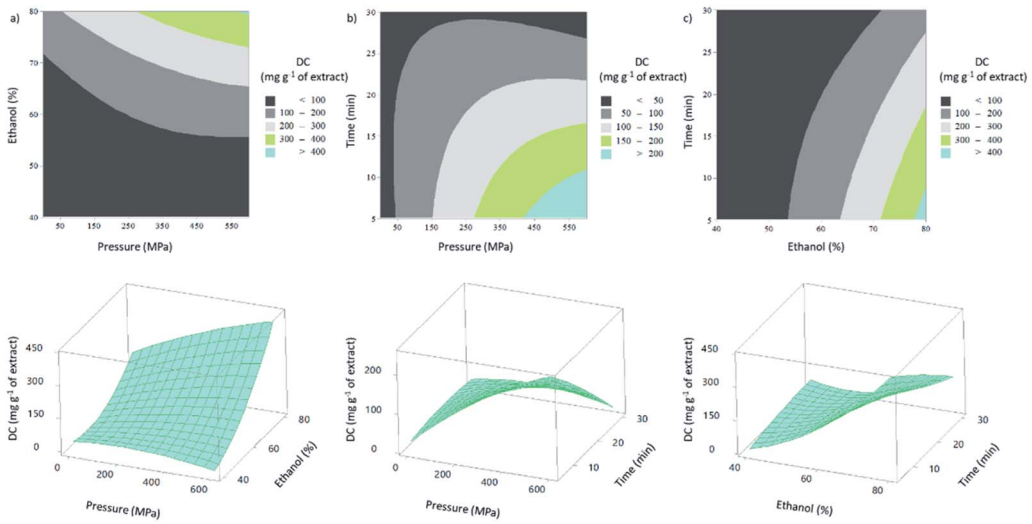

Fig. 2 Contour and surface response plots of diterpenes content (mg g^{-1} of extract), as a function of (a) percentage of ethanol (%) and pressure (MPa) (time = 17.5); (b) time (min) and pressure (MPa) (ethanol = 60%); (c) time (min) and percentage of ethanol (%) (pressure = 300 MPa).

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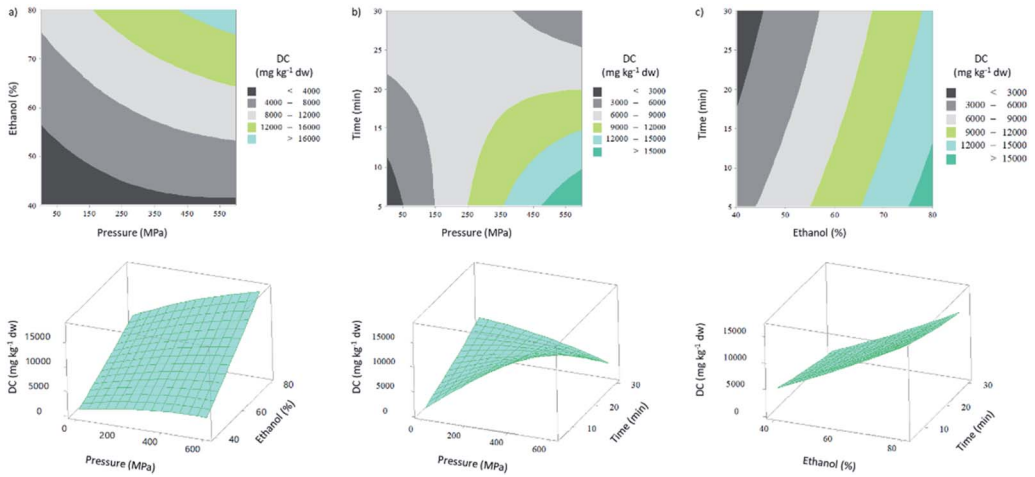

Fig. 3 Contour and surface response plots of diterpenes content (mg kg^{-1} dw), as a function of (a) percentage of ethanol (%) and pressure (MPa) (time = 17.5); (b) time (min) and pressure (MPa) (ethanol = 60%); (c) time (min) and percentage of ethanol (%) (pressure = 300 MPa).

of cracks,[19] resulting in more solvent inside the cells and consequently more compounds permeate the damaged cell membrane.[40]

Notwithstanding, the most important effect to achieve higher amounts of diterpenes was the ethanol percentage, which had a F value of 269.12 (linear effect), represented in Table 3S.† DC (mg kg^{-1} dw) increased 79%, when the ethanol percentage was changed from 40% (3608 mg kg^{-1} dw) to 80% (17 470 mg kg^{-1} dw).

Pressure : time interaction also had a significant effect (F value of 124.54) (Table 3S†) on the DC in a dw basis. According to the model, the maximum predicted DC (25 625.2 mg kg^{-1} dw) is achieved at X_1: 600 MPa; X_2: 80%; and X_3: 5 min. At these extraction conditions, there is a large pressure differential between the intra- and extracellular medium, which will lead to a rapid permeation of the compounds obtaining the equilibrium in a shorter time.[40]

The R^2 and R_{adj}^2 of the predicted model were 0.990 and 0.973, respectively, which did not differ significantly. In agreement with these statistical parameters, the P-value of lack-of-fit was 0.84 (P-value > 0.05) (Table 3S†), which is another evidence that the model equation for DC (mg kg^{-1} dw) was adequate to predict the respective values under any sets of combination within the range of experimental values. Additionally, only 27% of samples showed a variation between values higher than 10% (Table 3).

3.4 Optimization of high-pressure extraction conditions

The optimization of HPE of diterpenes from *B. bifurcata* was performed in order to maximize the DC (expressed as mg g^{-1} of extract and mg kg^{-1} dw) and not EY, because an increase in the EY might mean an increase of co-extracted polysaccharides. The optimized extraction pressure, ethanol percentage and extraction time were 600 MPa, 80% and 5 min, respectively. According to the models, the predicted results in extract and dw basis under these conditions were 593.5 mg g^{-1} of extract and 25 625 mg kg^{-1} dw, respectively. The experimental results achieved at this point were 612.2 ± 10.6 mg g^{-1} of extract and 38 954 ± 633 mg kg^{-1} dw (Table 4). The difference between the predicted and experimental values was satisfactory, being the variation lower than 5% for DC (mg g^{-1} of extract), which validates the respective model.

3.5 Comparative perspective between HPE at optimal conditions and conventional extraction

3.5.1 Extraction yield and diterpenes content. As mentioned before, the optimal HPE conditions were determined to maximize the DC, in both extract and dw basis. Actually, the optimal conditions determined for EY were different (X_1: 600 MPa; X_2: 40%; X_3: 30 min). For this reason, the experimental yield obtained at the optimal HPE conditions (6.9 ± 0.6% (w/w)) was lower than the Soxhlet EY (9.4 ± 0.1% (w/w)), as shown in Table 4.

Concerning the amount of diterpenes in the extract, HPE allowed obtaining 612.2 ± 10.6 mg g^{-1} of extract, which is 12.2-fold higher than conventional extraction (50.1 mg g^{-1} of extract). In the same way, the DC in a dw basis in HPE accounted for 38 954 ± 633 mg kg^{-1} dw, which is considerably higher (6.1-fold) than that obtained with Soxhlet extraction (6395 mg kg^{-1} dw).

Table 4 Experimental values of extraction yield (EY) and diterpenes content (DC) obtained in *B. bifurcata* Soxhlet and optimized HPE extracts[a]

	Responses variables		
Optimal conditions	EY (w/w, %)	DC (mg g^{-1} of extract)	DC (mg kg^{-1} dw)
Soxhlet extraction	9.4 ± 0.1	50.1 ± 13.2	6394 ± 767
HPE	6.9 ± 0.6	612.2 ± 10.6	38 954 ± 633

[a] HPE – high pressure extraction; dw – dry weight.

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3.5.2 SEM analysis. Once the optimal HPE conditions were achieved, and in order to verify the effect of this methodology on biomass structure, SEM analysis was performed. As mentioned before, the basis of this extraction methodology is the increase permeability of pressurized cells.[19] At high pressure, the surface area increases since pores and gaps are formed in the cells structures (cellular membranes and organelles membranes), which facilitate the solvent entry on the cell.[17,19]

Macroalgae before (Fig. 4a) and after conventional (Fig. 4b) and HPE at optimal conditions (Fig. 4c) were analysed by SEM. The higher magnification images (×2.5k) showed the biggest differences at the cell surface level, between the three samples. The image of the initial macroalga shows a regular surface, whereas the image correspondent to macroalga after Soxhlet extraction present already some damages. Nonetheless, the most evident surface damages are present in the macroalga after HPE, where gaps can be observed in the cell structure.

The SEM images of these samples corroborated the results reported above, notably the higher amount of diterpenes extracted in HPE, since greater damage in the cell structure leads to a reduction of mass transfer resistance and, consequently, to a higher amount of compounds extracted.

3.5.3 Antioxidant activity evaluation. The antioxidant activity of *B. bifurcata* extracts, obtained by Soxhlet and HPE at optimal conditions was studied *in chemico* by the DPPH assay and the results expressed as IC_{50}.

Previous antioxidant activity results of solid–liquid and Soxhlet *B. bifurcata* extract against DPPH$^{\bullet}$, reported for a wild sample collected from Peniche Coast Portugal (345 μg mL^{-1} (246.10–482.80))[3] and for a sample from an integrated multi-trophic aquaculture from Portugal (366 ± 10 μg mL^{-1}),[5] respectively, showed quite similar IC_{50} values. In the present work, the Soxhlet extract of *B. bifurcata* collected in the Portuguese north coast, presented an IC_{50} value of about 777 ± 16 μg mL^{-1}, demonstrating a lower antioxidant activity than those reported before.

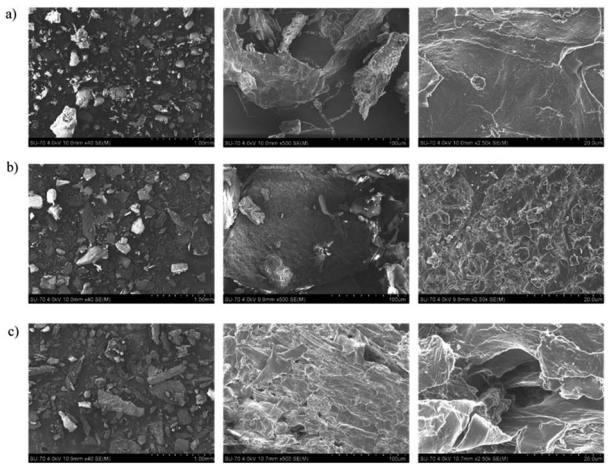

Fig. 4 SEM micrographs of macroalgae (a) before extraction, (b) after Soxhlet extraction and (c) after HPE for three different magnifications (×40, ×500 and ×2.50k).

On the contrary, the antioxidant activity of *B. bifurcata* HPE extract obtained at the optimal conditions accounted for 28 ± 2 μg mL^{-1}, which is a noticeably improved result compared to the Soxhlet extract obtained from the same macroalgae sample as well as from those previously reported in the literature. In addition, this IC_{50} value is in the same range of those reported in the literature for extracts rich in antioxidant compounds (*e.g.* phenolic compounds),[44] which could emerge as a consequence of using H_2O and ethanol as solvents, due to their higher polarity. Thus, these compounds not extracted with DCM can also contribute to an improvement of antioxidant activity. Although, these antioxidant activity results clearly demonstrate the potential of HPE in this context. This difference may be related with the higher abundance of diterpenes observed in HPE extract. In fact, diterpenes have been well recognized by their bioactivities including antioxidant.[5]

3.5.4 Antibacterial activity. The antibacterial activity of the *B. bifurcata* Soxhlet extract and HPE extract obtained at the optimal conditions were evaluated against *Staphylococcus aureus* ATCC® 43300 and expressed as MIC and MBC values, which are present in Table 5. This strain has one of the highest antibiotic resistance, which is an important health concern, resulting in a demand for new therapeutic strategies to overcome it.[5] Thus, in this study, the synergist effect of *B. bifurcata* HPE extract, obtained at optimal conditions, with four distinct antibiotics were evaluated, similarly to that studied previously for a dichloromethane *B. bifurcata* extract.[5]

The antibiotics used represent drug families of major clinical importance, such as aminoglycosides (Gent: gentamicin), tetracyclines (Tetra: tetracycline), macrocyclics (Rif: rifampicin), and β-lactams antibiotics, such as aminopenicillins (Amp: ampicillin). The results are expressed in MIC and FICI (Table 6). The antibacterial activity observed for the *B. bifurcata* Soxhlet extract (MIC = 2048 μg mL^{-1}) matched that obtained by Santos *et al.*[5]

Whereas the activity of *B. bifurcata* HPE extract (MIC = 1024 μg mL^{-1}) was 2-fold higher than that of Soxhlet extract. Furthermore, MBC determination showed that *B. bifurcata* HPE extract has bactericidal effect against *S. aureus* ATCC® 43300 (MBC = 2048 μg mL^{-1}), which indicates that HPE method is more efficient than Soxhlet in what concerns antibacterial potential of the ensuing diterpene rich extracts. The higher content in diterpenes may explain the antibacterial activity obtained.

Table 5 Antibacterial activity of *B. bifurcata* Soxhlet and optimized HPE extracts expressed in MIC and MBC (μg mL^{-1})[a]

	S. aureus ATCC® 43300	
	MIC (μg mL^{-1})	MBC (μg mL^{-1})
Soxhlet extract	2048	>2048
HPE extract	1024	2048

[a] HPE – high pressure extraction; MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration.

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Table 6 Synergistic potential between *B. bifurcata* HPE extract and antibiotics: rifampicin, gentamicin, tetracycline and ampicillin, in a concentration range from 2 to 256 μg mL^{-1}, expressed as MIC (μg mL^{-1}) against *Staphylococcus aureus* ATCC® 43300[a]

	MIC (μg mL^{-1})	FICI
Rif	16	<0.125 (S)
Rif + HPE ext	<2	
Gent	>256	<0.125 (S)
Gent + HPE ext	32	
Tetra	>256	<0.125 (S)
Tetra + HPE ext	<2	
Amp	128	0.125 (S)
Amp + HPE ext	16	

[a] HPE ext – high pressure extract; Rif – rifampicin; Gent – gentamicin; Tetra – tetracycline; Amp – ampicillin; MIC – minimal inhibitory concentration; FICI – factorial inhibitory concentration index; synergistic (S) if FICI ≤ 0.5; partially synergistic (PS), if 0.5 < FICI < 1; additive (ADD), if FICI = 1; indifferent (IND), if 1 < FICI ≤ 4 and antagonistic (ANT), if FICI > 4.

The combination of the HPE extract with distinct antibiotics resulted in a considerable decrease of antibiotic MICs values against the *S. aureus* ATCC® 43300. Outstanding decreases were observed with gentamicin and tetracycline, which MIC values decreased from >256 μg mL^{-1} to 32 and <2 μg mL^{-1}, respectively. Regarding FICI values, performed according to eqn (3), *B. bifurcata* HPE extract with antibiotics resulted in a synergistic effect against *S. aureus* ATCC® 43300. HPE extract, obtained at optimal conditions, shows thus high potential to be further studied as a possible strategy to eradicate *S. aureus*.[5]

The possible use of natural compounds as coadjuvants in conventional antibiotherapy has already been described.[45,46] Since their structures are quite different from those of antibiotics, the mechanism of action and/or target may be different and, therefore, other pathways/targets might be involved in bactericidal effect. This fact leads to better outcomes such as enhanced efficacy, decreased dosage and delayed development of drug resistance.[45] Given the favourable results obtained, it would be of interest to better understand the mechanism against bacterial cells as well as to assess effectiveness over time of the combinations used.

4. Conclusions

In this study it was demonstrated the feasibility of HPE to enhance extraction of linear diterpenes from *B. bifurcata*. In addition, HPE showed to be more selective and efficient than conventional Soxhlet extraction with dichloromethane. The effect of pressure, ethanol percentage and time of extraction were evaluated and the HPE conditions were optimized using RSM. HPE optimal conditions achieved were: extraction pressure – 600 MPa; ethanol percentage – 80%; and extraction time – 5 min. At these conditions, the *B. bifurcata* extract presented a DC of 612.2 ± 10.6 mg g^{-1} of extract, which is 12.2-fold higher than Soxhlet extraction (50.1 mg g^{-1} of extract). In the same way, the DC in a dw basis was considerably higher in HPE, accounting for 38 954 ± 633 (mg kg^{-1} dw), which is 6.1-fold than that obtained with Soxhlet extraction (6394 mg kg^{-1} dw). HPE at optimal conditions also resulted in an extract with considerably higher antioxidant and antibacterial activities and a synergism with distinct antibiotics was also observed. Therefore, HPE shows to be a promising technique to obtain *B. bifurcata* extracts with potential to be used in pharmaceutical or biomedical applications, particularly in the management of antibiotic-resistant pathogenic bacteria.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Thanks are due to the University of Aveiro and FCT/MCT for the financial support for the CICECO (FCT UID/CTM/50011/2019) and QOPNA (FCT UID/QUI/00062/2019) research Units through national founds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement. This work was also funded through FCT under the Project UID/AGR/00115/2019 to ICAAM. S. A. O. Santos thanks the Agro-ForWealth: Biorefining of agricultural and forest by-products and wastes: integrated strategic for valorisation of resources towards society wealth and sustainability project (CENTRO-01-0145-FEDER-000001), funded by Centro2020, through FEDER and PT2020 for the contract. P. A. B. Ramos acknowledges the "MultiBiorefinery" project (POCI-01-0145-FEDER-016403) for her post-doctoral grant.

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