Highlights

Response surface methodology was used for microwave extraction of TPC from *S. holoschoenus.*

The optimal extraction conditions were found to be 56% acetone, 600 w and 69 s.

Only ethyl acetate fraction from the phenolic extract showed antipseudomonal effect.

A mixture of ethyl acetate fraction and EO of *T. fontaneseii* gave the highest activity.

The antibacterial effect was most important at low temperature.

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Optimized microwave-assisted extraction of phenolic compounds from *Scirpus holoschoenus* and its antipseudomonal efficacy, alone or in combination with *Thymus fontanesii* essential oil and lactic acid.

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Abstract

Phenols extracted from *Scirpus holoschoenus* rhizomes, alone or in combination with other substances, have interesting antipseudomonal properties. Maximum extraction efficiency was achieved by using parametric optimization methods. In this study, the microwave-assisted extraction of phenolic compounds from *Scirpus holoschoenus* L. rhizome was performed with the help of Response Surface Methodology, and the optimal parameters were found to be 56% acetone, 69 s time and 600 W for power, with a TPC value of 30.70 ±1.22 mg GAE /g dry weight (dw). The ethyl acetate fraction (EA) of the optimized extract was tested in combination with diluted essential oil of *T. fontanesii* (EO_d) and lactic acid (LA) on *Pseudomonas aeruginosa*. Mixture design indicated that at 7°C, the 75% EO_d, 25% EA and 0% LA mixture gave the largest diameter inhibition zone (63 ± 2 mm). A mixture of 83.7% EO_d and 16.28 % EA was the most effective at 37°C (24.16 ± 1.04 mm).

Keywords: *S. holoschoenus*, *T. fontanesii*, lactic acid, *P. aeruginosa*, antibacterial activity, optimization, temperature

1. Introduction

The preservation of foods by low temperature is one of the most widely used practices for keeping foods fresh (Zanoni and Zavanella, 2012). However, it is not always sufficiently effective, since psychrotolerant bacteria, thanks to their ability to grow at low and moderate temperatures, colonize a wide range of products (Moschonas, Bolton, McDowell, & Sheridan, 2011), especially when the cold chain is broken.

This group of bacteria has been shown to affect food quality and safety, leading to industrial losses and in some cases, presenting a danger to human health (Huss, 1997; Szabo, Scurrah, & Burrows, 2000). *Shewanella putrefaciens*, *Xanthomonas campestris* and *Pseudomonas* species (*P. fluorescens*, *P. fragi*, *P. lundensis*, and *P. viridiflava*) are among the most food spoilers (Rawat, 2015), and according to Arslan, Eyi, and Ozdemir (2011), psychrotrophic *Pseudomonads* are classed as major spoilage microorganisms due to their several extracellular enzymes. *P. aeruginosa* is a pathogenic bacterium which is responsible for nosocomial infections and has been shown to possess a remarkable capacity to resist antibiotics (Banu et al., 2016) either intrinsically or following acquisition of resistance genes.

A large number of phenolic compounds and essential oils have been identified as antimicrobial substances (Petti and Scully, 2009; Prasad et al., 2011; Sakanaka et al., 2014). Some of them are classed as GRAS (Generally Recognized As Safe), which creates interest in their incorporation into food products. However, the use of individual aromatic compounds as food preservatives requires a high concentration which often contributes an unwanted flavour and sometimes causes irritation and toxicity (Burt, 2004). Fortunately, some of these compounds exhibit a synergistic effect when used in combination (Burt, 2004; Kumar et al., 2015; Passereiter et al., 2004). Studies have shown the positive effect of essential oils in combination with organic acids (Alakomi et al., 2000; Dimitrijević et al., 2007).

For a better exploitation of natural substances of vegetable origin, advanced techniques have been developed, starting with extraction tools and it has been seen that non-conventional methods can save time and solvent (Koubaa et al., 2015; Roselló-Soto et al., 2015). Researchers made use of a microwave-assisted process for extracting bioactive substances from the plant matrix (Bouras et al., 2015; Proestos and Komaitis, 2008; Wang et al., 2006; Zohar et al., 2004). However, since the nature of bioactive substances differs from one plant to another, they do not respond in the same way to extraction techniques and conditions. Thus, careful extraction aided by parametric optimization has been a useful step towards the success of the following experiments.

If the studies on *Thymus fontanesii* are numerous (Boukraâ et al., 2013; Dob et al., 2006; Ghannadia et al., 2004), this is not the case for *S. holoschoenus*, which is a plant from the *Cyperaceae* family, the decoction of whose rhizomes has been empirically used for the protection of the liver in Romania (Popescu et al., 2016) and in North Africa to treat haemorrhoids. In Spain, an infusion of its inflorescences is used to treat catarrh, coughs and whooping cough (Gonzalez-Tejero et al., 1995), and its shoots as a hypotensive agent (Rivera et al., 2005). With respect to the chemical composition, a previous study on the phenolic compounds of the *S. holoschneus* rhizome identified vanillic, chlorogenic, caffeic, cinnamic and gallic acids and E and Z resveratrol (Popescu, 2011). Abdel-Mogib et al. (2001) isolated 2-prenyl-3,5,4'-trimethoxystilbene, 2-prenyl-3-hydroxy-5,4'-dimethoxystilbene, 2-prenyl-3,4'-dihydroxy-5-methoxy-stilbene and 3,5,4'-trimethoxystilbene from tubers of this plant, all of which are derivatives of reservatrol. Recently, a significant 1,1-diphenyl- 2-picrylhydrazyl (DPPH) radical scavenging effect of hydroacetone extract of *S. holoschoenus* has been reported (Oussaid et al., 2017) and its hydroethanol extract has been shown to have an antioxidant capacity (Popescu et al., 2016). Additionally, phenolic extract from this plant showed antibacterial activity against *B. subtilus* and *S. aureus* (Oussaid et al., 2017). To the

best of our knowledge, all studies conducted on the extraction of phenolic compounds from *S. holoschoenus* rhizome have been carried out using conventional approaches.

This study aims to (i) optimize the microwave extraction of phenolic compounds from the *S. holoschoenus* rhizome by applying Response Surface Methodology (RSM), (ii) to test the individual effects of lactic acid, essential oil of *T. fontanesii*, phenolic extract of *S. holoschoenus* and its fractions against *Pseudomonas aeruginosa* ATCC 27853 and (iii) to determine the optimum mixture proportions of the samples cited above for antibacterial activity at mesophilic and low temperature by using a simplex centroid design.

2. **Material and methods**

2.1.Chemicals

Sodium bicarbonate (Na_2CO_3) and Folin-Ciocalteu phenolic reagent were obtained from Prolabo (Loire, France). Gallic acid and dimethylsulphoxide (DMSO) were purchased from Biochem-Chemopharma (Loire, France).

2.2.Plant materials and Microbial strain

The two plants studied were harvested in May in northern Algeria and underwent several treatments. The underground part of *S. holoschoenus* was cleaned with distilled water, cut into small pieces and dried in an oven at 40 °C until the weight was stable. After grinding, the powder was protected from light and moisture. The aerial parts of *T. fontanesii* were dried in the shade.

Antibacterial activity was tested against *P. aeruginosa* ATCC 27853, obtained from the American Type Culture Collection. The test strain was grown in Brain Heart Infusion Broth (BHIB) for 24 h at 37 °C, then streaked on BHI-agar and incubated at 37 °C for 24 h.

2.3. Microwave-assisted extraction of phenolic compounds from S. holoschoenus

Phenolic compounds were extracted by a MAE technique using a modified microwave oven (ME8123ST, Samsung, Malaysia, UPC). For the optimization of the MAE process, the experimental approach was carried out in two steps:

2.3.1. Single-factor experiments

In the first stage, a preliminary study was carried out to determine the range of extraction variables. One gram of the *S. holoschoenus* powder sample was mixed with 20 mL of solvent (Proestos and Komaitis, 2008) and subjected to microwave irradiation for a certain time. A single factor test was performed and the parameters considered were the nature of the solvent (acetone, methanol and ethanol), extraction time, solvent-water ratio and microwave power (Table 1). Each variable was coded at three levels: low (-1), middle (0) and high (+1) (Table 2).

2.3.2. Experimental design and response surface approach

A total of 15 trials, with three replications at the central point (runs 14 and 1 are repetitions of run 5), were carried out according to the chosen design variables: extraction time (30-120 s, x_1), acetone ratio (0-90%, x_2) and the microwave power (300-900 w, x_3), while maintaining a constant liquid-to-solid value (20:1, v: w). A Box-Behnken design, which is a specific set of experiments defined by a matrix composed of different combinations of the variables, was used. The RSM was applied by JMP software for data analysis and model construction (Table 3).

The generalized second-order polynomial model was as follows：

$$Y = B_0 + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} B_{ii} x^2 + \sum_{i>j}^{k} B_{ij} x_i x_{ij} + E \qquad (1)$$

where B_0, B_i, B_{ii}, and B_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and xi, and x_j are the independent variables.

2.3.3. Determination of total phenolic compounds (TPC)

The TPC was determined by the Folin-Ciocalteu colorimetric method (Georgea et al., 2005). A volume of 1 mL of Folin-Ciocalteu reagent (diluted ten times with water) was mixed with 100 µL of the extract. After 5 min, 1 mL of a 7.5% aqueous solution of sodium carbonate (Na_2CO_3) was added. After 15 min in a water bath at 50 °C, the absorbance was measured at 760 nm. Gallic acid is used as a standard and the TPC are expressed in mg gallic acid equivalent / g of the dried powder (mg GAE/ g dw).

2.4. Fractionation of optimized crude extract

Five extractions were carried out as described in a previous section in order to increase the amount of optimized extract. The filtrates were combined and the solvent was evaporated to obtain a dry extract. The latter was weighed before being dissolved in 50 mL of distilled water. The aqueous solution was fractionated as follows: the extract was treated first with 3 x 25 mL of petroleum ether; then several times with ethyl acetate (1: 1, v / v) until a clear solution of ethyl acetate was obtained. In all, four solutions were obtained; those extracted with petroleum ether, ethyl acetate, an interphase solution formed between water and petroleum ether and finally, aqueous residues.

The different samples were concentrated with a vacuum evaporator and then subjected to evaporation in a ventilated oven (50 °C) (Safaei-Ghomi et al., 2009). The dry residues of the various phases recovered were reconstituted in methanol and designated as a petroleum

ether fraction (PE), an intermediate fraction (IN), an ethyl acetate fraction (EA) and an aqueous fraction (AQ). The same steps were repeated but with reconstitution in DMSO at a concentration of 100 mg mL^{-1}. Another crude extract (CE) without fractionation was also prepared (Fig. 1). The TPC value in each fraction was determined as described previously.

2.5. Extraction of T. fontanesii essential oil

The extraction of essential oil (EO) was carried out by a hydrodistillation process in a Clevenger apparatus by boiling 100 g of dried vegetable material with distilled water for 3 h. The EO thus obtained was dried over anhydrous sodium sulphate and maintained at 4 ° C.

2.6. Antibacterial activity

2.6.1. Agar disc-diffusion method

The disc diffusion method was used to evaluate the antibacterial activity against *P. aeruginosa*. Stock solutions of EO (90% v / v), LA (50% v / v), CE of *S. holoschoenus* and its four fractions (100 mg mL^{-1}) were prepared in DMSO. Various concentrations of antibacterial agents: EO (50, 25, 12.5, 8, 6.25 and 5%), EA (90, 80, 70, 60, 50, 40, 30, 20 and 10 mg mL^{-1}) and LA (40, 35, 30, 25, 20, 15, 10 and 5%) were prepared from stock solution in BHIB. 20 µL of each sample and its dilutions were used to impregnate sterile discs (6 mm diameter), which were deposited on Muller Hinton agar plates (14 cm in diameter), previously inoculated from the 10^6 CFU / mL suspension (NCCLS, 2001). The negative control consisted of a disc impregnated with 20 µL of DMSO only. After incubation for 2 h at 4 °C, the plates were incubated at 37 °C for 18 h and the diameters of the zone of inhibition (DZI) in mm were measured.

2.6.2. Mixture design

For the determination of optimal combinations, an augmented simplex centroid design was used. The independent variables were EO at 12.5% (α_1), EA 70 mg mL^{-1} (α_2) and LA at 40 % (α_3). The factors represent the fraction of each sample in the mixture, which ranges from 0 to 1. A total of 14 preparations with these variables were prepared: 3 single-fraction, 7 two-fraction and 4 three-fraction mixtures. The effect of each formulation was tested as explained previously, with two replications, one pair of which was incubated at 37 °C for 24 hours while the other at 7 °C for 7 days. The RSM was used to construct a mathematical model to explain how the antibacterial substance (alone and in binary/ternary combinations) affected antibacterial activity and a second order polynomial equation (2) was used to fit experimental data.

$$Y = b_1\alpha_1 + b_2\alpha_2 + b_3\alpha_3 + b_1b_2\alpha_1\alpha_2 + b_1b_3\alpha_1\alpha_3 + b_2b_3\alpha_2\alpha_3 + b_1b_2b_3\alpha_1\alpha_2\alpha_3 \quad (2)$$

Where Y is the estimated response; b_1, b_2 and b_3 are the constant coefficients for linear and non-linear terms, and α is the ratio of the volume of components to the total volume of mixture.

2.6.3. Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

The MIC values were determined according to the protocols reported by Djenane et al. (2011). A bacterial suspension of 10^5 CFU / mL was prepared from 24h inocula. The serial twofold dilutions of the three stock solutions were carried out with different concentrations of EO and EA in BHIB (Brain-Heart Infusion Broth). In each well of a microwell were distributed 100 µl of each dilution of the sample, 5 µL of a bacterial suspension and 95 µL of BHIB. A positive control was carried out by replacing the sample with the culture medium. After incubation for 24 h at 37 °C, the MIC corresponds to the first concentration which does not exhibit bacterial growth. A swab seeding of each concentration lower than the MIC was

made on nutrient agar. The lack of development (99.9 % decrease in growth with respect to the control) after 24 h indicates the MBC value.

2.7. Statistical analysis and modelling of experimental data

All tests were carried out in triplicate. The differences were statistically evaluated by the Tukey's test with a 95% confidence level with JMP software.

3. Results and discussion

3.1. Effect of microwave assisted extraction on polyphenols recovery

The extraction from the plant matrix is influenced by several parameters: the type of solvent used (Periago et al., 2004), the number and duration of extractions, temperature, power intensity and size of the particles (Chan et al., 2014), which determine both the amount and the type of substances extracted. Thus, it was necessary to first determine the appropriate experimental range of these parameters by studying the individual effect of intrinsic (time) and non-intrinsic factors (type of solvent, solvent/water ratio) on the TPC of the *S. holoschoenus* rhizome. The results of the preliminary study are presented in Table 1.

3.1.1. *Single-factor experiments*

\- *Influence of type of solvent*

The choice of extraction solvents was not random. Referring to the literature, acetone, methanol and ethanol combined with water are the solvents most commonly used in the extraction of phenolic compounds (Tsao, 2010). Dahmoune et al. (2015) obtained better retention of phenolic compounds from Myrtle leaves in 60 s, with 40% ethanol (v/v), 500W microwave power level and 20 mL/g liquid-to-solid ratio as fixed parameters. The three

solvents cited above were tested individually at first, with fixed extraction time at 60 s and irradiation power at 500 W. As can be seen in Table 1, the acetone extract had the highest yield of TPC (6.53 ± 0.007 mg GAE / g dw), followed by the methanol extract (4.09 ± 0.06 mg GAE / g dw) and then the ethanol extract (3.63 ± 0.09 mg GAE / g dw). The selection of acetone as the best extraction solvent for the MAE method was confirmed by Proestos and Komaitis (2008), who explain that the solvents transparent to microwaves (acetone) are better than micro-absorbents (methanol). Bouras et al. (2015) found that the yield is slightly better with ethanol than with methanol. In addition, 70% acetone was found to be the most effective extractor solvent for TPC from *S. holoschoenus*, better than 70% methanol and 70% ethanol when using a maceration process (Oussaid et al., 2017). So, 98% acetone was chosen for the determination of the optimum extraction time.

- Influence of extraction time

During extraction by microwave, the prolongation of the extraction time can lead to the degradation of polyphenols (Dahmoune et al., 2015), hence the importance of this variable. The effects of irradiation time on the yield of TPC were evaluated at levels ranging from 30 to 150 s, with fixed solvent (98% acetone) and 500W microwave power level. A maximum yield of TPC was obtained in 60 s (6.53 mg GAE / g dw), but the yield fell with further increases in MAE irradiation time. At 120 s, the amount of TPC obtained was 2.33 (mg GAE / g dw). These results are in agreement with those obtained by Dahmoune et al. (2015). (Bouras et al., 2015) suggested that a few minutes were sufficient to cause an unwanted rise in temperature when microwave radiation was applied.

-Effect of acetone concentration

The polarity of the solvents influences the phenolic compounds extracted by MAE. Solvents with a high dielectric constant, such as water, absorb more microwave energy (Proestos and Komaitis, 2008). Therefore, a solvent mixture with water gives better yields than the solvent alone, which is evidently beneficial (Prasad et al., 2011). However, water, with its lower dissipation factor, generates an overheating effect (Proestos and Komaitis, 2008), resulting in a degree of unwanted TPC degradation (Liazid et al., 2007). In our study, the effect of the acetone/water ratio was investigated using an extraction time of 60s at 500 W. The hydroacetone containing 50%, 30% and 10% water gave the best yields of TPC without significant differences between the different acetone concentrations (10.08 ± 0.003, 10.31 ± 0.00 and 10.19 ± 0.005 mg GAE / g dw, respectively). When the acetone concentration decreased to 30%, there was a decrease in TPC (08.70 ± 0.002 GAE / g dw), while the lowest amount was obtained with 100% water (04.77 ± 0.001GAE / g dw). These results are in agreement with those obtained by Bouras et al. (2015), who achieved better recovery of polyphenols when methanol and ethanol were mixed with water. Popescu et al. (2016) studied the effect of water and organic solvents on the extraction of phenolic compounds from *S. holoschoenus* and obtained the highest yield using 50% ethanol/water and lowest yield using only water after 6 hours reflux in both cases.

- *Influence of microwave power*

The effect of microwave power was examined at levels between 300 W and 900 W. The best performance was obtained with 500 W (10.31 GAE / g dw), but there was no significant difference between this and 300, 400, 600 and 700 W power levels. However, a significant decrease in the TPC was observed with the increase in power to 900 W (7.74 ± 0.33 mg GAE / g dw).

Microwave heating (Wang et al., 2006) influences the extraction of bioactive substances, and in particular it has been reported (Koubaa et al., 2015) that heat transfer was faster using microwave radiation than in the conventional method, which can have a negative impact on thermolabile compounds (Roselló-Soto et al., 2015).

The use of high power leads to overheating (Wang et al., 2006), which leads to the degradation of certain phenolic compounds. It is reported that catechin and resveratrol are readily oxidizable under these conditions (Pan, 2000). Temperatures below 100 °C are not sufficient to extract epicatechin (He and Sun, 1995). Liazid et al. (2007) studied the stability of 22 phenolic compounds extracted by microwave and observed that the three molecules cited above were stable at 100 °C, beginning to be degraded above this temperature. The other polyphenols tested by these authors began to degrade at 125 °C. They also found that the polyphenol compounds with a greater number of hydroxyl substituents in the aromatic ring were the most affected by a temperature of extraction of 150 °C (Liazid et al., 2007).

3.1.2. Optimization of MAE conditions

Based on the results of the preliminary tests on the individual effect of the parameters, an RMS, using a Box-Behnken design, was performed to study the impact of the combination of the following parameters: extraction time (30-120 s, x_1), acetone ratio (0-90%, x_2) and microwave power (300-900 W, x_3).

The design was applied by running 15 experiments, with three replications at the centre point (x_1 = 90s, x_2 = 45%, and x_3 = 600w) and the TPC yields obtained in the cubic model tests are reported in the response surfaces (Fig. 2 and Table 3). In order to verify the validity of the model, the measured responses were compared with those predicted by the estimation of the difference and the experimental error (Table 4). An ANOVA analysis had been performed to analyse the statistical significance of the coefficients of the experimental

models. It was shown that the independent variable x_2, and quadratic terms x_1, x_1^2, x_2^2 and x_3^2 significantly affected the extraction. So, neglecting the non-significant (p > 0.05) terms, the following polynomial equation was obtained:

$$Y_{TPC} = 30.05 + 4.02\, x_2 - 4.48\, x_1^2 - 8.82\, x_2^2 - 8.87\, x_3^2 \quad (3)$$

In this framework, the coefficient of regression, R^2, was 0.93. The significance ($P< 0.05$) *P*-value was 0.0214, the high value of Sum of square (722.88) and F-value (7.18) indicated the suitability of the model.

Applying the maximum desirability approach, the optimum conditions for the highest TPC yield were 69 s, 56% acetone and 600 W irradiation power, with a predicted value of 30.6 ± 4.85 mg GAE / g dw and a predicted R^2 of 0.86. The TPC obtained experimentally under these optimal conditions was 30.70 ± 1.22 mg GAE / g dw), which is close to the predicted value. This result confirms the validity of the experimental design used.

However, the quantity of TPC obtained with MAE was smaller than that obtained by conventional solvent extraction, using 1 cycle of 24 h or three cycles of 1 h, which was 182.29 ± 0.22 mg GAE / g of dry extract (43.20 ± 0.05 mg GAE /g dw of the plant dust) and 236.02 ± 1.24 mg GAE / g of dry extract (59 ± 00.31 mg GAE /g dw of the plant dust), respectively (Oussaid et al., 2017) .

3.2. Extraction yield of thyme EO, CE and fractions of S. holoschoenus

The *T. fontanesii* EO extraction rate was 4.9 ± 0.13% (v / w) and was significantly higher than the rates obtained by Dob et al. (2006), Ghannadia et al. (2004) and (Boukraâ et al., 2013), which were 0.9%, 1.9% and 2.39%, respectively. The extraction rates of CE and its fractions are shown in Table 5. Among the four fractions, the AQ had the highest TPC yield

(14.67 ± 0.16 GAE mg / g dw), while the PE was the least rich (0.202 ± 0.007 mg GAE / g dw).

3.3. *Antibacterial activity*

3.3.1. Disc-diffusion assay

As can be seen in Fig. 3, *P. aeruginosa* was very sensitive to 90% EO (40 ± 1 mm) and 50% LA (25.66 ± 0.57 mm). However, among the CE and fractions of *S. holoschoenus*, only EA showed an antibacterial effect (15.66 ± 0.5 mm) and there was no significant difference between a concentration of 70 and 100 mg mL^{-1}. In addition, essential oils (12.5%) and lactic acid (40%) did not show a significant difference ($P > 0.05$) in DZI (23.5 ± 0.8mm and 26.33 ± 0.5mm, respectively). The role of phenolic compounds in resistant plants is widely documented. Phenolic acids (Petti and Scully, 2009) and flavonoids (Treutter, 2006) showed an increase in production during plant stress or infection. In addition, various studies have shown that EOs with the greatest antibacterial activity against dietary pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol (Burt, 2004; Safaei-Ghomi et al., 2009).

T. fontanesii was found to be active against *P. aeruginosa* (Boukraâ et al., 2013). Despite its high antibacterial potential, *T. fontanesii* is characterized by a strong flavour, limiting its use as a food preservative, hence the importance of its incorporation at low concentrations.

Alakomi et al. (2000) demonstrated the effect of lactic acid on *P. aeruginosa* and other Gram-negative bacteria. Our previous study showed that the EA fraction from *S. holoschoenus* has a higher effect against *B. subtilus* and *S. aureus* than PE and crude extract obtained with maceration (Oussaid et al., 2017). Published work on the antibacterial potential

of plants belonging to the genus Scirpus revealed the efficacy of *S. fluviatilis* extract against *S. aureus* (Borchardt et al., 2008a) and of *S. americanus* versus *S. aureus*, *E. coli* and *P. aeruginosa* (Borchardt et al., 2008b). In addition, cis-stilbenoids isolated from *S. yagara* had an anti-staphylococcal effect.

As has been reported in the literature, the tolerance of bacteria to polyphenols depends essentially on the species. Reduction of the permeability to hydrophobic antibacterial agents of the outer membrane surrounding the cell wall caused by the lipopolysaccharides of Gram negative bacteria may promote resistance to these agents. Antibacterial activity is also associated with the site and the number of hydroxyl groups on the phenolic ring (Ultee et al., 2002).

It has been reported that fractionation improved the antibacterial potency of the crude extract. Sakanaka et al. (2014) studied the antibacterial activity of methanol green tea extract against three Streptococcus strains and found that the ethyl acetate fraction was the only active sample.

In the same study, (+) gallocatechin isolated from the ethyl acetate fraction, was found to be more effective antibacterial compound than epigallocatechin and epigallocatechin gallate. Also, in this study, the epigallocatechin gallate showed twice as much growth inhibition in a meat extract medium as in the BHIB agar medium. In another study, the ethyl acetate fraction of *Ficus microcarpa* bark extract exhibited a high level of antibacterial activity, similar to that of the ethanol, hexane and aqueous fractions (Ao et al., 2008).

Currently, many authors are interested in using mixture designs to evaluate the combined effect of extracts against bacteria (Granato et al., 2016; Herrero et al., 2006). In this work, the centroid design coupled with the disk agar approach was applied to determine a possible interaction between the samples studied previously at two temperatures. The results are shown in Table 6, the response contours in Fig. 4 and the result of all the dependent

variables is given in Table 7. It emerged from ANOVA analysis that EO_d is the most influential variable. A negative interaction has been seen between EO_d and LA and between LA and EA. A synergy effect was recorded between EO_d and EA at 7 °C. The fitted equations for the responses are given as follows:

$$Y_{37°C} = 20.84\ \alpha_1 + 23.5\ \alpha_2 + 12.83\ \alpha_3 - 62.11\alpha_1\alpha_2 - 48.27\ \alpha_1\alpha_3 \quad (4)$$

$$Y_{7°C} = 18.13\ \alpha_1 + 46.39\ \alpha_2 + 17.11\ \alpha_3 \quad (5)$$

The values of the correlation coefficients for DIZ_{37} (R^2= 0.824) and DIZ_7 (R^2= 0.844) indicated an adequate fit. The contour of the response surface obtained with JMP V7 indicates that the areas of inhibition against *P. aeruginosa* tend to expand as the amount of EO_d increases. From the prediction profiler plot, the best mixture was composed of 83.7% EO_d and 16.3% EA for DIZ_{37} and 75% EO_d and 25 % EA for DIZ_7. The predicted values with these mixtures were 23.9 ± 5.85 mm and 49.85 ± 11.07 mm with the composite desirability of 0.7 and 0.68, respectively for DIZ_{37} and DIZ_7. The experimental results with these mixtures were 24.16 ± 1.04 and 63 ± 2 for DIZ_{37} and DIZ_7, which are close to the predicted values.

Several studies have been interested in the effect of interaction between bioactive molecules. It has been shown that the effect may be additive, synergistic or even antagonistic. According to Ultee et al. (2002), cymene applied individually is unable to cross the cytoplasmic membrane of *Bacillus cereus*, while it becomes active when used in conjunction with carvone.

It is also important to note that the extent of interaction is not the same in all strains and the study by Herrero et al. (2006) confirms this. They found that the regression coefficient for the MIC value of *Origanum compactum* and the mixture of *Origanum majorana* EO against *B. subtilis* was different: being antagonistic for *S. aureus* and synergistic for *E. coli*.

The antilisteric activity of lactic acid combined with EO has been studied (Dimitrijević et al., 2007). The results of this study showed that this acid accentuated the effect of *Thymus*

vulgaris and *Rosmarinus officinalis*, mainly when the concentration of EO was low. In their study Alakomi et al. (2000) found that in addition to its antibacterial effect achieved by lowering the pH level and its ability to release lipopolysaccharides, lactic acid enhanced the permeability of the outer membrane, which may improve the effect of other antibacterial substances.

It is most remarkable that more appreciable antipseudomonal effect produced by the antibacterial agents was determined at temperatures around 7 °C. Incubation at temperature affects the potency of bioactive substances, as was found by Smith-Palmer et al. (1998), who were interested in the antibacterial effect of five EOs on *Listeria monocytogenes*, as a model of psychrotrophilic bacteria. It was found that, unlike cinnamon EO, bay and nutmeg EOs were less inhibitory at 4 °C than 35 °C, while the MBC values of cloves and thyme were independent of temperature. In their study, they found a synergy effect between thymol and refrigeration temperatures inferior or equal to 8 ºC against boreal spores (Valero and Frances, 2006).

Ting and Deibel (1991), for their part, showed that low temperature improved the effect of sage. Smith-Palmer et al. (1998) attribute the variation in activity to low oil penetration of the bacterial membrane, resulting from its alteration or from changes in the active sites of low temperature oils or to a greater release of volatile substances at high temperatures.

3.3.2. MIC and MBC determination

The MIC and MIB of EA, EO_d and their optimized combination were determined at the two temperatures tested. The results of the MIC determination show a variability in the susceptibility of the strain to the samples tested (Table 7). The MIC value of *T. fontanesii* was 0.625 µL mL^{-1} at both temperatures, which is lower than that noted by Boukraâ et al. (2013), which was 5 µL mL^{-1}. The EOd / EA combination gave a MIB value of 2.5 µL mL^{-1} at both

temperatures, which is equal to that obtained with the individually applied EOd. As is the case in our study, essential oils of Thymus species showed positive interactions with other phenolic extracts. Indeed, Boukraâ et al. (2013) combined five varieties of honey with *T. fontanesii* EO and their results showed a significant decrease in MIC versus *P. aeruginosa*, which suggests synergism. In a study of the antibacterial extract and essential oils of *Thymus vulgaris* and *Pimpinella anisum*, Al-Bayati (2008) noted the resistance of *P. aeruginosa* (MIC> 500 µg mL^{-1}) when EO or extracts from the two plants were applied individually, and the additive effect (MIC = 500 µg mL^{-1}) that was seen when they were applied in combination.

4. Conclusion

Storage at low temperatures is insufficient to limit the growth of psychrotrophic microorganisms and finding a natural alternative to synthetic substances is a challenge. In this study, the effect of *S. holoschoenus* and *T. fontanesii* against a very widespread psychrotrophic bacteria, *P. aeruginosa*, was investigated.

The largest amount of phenolic compounds was extracted from the *S. holoschoenus* rhizome with 56% acetone under an irradiation power of 600 W for 69 s. The combined effect of *T. fontanesii*, lactic acid and *S. holoschoenus* on *Pseudomonas* at 7 and 37 ºC was investigated using simplex centroid design. The SCMD used allowed modelling of the combined effects of the ethyl acetate fraction (70 mg. mL^{-1}) with essential oil from *T. fontanesii* (12.5%) and lactic acid (40%). The optimum mixtures were 75 % EO_d and 25% EA at 7 °C. At 37 °C, the best mixture was 83.7% EO_d and 16.28% EA. Therefore, it would be interesting to test this combination in a food model and it is necessary to identify the bioactive molecules responsible for the antibacterial activity and interaction between molecules.

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Tables

Table 1. Results on TPC of single-factor experiments, solvent, acetone concentration (%), extraction time and microwave power

Fixed variables	Extraction time: 60 s Microwave power: 500 W		Solvent: 98% acetone Microwave power: 500 W		Extraction time: 60 s Microwave power: 500W		Solvent: 70% acetone Extraction time: 60 s	
Constant variable	Solvent type	TPC (mg_{GAE}/g_{dw})	Extraction time (s)	TPC (mg_{GAE}/g_{dw})	Acetone ratio (%)	TPC (mg_{GAE}/g_{dw})	Microwave power (W)	TPC (mg_{GAE}/g_{dw})
	98% ethanol	4.09 ± 0.006^{a}	30	03.58 ± 0.078^{a}	0	$04. 77 \pm 0.001^{a}$	300	$09. 86 \pm 0.120^{ab}$
	98% methanol	3.63 ± 0.009^{b}	60	06.53 ± 0.007^{b}	30	$08. 70 \pm 0.002^{b}$	400	$09. 68 \pm 0.061^{ab}$
	98% acetone	6.53 ± 0.007^{c}	90	04.17 ± 0.003^{a}	50	$10. 08 \pm 0.003^{c}$	500	$10. 31 \pm 0.000^{b}$
			120	02.33 ± 0.000^{c}	70	$10. 31 \pm 0.000^{c}$	600	$09. 82 \pm 0.072^{ab}$
			150	03.12 ± 0.005^{ac}	90	$10. 19 \pm 0.005^{c}$	700	08.72 ± 0.057^{ac}
							800	$07. 35 \pm 0.061^{c}$
							900	$07. 74 \pm 0.033^{c}$

TPC : Total phenolic contents ; GAE : Galic acid equivalents ; Values are expressed as mean ± standard deviation ($n = 3$). Means with different letters were significantly different at the level of $p < 0.05$.

Table 2. The three levels code of independent variables

Independent variables	Levels		
	Low	Middle	High
x_1: Extraction time (s)	30	90	120
x_2: Acetone ratio (%)	0	45	90
x_3: Microwave power (W)	300	600	900

Table 3. Box–Behnken design matrix with the observed responses and predicted values for total phenolic compounds (TPC)

				Responses	(TPC (mg EGA/gdw))
Run order	Extraction time (s)	Acetone ratio (%)	Microwave power (W)	Experimental	Predicted
1	75	45	600	30.05	30.05
2	75	90	900	19.20	16.63
3	30	45	300	18.23	18.84
4	30	0	600	15.73	012.5
5	75	45	600	30.05	30.05
6	75	0	300	06.78	09.35
7	75	0	900	04.74	07.32
8	120	45	300	15.93	15.33
9	120	45	900	16.71	16.11
10	30	90	600	20.93	22.90
11	30	45	900	15.93	16.53
12	120	90	600	15.46	18.63
13	120	0	600	14.87	12.89
14	75	45	600	30.05	30.05
15	75	90	300	18.70	16.13

Table 4. Analysis of mean square deviation of the quadratic model terms (Eq. (1)) applied to the experimental values of total phenolic yields obtained with microwave assisted extraction.

Term	Estimate	STD Error	T Ratio	Prob > ItI
Intersept	30.05	1.93	15.56	<**0.001**
x_1 (30, 120)	-0.98	1.18	-0.83	0.44
x_2 (0, 90)	4.02	1.18	3.4	**0.019**
x_3(300, 900)	-0.38	1.18	-0.32	0.75
x_1*x_1	-4.48	1.74	-2.57	**0.04**
x_1*x_2	-1.15	1.67	-0.69	0.52
x_2x_2	-8.82	1.74	-5.07	**0.003**
x_1*x_3	0.77	1.67	0.46	0.66
x_2*x_3	0.63	1.67	0.38	0.72
x_3*x_3	-8.82	1.74	-5.09	**0.003**

RSquare = 0.93 *RSquare Adjuted = 0.8*

Table 5. Extraction yield and TPC, total flavonoids and tannins for optimized extract and its fractions

Sample	*T. fontaneseii*	*Scirpus holoschoenus*				
		CE	PE	IN	EA	AQ
Extraction yield (%)	4.9 ± 0.13	15.91 ± 0.09	01.22 ± 0.23	0.526±0.025	7. 59 ± 0.26	7.91 ± 0.11
TPC (mg GAE/ g_{dw})	*ND*	30.70 ± 1.22	0.202 ± 0.007	01.29 ± 0.03	13.02 ± 0.48	14.67 ± 0.16

ND: No Determined

Table 6. The design matrix and experimental responses (inhibition zone diameter (mm) obtained with lactic acid, essential oil and ethyl acetate fraction

Run	LA (40%)	EO_d (12.5%)	EA (70 mg mL^{-1})	Responses (Inhibition zone diameter (mm))			
				7 °C		37 °C	
				experimental	predite	experimental	predite
1	0.5	0.5	0	25.00	18.23	06.00	6.648
2	0.5	0	0.5	09.00	08.70	08.00	4.765
3	0	1	0	43.00	46.38	23.5	23.51
4	0	0.5	0.5	38.00	46.13	21.00	23.5
5	0	0	1	17.7	17.08	15.5	12.83
6	0.3333	0.3333	0.3333	25.00	20.23	11.00	09.18
7	1	0	0	21.00	18.13	26.33	20.84
8	0.75	0.25	0	09.00	14.67	09.50	9.986
9	0.75	0	0.25	09.50	11.29	06.00	9.785
10	0.167	0.167	0.67	23.33	21.68	06.00	10.97
11	0.167	0.67	0.167	26 .00	34.59	14.00	15.13
12	0	0.75	0.25	63.00	49.85	30.00	23.88
13	0.67	0.167	0.167	10.66	12.54	11.00	08.35
14	0	0.25	0.75	34.66	35.20	16.00	18.46

Table 7. Minimal inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of extracts and their mixtures.

Sample	MIC		MBC	
	7°C	37°C	7°C	37°C
EO_d ($\mu L\ mL^{-1}$)	0.625	1.25	0.625	2.5
EA ($mg\ mL^{-1}$)	1.093	2.18	1.093	2.18
M_1 ($\mu L\ mL^{-1}$)	-	0.625	-	2.5
M_2 ($\mu L\ mL^{-1}$)	0.625	-	2.5	-

$M_1 = 83.7\% EO_d + 16.28\% EA$ *$M_2 = 75\% EO_d + 25\% EA$*

Figure captions

Figure 1: Schematic diagram of the preparation of extract and fractions of *S. holoschoenus*

Figure 2: Response surface analysis for the total phenolic yield from *S. holoschoenus* with respect to microwave power and acetone percentage (A); microwave power and extraction time (B); extraction time and microwave power (C).

Figure 3: Antimicrobial activity (zone of inhibition, mm) of various concentrations of ethyl acetate fraction (a), essential oil (b) and lactic acid (c).

Figure 4: Response-surface contour plots for the effect of different combinations of studied extract essential oil and lactic acid on zone inhibition diameter values against *P. aeruginosa* (a) at 37°C and 7 °C (b).

Figure 1

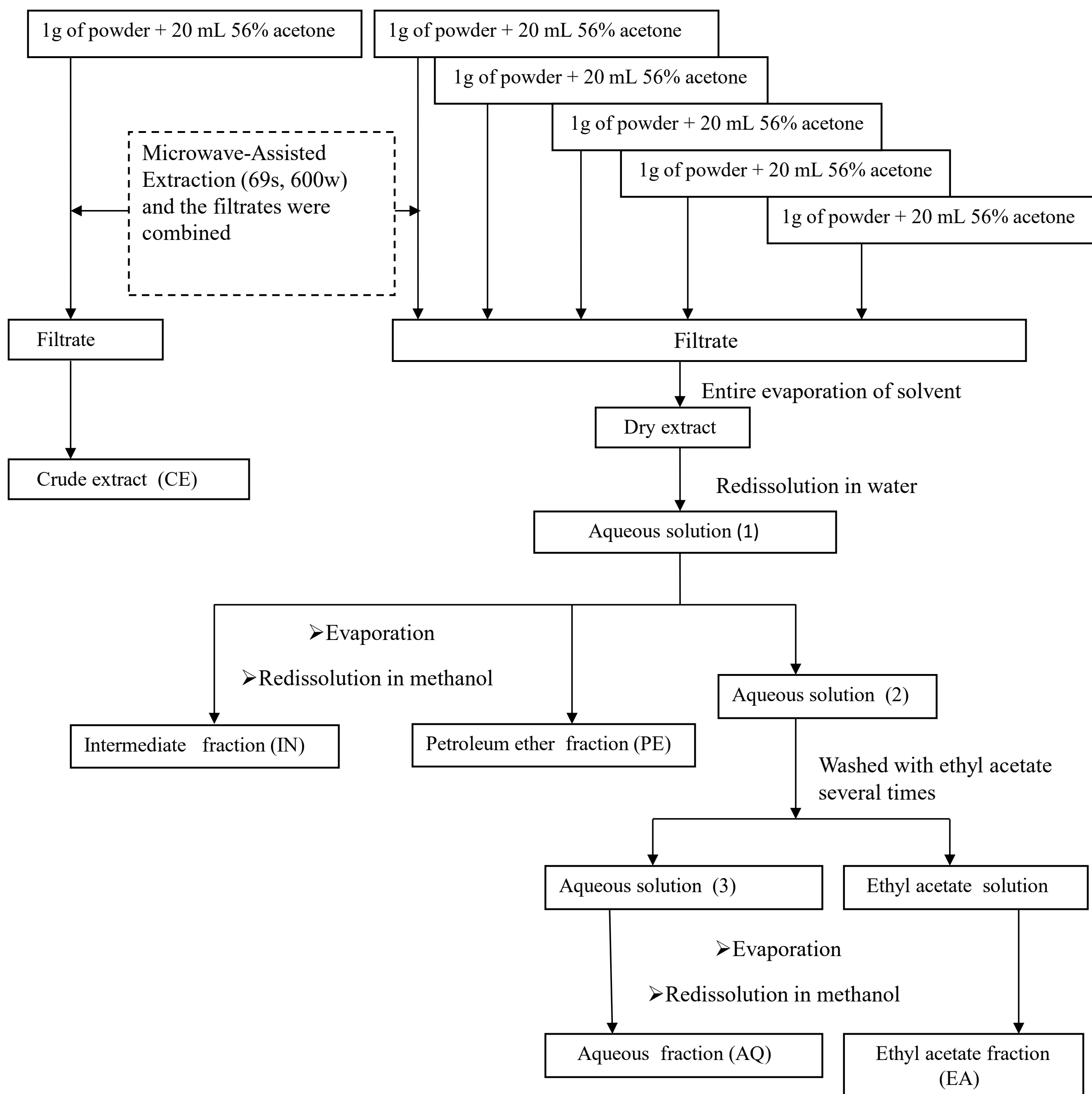

Figure 1

Figure 3

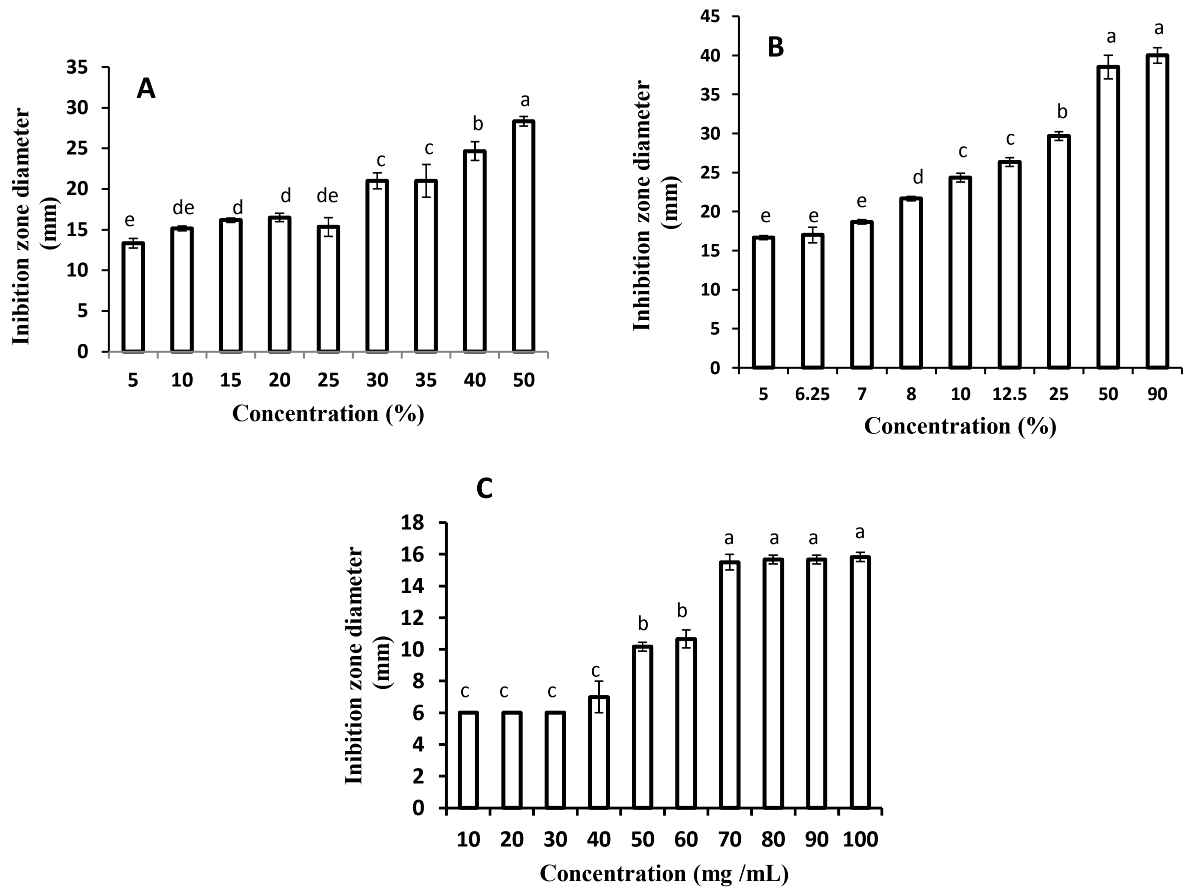

Figure 3

Figure 4

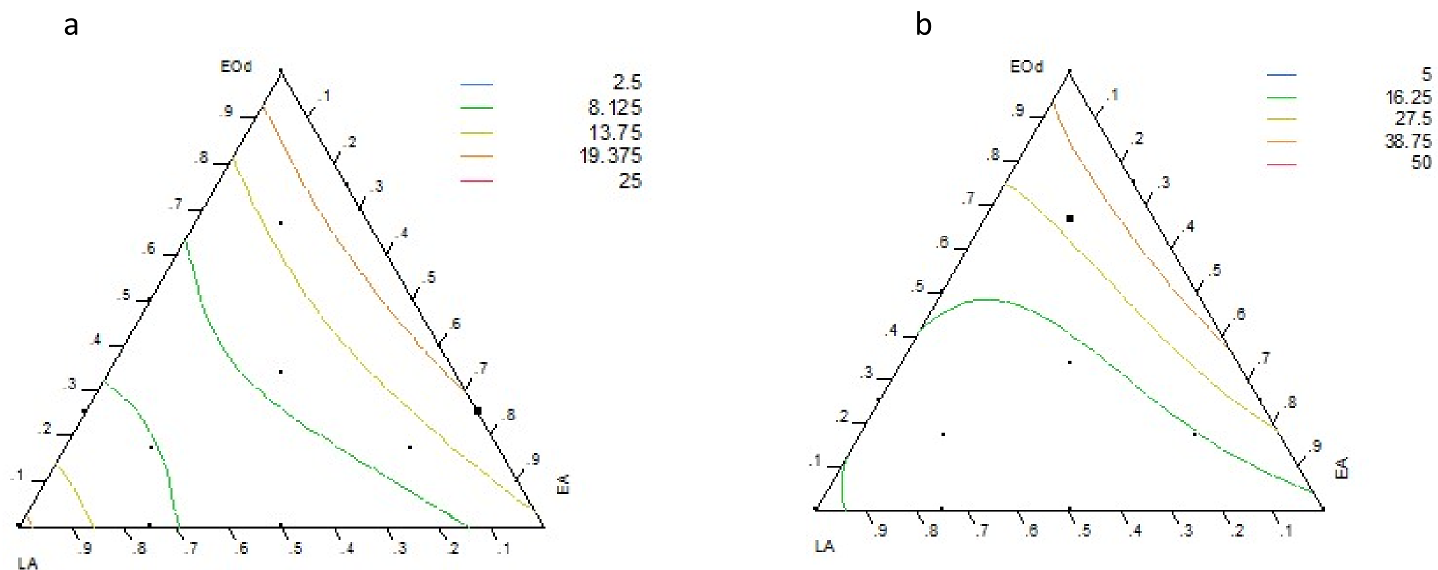

Figure 4

Figure 2

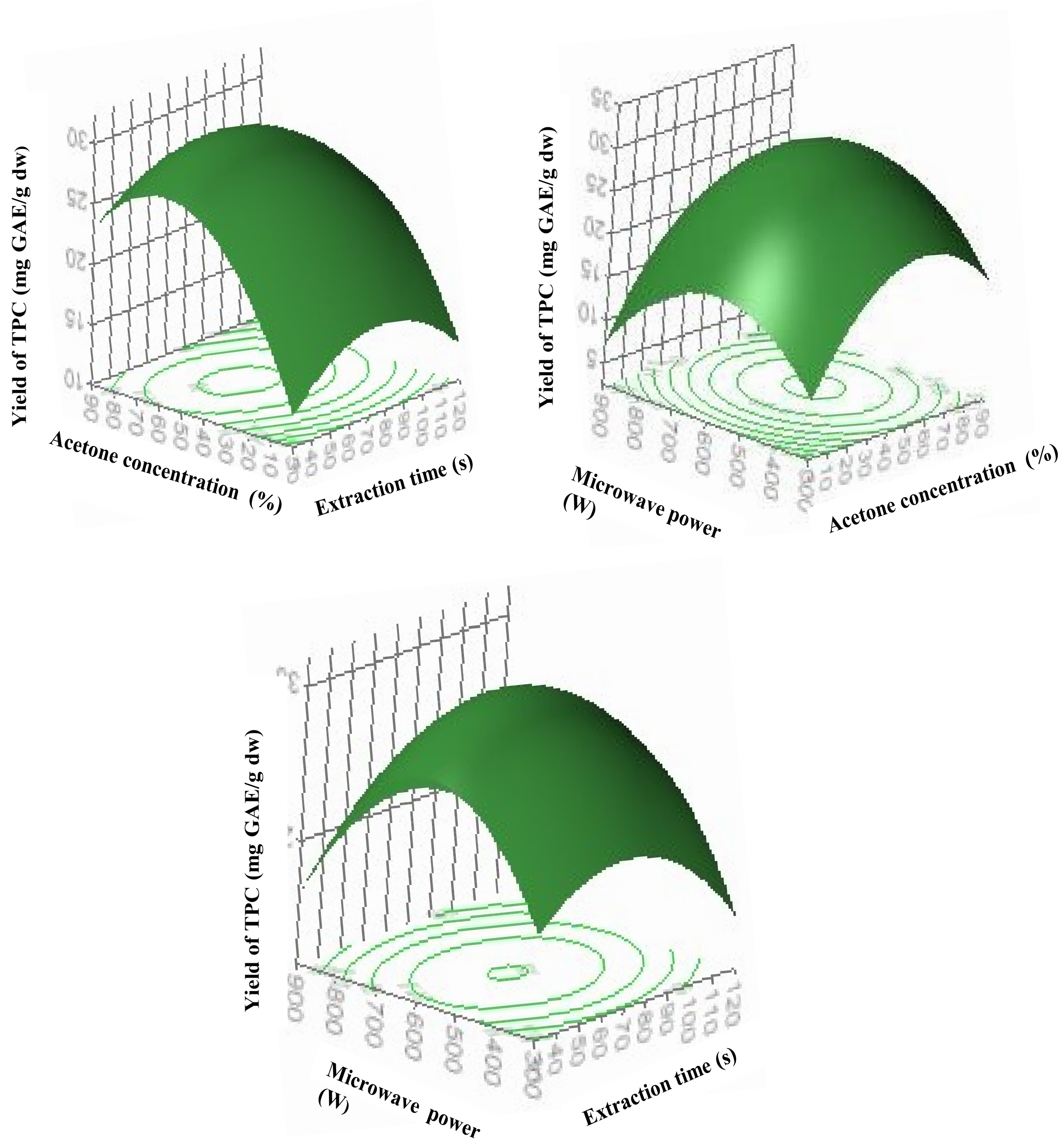

Figure 2