Accepted Manuscript

Optimization of the extraction of phenolic compounds from *Scirpus holoschoenus* using a simplex centroid design for antioxidant and antibacterial applications

Saliha Oussaid, Mohamed Chibane, Khodir Madani, Tahar Amrouche, Sabiha Achat, Farid Dahmoune, Karim Houali, Manuel Rendueles, Mario Diaz

PII: S0023-6438(17)30634-5

DOI: 10.1016/j.lwt.2017.08.064

Reference: YFSTL 6486

To appear in: *LWT - Food Science and Technology*

Received Date: 17 March 2017

Revised Date: 11 August 2017

Accepted Date: 20 August 2017

Please cite this article as: Oussaid, S., Chibane, M., Madani, K., Amrouche, T., Achat, S., Dahmoune, F., Houali, K., Rendueles, M., Diaz, M., Optimization of the extraction of phenolic compounds from *Scirpus holoschoenus* using a simplex centroid design for antioxidant and antibacterial applications, *LWT - Food Science and Technology* (2017), doi: 10.1016/j.lwt.2017.08.064.

Optimization of the extraction of phenolic compounds from *Scirpus holoschoenus* using a simplex centroid design for antioxidant and antibacterial applications.

Oussaid Saliha [1, 2], Chibane Mohamed [3], Madani Khodir[1], Amrouche Tahar[4], Achat Sabiha[1], Dahmoune Farid[1], Houali Karim[2], Rendueles Manuel[5], Diaz Mario[5]*

[1]Laboratoire de Biomathématiques, Biophysique, Biochimie et Scientométrie, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria

[2]Laboratory Analytical Biochemistry & Biotechnology Research (LABAB), Faculty of Biological Sciences and Agricultural Sciences, Mouloud Mammeri University of Tizi-Ouzou, Tizi Ouzou, Algeria

[3]The Management Laboratory and Development of Natural Resources and Quality Assurance, AMO University Bouira, Algeria

[4]Laboratory of quality and food safety, Faculty of Biological sciences and Agronomy, M. Mammeri University, Tizi Wezzu, Algeria

[5]Department of Chemical Engineering and Environmental Technology, University of Oviedo, C/Julián Clavería s/n, 33071 Oviedo, Spain.

(*)Corresponding author. Tel: 0034-985103439
E-mail addresses: mariodiaz@uniovi.es

Abstract

A simplex-centroid mixture design (SCMD) approach was used to select the best solvent for the extraction of the phenolic compounds from *Scirpus holoschoenus* L. rhizome. The optimized crude acetone extract (CE) and its ethyl acetate (EA) and petroleum ether (PE) fractions were investigated for their antioxidant and antibacterial properties. The EA fraction showed the highest antioxidant activity and antibacterial effect, with minimal inhibitory concentration (MIC) values of 0.4 and 0.6 mg mL^{-1} for *Staphylococcus aureus* and *Bacillus subtilis*, respectively. The antibacterial activity was evaluated by SCMD and the results

indicated that antagonist binary extract effects between the PE–EA and PE–CE pairs are found.

Keywords*: Scirpus holoschoenus*, phenolic compounds, optimisation, antioxidant activity, antibacterial activity.

1. Introduction

Antioxidant and antimicrobial agents have been added to foods to extend their shelf life and were shown to prevent lipid peroxidation and foodborne illness due to pathogen growth. Harmful effects of the use of chemical preservatives (Gulcin, 2004) encourages the use of natural products as biopreservatives (Owen & Palombo, 2007). In addition, the phenomenon of bacterial resistance is becoming more important mainly due to the excessive use of antibiotics. Nowadays there is a worldwide trend towards employing new substitutes to control rancidity and foodborne diseases, promoting the use of methods without negative side effects on human health (Nedorostova, Kloucek, Kokoska, Stolcova, & Pulkrabek, 2009). Some scientific research is focused on the assessment of the effects of plant extracts as antioxidant or/and antimicrobial agents in food preservation (Burt, 2004). Among investigated phytochemicals, polyphenols seem to be among the more interesting due to their varying structures and biological activities (Vaquero, Alberto, & de Nadra, 2007; Viswanath, Urooj, & Malleshi, 2009). Indeed, their antioxidant and antimicrobial properties are highly valued in the food, cosmetics and pharmaceutical fields (Bento, Torres, Fialho, & Bononi, 2013).

Scirpus holoschoenus is a perennial Cyperaceae (Abdel-Mogib, Basaif, & Sobahi, 2001). Its rhizome has been used as a traditional medicine to eliminate kidney stones (Morales, Pardo-De-Santayana, & Tardio, 2006) and for liver protection (Popescu, 2011).

It is known that the rhizome of *S. holoschoneus* is rich in a range of phenolic compounds such as 3,5,4'-trimethoxystilbene, 2-prenyl-3,5,4'-trimethoxystilbene, 2-prenyl-3-hydroxy-5,4'-dimethoxystilbene, 2-prenyl-3,4'-dihydroxy-5-methoxystilbene, which are all acetophenone derivatives (Abdel-Mogib et al., 2001; Popescu, 2011), vanillin , E-resveratrol, Z-resveratrol, chlorogenic acid, caffeic acid, cinnamic acid and gallic acid (Popescu, 2011).

S. holoschoenus is widely distributed in Kabylia (Northeast Algeria) and its root is locally used by decoction to treat haemorrhoids. To the best of our knowledge, there have been no studies carried out on the extraction of phenolic compounds from *S. holoschoenus* rhizome and on its antibacterial activity using an SCMD approach. Therefore, the objectives of the present work were to (i) optimize solvent extraction for a higher total phenolic content (TPC) of the extract using SMCD, (ii) optimize extraction time for the selected solvent, (iii) determination of TPC, flavonoids, tannins and antioxidant activity of crude extract (CE) and its fractions obtained with ethyl acetate (EA) and petroleum ether (PE) and then (iv) use an SMCD to optimize the combination effect of CE, EA and PE against *S. aureus* and *B. subtilis*, which were chosen as representatives of bacterial food contaminants.

2. Materials and methods

2.1. Chemicals

Sodium bicarbonate (Na_2CO_3), Folin–Ciocalteu phenol reagent, disodium hydrogen phosphate (Na_2HPO_4) and aluminium chloride ($AlCl_3$) were obtained from Prolabo (Loire, France), butylated hydroxyanisole (BHA) and 1-diphenyl-2-picryl-hydrazil (DPPH) from Sigma Aldrich (Germany). Gallic acid, ferric chloride ($FeCl_3 \cdot 6H_2O$), potassium ferricyanide

74 ($C_6N_6FeK_3$), dodecylsulfate de sodium (SDS), trichloroacetic acid, and dimethylsulfoxide
75 (DMSO) were purchased from Biochem-chemopharma (Loire, France).

76 The antibacterial activity against *S. aureus* ATCC 25223 and *B. subtilis* ATCC 6633, obtained
77 from the American Type Culture Collection, was screened.

78 *2.2. Optimization of sample preparation*

79 *Scirpus* rhizomes were collected from Chemini (Bejaia, east of Algeria), in spring during the
80 flowering stage and kept preserved by drying under a forced air oven at 40 °C until constant
81 mass was obtained. The dried material was crushed to prepare powder, which was milled
82 through a 63 µm sieve (final powder size <63 µm). The effect of 3 solvents in water: 70%
83 acetone (α_1), 70% ethanol (α_2) and 70% methanol (α_3), and their mixtures considering the
84 TPC of the extracts as the optimizing parameter were tested according to a SCMD. This
85 method gives the optimal proportion of the variables (in this case the proportion of each
86 solvent, α_1, α_2 and α_3) selecting the best possible combination. The sum of the three variables
87 must be 100.

88 Fitting response values was done using the cubic model:

89 $$Y= b_1\alpha_1+ b_2\alpha_2 + b_3\alpha_3 +b_1b_2\ \alpha_1\alpha_2 + b_1b_3\ \alpha_1\alpha_3 + b_2b_3\ \alpha_2\alpha_3 + b_1b_2b_3\alpha_1\alpha_2\ \alpha_3 \quad \text{(eq. 1)}$$

90

91 The extraction was performed at solid / liquid ratio of 1/50 (w/v) (Djeridane, Yousfi,
92 Boutassouna, Stocker, & Vidal, 2006). Dried sample (0.5 g) was macerated with 25 mL of
93 solvent, following the 10 formulations specified in Table 1. The maceration was carried out
94 under shaking at 300 rpm for 24h at room temperature. After filtration through filter paper,
95 the solvent was entirely removed in a rotary evaporator (Buchi R 210, Switzerland). The dry
96 extract was weighed and then re-dissolved in 5 mL of methanol to obtain a solution with

known concentration. The Extraction yield was calculated on a dry weight basis from the formula given below:

$$E_y(\%) = \frac{W_2 - W_1}{W_0} \times 100$$

Where, E_y is the Extraction yield, W_2 is the weight of the extract and the container, W_1 is the weight of the container alone and W_0 is the weight of the initial dried sample.

Using the optimal solvent type selected, samples were extracted using varying maceration times (1h, 2 h or 3 h), in 25 mL of solvent, and with 3 steps, each step lasting 1 hour in 10, 10 and 5 mL volume respectively (3x1h).

The extract obtained under the optimal conditions constitutes the crude acetone extract (CE).

2.2.1. Extraction liquid-liquid

To increase the amount of optimized extract, ten extractions were carried as described in the previous section and the sum of the filtrates was subjected to complete evaporation of the acetone in a rotary evaporator (Buchi R 210, Switzerland) at 40 °C. The remaining aqueous sample was treated three times with the same volume of petroleum ether, then six times with ethyl acetate containing 20% ammonium sulphate and 2% meta-phosphoric acid solution. The residual water in the ethyl acetate fraction was eliminated by adding a sufficient amount of anhydrous sodium sulphate (Djeridane, Yousfi, Boutassouna, Stocker, & Vidal, 2006). The petroleum ether and ethyl acetate of the resulting solutions were completely evaporated on a rotary evaporator. The dry extracts were dissolved in methanol and designated, respectively, as EA (ethyl acetate fraction) and PE (petroleum ether fraction) (Fig.1).

121

122 *2.2.2. Determination of total phenolic content*

123 TPC of CE, EA and PE were determined by the Folin–Ciocalteu method (Gil, Toms-
124 Barbern, Hess-Pierce, Holcroft, & Kader, 2000). 100 µL of each sample was diluted in 6 mL
125 of distilled water and mixed with 0.5 mL of Follin-Ciocalteu reagent (2 N) and 1.5 mL of the
126 20% (w/v) sodium bicarbonate solution. Total volume was adjusted to 10 mL with distilled
127 water. After incubation for 2 h in the dark at room temperature, the absorbance was measured
128 at 760 nm using a spectrophotometer (SpectroScan 50, Nkesia, Cyprus). The experiment was
129 carried out in triplicate and the concentration of TPC in the extract was expressed, based on a
130 gallic acid standard curve, as mg gallic acid equivalent (GAE) per gram of dry extract (DE)
131 i.e., mg GAE/g DE.

132 *2.2.3. Determination of total flavonoids*

133 Total flavonoid content was determined using the aluminium trichloride method
134 (Bahorun et al., 1996). 1.5 mL of extract was mixed with the same volume of 2% aluminium
135 trichloride solution ($AlCl_3$) in methanol. The mixtures were left to stand for 10 min at room
136 temperature, and then the absorbance was determined using a spectrophotometer
137 (SpectroScan 50, Nkesia, Cyprus) at 415 nm. Quercetin was used to plot the calibration curve.
138 The experiment was carried out in triplicate and total flavonoid content was expressed as
139 milligrams quercetin equivalent (QE) per gram of dry extract i.e., mg QE/g DE.

140

141 *2.2.4. Determination of tannins*

142 Tannins were determined by protein-precipitation assay (Hagerman & Butler, 1978),
143 and a calibration curve was plotted with tannic acid. A volume of 0.5 mL of each sample was
144 mixed separately with 1 mL of Bovine serum albumin (BSA) solution (1 mg BSA mL^{-1}

dissolved in a buffer of 0.2 M acetic acid and 0.17 M sodium chloride adjusted to pH 4.9) for 24 h at 4°C. After centrifugation for 10 min at 14000×g rpm, the pellet was dissolved in 2 mL buffer (containing 5% (w/v) sodium dodecylsulfate (SDS) and 5% (v/v) triethanolamine and adjusted to pH 9.4 with HCl), then added to 0.5 mL of ferric chloride solution (0.01 M in 0.01 M HCl). After 15 min, the absorbance was measured at 510 nm. The experiment was carried out in triplicate and the tannin contents were expressed as milligrams tannic acid equivalent (TAE) per gram of dry extract i.e., mg TAE/g DE.

2.2.5. Determination of antioxidant activity

The radical-scavenging activity (RSA) of samples was evaluated by the $DPPH^{\bullet}$ assay (Shirwaikar, Shirwaikar, Rajendran, & Punitha, 2006). 2 mL of each sample at different concentrations was added to 2 mL of $DPPH^{\bullet}$ solution (0.1 mM in methanol). A control containing 2 mL of methanol and 2 mL of the DPPH• solution was prepared and BHA was used as the control standard. After incubation at 37 °C in the dark for 20 min, the absorbance was measured at 517 nm. The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC_{50}) was calculated graphically. Radical scavenging activity was calculated using the following formula:

$$\%\text{DPPH inhibition} = [(Abs_{control} - Abs_{sample})/ Abs_{control}] \times 100$$

Where $Abs_{control}$ was the absorbance of control and Abs_{sample} was the absorbance of sample.

2.2.6. Antibacterial activity

For the antibacterial tests, the samples were prepared as indicated previously in section 2.2 but in this case the samples were reconstituted in DMSO.

2.2.6.1. *Agar Diffusion Tests*

The antibacterial activity of samples was evaluated by a diffusion test according to the National Committee for Clinical Laboratory Standards (NCCLS, 2001), using Mueller–Hinton agar previously inoculated with 100 µL of 10^6 CFU mL^{-1} bacterial suspensions of *S. aureus* ATTCC 25928 or *B. subtilis* ATCC 6633. Sterilized paper discs (6 mm) were impregnated with 20 µL of different extracts in DMSO (90.2 mg mL^{-1}) and applied to the surface of the agar. Plates were kept for 2h at 4 °C to allow diffusion of the active compounds in the medium, then incubated at 37 °C for 24h. DMSO and Chloramphenicol (30 µg /disc) were used as negative and positive controls, respectively. The antibacterial activity was expressed as the diameter of the inhibition zones (DIZ) produced and measured in mm unit. The effect of extract concentration (1.62, 2.02, 2.42, 2.82, 5.63, 11.27, 22.55 and 45.1 mg mL^{-1}) was also tested.

2.2.6.2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The MIC values were determined as the lowest extract concentration at which no growth was observed. One mL of each of extract concentration (0.02-90.2 mg mL^{-1}) was mixed with 9 mL of Muller Hinton medium and poured into Petri plates. Immediately after solidification, 10 µL of suspension of each strain containing 10^4 CFU mL^{-1} was spot inoculated onto the surface of the agar and incubated at 37 °C for 24h (Taguri, Tanaka, & Kouno I., 2004).

193 To determine the MBC, samples were taken from spot inoculation points which did not
194 show any growth and used to inoculate nutrient broth tubes. The mixture was incubated at 37
195 °C for 24h. The lowest concentration of the extract with no visible growth after incubation
196 was taken as MBC.
197
198 *2.2.6.3. Antimicrobial effects of different combinations of CE, EA and PE*
199 A simplex centroid mixture design (SCMD) was used to evaluate the antibacterial
200 effects of different combinations of CE (X_1), EA (X_2) and PE (X_3), each at the same
201 concentration (90.2 mg/mL), on *S. aureus* and *B. subtilis*. The optimum combination was
202 determined by measuring the DIZ (mm). The complete experimental design for each
203 bacterium consisted of 7 experiments with three replicate runs at the centre point (Table 2).
204 *Statistical analysis and modelling of experimental data*
205 Fitting response values was done using the cubic model (Eq. 2).
206 $$Y = b_1 X_1 + b_2 X_2 + b_3 X_3 + b_1 b_2 X_1 X_2 + b_1 b_3 X_1 X_3 + b_2 b_3 X_2 X_3 + b_1 b_2 b_3 X_1 X_2 X_3 \quad \text{Eq. 2}$$
207 Where Y is the estimated response; b are the constant coefficients for linear and non-
208 linear terms, and X is the proportion of real-components. The analysis was performed using
209 uncoded units.
210
211 ANOVA with Tukey's test at $P < 0.05$) was used to evaluate the statistical significance
212 of each equation. The computational work, including ternary contour graphical presentations
213 of the model, was accomplished using JMP statistical package software (trial Version 10.0.0,
214 SAS Institute. Inc. Cary, NC) and used to compute the predicted equations.
215
216

3. Results and discussion

3.1. Optimization of sample preparation

The recovery of phenolic contents in different samples is influenced by the polarity of the extracting solvents and the solubility of the particular compound in the solvent used for the extraction process (Abozed, El-kalyoubi, Abdelrashid, & Salama, 2014). In fact, the selection of extraction solvents is critical for the plant matrices and it is known that acetone, ethanol, methanol (Chan, Lee, Yap, Wan Aida, & and Ho, 2009; Dai & Mumper, 2010; Naczk & Shahidi, 2004) and their combinations (Dai & Mumper, 2010) are the most commonly employed for phenolic extraction from botanical materials. By increasing the proportion of water, the solvent system is able to extract phenolic substances from both ends of the polarity range (high polarity substances and low polarity substances), as well as those of moderate polarity (Uma, Ho, & Aida, 2010). In addition, the highest amount of total phenols (Bohr, Meier, & Sticher, 2000) and tannins (Shahidi & Naczk, 2011) were obtained using 70% acetone. The extraction yield was 23.7, 23 and 22.3% of plant powder for 70% acetone, 70% methanol and 70% ethanol, respectively. The coefficients of determination were R^2 = 0.896, indicating a high degree of correlation between the observed and predicted values (Table 1).

The response surface for TPC with respect to the percentage composition of 70% acetone (α_1), 70% ethanol (α_2) and 70% methanol (α_3) is shown in Fig.2. The TPC was affected more significantly (P<0.01) by 70% acetone and 70% methanol ($p < 0.0001$). The highest (182.29 ± 0.22 mg g^{-1} DE) and the lowest TPC (98.32 ± 2.63 mg g−1 DE) were obtained for 70% acetone and the binary mixture ($\alpha_1\alpha_2$), respectively (Table 1). The binary ($\alpha_2\alpha_3$) and ternary interactions ($\alpha_1\alpha_2\alpha_3$) were not significant (P= 0.6 and 0.69, respectively). The equation to calculate the TPC is given by:

$$TPC = 184.58\,\alpha_1 + 128.83\,\alpha_2 + 177.65\,\alpha_3 - 317.356\,\alpha_1\alpha_3 - 201.81\,\alpha_1\alpha_2 \quad \text{(eq. 3)}$$

241 The *p*-value was equal to 0.0234 (<0.05), indicating that the main effect of regression was
242 statistically significant. The solvent composed only of 70% acetone was the most suitable for
243 TPC extraction, with a composite desirability of R^2 = 0.929. The observed (182.29 ± 0.22 mg
244 g^{-1} DE) and predicted (184.58 ± 35 mg g−1 DE) values for TPC were found to be
245 comparable.
246 Extraction time and the number of extraction steps were other factors which contributed to
247 the efficiency of extraction (Chirinos, Rogez, Campos, Pedreschi, & Larondelle, 2007). It is
248 reported that the extraction is more efficient with four cycles in 1 mL than one cycle in 4 mL
249 (Watson, 2014). In this study, the extraction procedure using three cycles of 1 hr (3x1h) was
250 more effective in terms of TPC, there being a statistically significant difference between this
251 and the one stage shaking procedure (1, 3 and 24h) (Table 1). For this reason, 70% acetone
252 with a three stage procedure (3x1h) was chosen as offering the optimal extraction conditions,
253 despite the fact that phenolic oxidation by a prolonged extraction process has been suggested
254 by Chan et al., (2009).
255 The yields of extraction with 70% acetone and its fractions were 25, 4.8 and 0.8% of
256 plant powder (PPW) for CE, EA and PE, respectively. The TPC of CE contains more tannins
257 (41. 38 ± 0. 65 %) than flavonoids (0.83 ± 0.03%) (Table 3). The same observation was noted
258 for the EA with values of 12.72 ± 0.85 and 0.54 ± 0.00% for tannins and flavonoids,
259 respectively. It is reported by Dai and Mumper (2010) that the concentration of phenolics in
260 the crude plant extract is low. So, to obtain and concentrate polyphenol-rich fractions, liquid-
261 liquid partitioning and/or solid phase extraction for the elimination of lipidic material, which
262 can be achieved by washing the crude extract with non-polar solvents to eliminate the non-
263 polyphenol compounds, is required (Dai & Mumper, 2010). However, in another study, the
264 washing of the CE with non-polar solvents led to losses of phenolic compounds (Moussi et

al., 2015) and indeed, in this investigation it was seen that the washing of the PE fraction led to a loss of phenolic compounds, with losses of 0.79% ± 0.01 and 0.175% ± 0.01 of TPC found in CE for tannins and flavonoids, respectively.

3.2. Antioxidant activity

The scavenging effect of fractions and CE from *Scirpus* rhizome was compared with that of BHA. The CE, EA and PE were shown to exhibit RSA (Fig. 3). IC_{50} values of BHA, EA, CE and PE were 21.77 ± 0.52, 24.76 ± 0.25, 32.4 ± 2.15 and 64.06 ± 4.02 µg/ml, respectively. A lower value of IC_{50} indicates a higher antioxidant activity, and so EA showed significantly higher scavenging efficiency than CE and PE ($p < 0.05$).

The antioxidant capacity observed is probably due to its high content in phenolic compounds. Plant-derived polyphenols display characteristic inhibitory patterns toward the oxidative reaction *in vitro* and *in vivo*. The molecular basis for the antioxidant properties of polyphenols is thought to have different mechanisms, arising from the direct reaction with free radicals, and from the chelation of free metals (Dangles, 2012; Leopoldini, Russo, & Toscano, 2011).

The activity of rhizome extracts may be related to the presence of compounds with high molecular weight, especially tannins, which were the main compounds quantified in these extracts. Indeed, this class of polyphenols has been reported to have potent antioxidative activities (Tian et al., 2009). Thus, EA was very rich in tannins and showed the highest levels of antioxidant activity. This trend was similar to that observed in other studies examining the antioxidant capacity of the ethyl acetate fraction (Moussi et al., 2015; Tian et al., 2009). Variations in antioxidant capacity of different extracts may be attributed to differences in their

chemical composition. Polyphenolic and antioxidant index is a combined measure of the quality and quantity of antioxidants in vegetables (Jayaprakasha & Patil, 2007).

3.3. Antibacterial activity

The activity of Chloramphenicol was 31.63 ± 0.55 and 30.06 ± 1.34 mm against *S. aureus* and *B. subtilis* respectively, while no effect of DMSO was observed. CE, EA and PE exhibited antibacterial activity towards tested microorganisms with the highest level for EA followed by CE and then PE (Table 4).

The experiments into the antibacterial effect on *S. aureus* and *B. subtilis* indicated that the inhibition was positively correlated with concentrations (Table 4). The MIC for EA was observed at values of 400 and 600 µg/mL, against *B. subtilis* and *S. aureus* respectively, for CE at 800 and 800 µg/mL and for PE at 800 and 1400 µg/mL (Table 5).

The MBC values of rhizome extract and its fractions against *B. subtilis* are lower than those for *S. aureus* (Table 5). It is reported that the ratio MBC/MIC allows a better evaluation of the antibacterial effect of bioactive compounds; a substance is bactericidal when the ratio MBC/MIC ≤2 and bacteriostatic if the ratio MBC/MIC > 2 (Biyiti, Meko, & Amvam Zollo, 2004). By these criteria, the CE exerts a bactericidal effect against *S. aureus* and *B. subtilis* (MBC/MIC = 1), while EA exhibits a bacteriostatic effect on *S. aureus* (MBC/MIC > 75) and a bactericidal effect against *B. subtilis* (MBC/MIC = 2). However, the PE exerts only a bacteriostatic effect on *S. aureus* and *B. subtilis* (MBC/MIC > 64 and 112, respectively).

The secondary metabolites of plants have been found to have antimicrobial properties (Bhalodia & Shukla, 2011) and the potential beneficial effect may be enhanced by using concentrated extracts. In addition, phenolic compounds are known to be synthesized by plants in response to infection by microorganisms (Doughari, 2008), which explains their *in vitro*

312 antimicrobial effect (Cowan, 1999). It has been reported that tannins have potent antibacterial
313 effects on various bacteria including *B. subtilis* and *S. aureus* (Taguri et al., 2004), indicating
314 that the observed activity of extracts, especially the ethyl acetate fraction, could be due to its
315 richness in tannins.

316 *S. aureus* has been reported to be sensitive to other genera of the Cyperaceae family.
317 The ethyl acetate and flavonoid oligomer extracts of *C. rotundus* were found by Kilani (2008)
318 to be the most active against *S. aureus* with an MIC value of 0.5 mg/mL for both. Luteolin, a
319 flavonoid found in *S. holoschoenus*, shows antibacterial activity against *S. aureus* (Su, Ma,
320 Wen, Wang, & Zhang, 2014). Additionally, the antibacterial properties of phenolic acids have
321 been investigated. The MIC values for cinnamic acid against *S. aureus* and *B. subtilis* were
322 found to be, respectively, 6.75 and 2 mM (Guzman, 2014). Chlorogenic acid has been
323 reported to inhibit *S. aureus* ATCC 25923 with an MIC value of 2.5 mg/mL (Li, Wang, Xu,
324 Zhang, & Xia, 2013).

325
326 Phenolic compounds can act at two different levels: the cell membrane and cell wall of
327 the microorganisms (Taguri, Tanaka, & Kouno, 2006). Electron microscopic observations
328 showed that the cell membrane of *S. aureus* was damaged by chlorogenic acid. It is concluded
329 that it inhibited the proliferation of this strain and destroyed the permeability of the cell
330 membrane (Li et al., 2013). In addition, phenolic compounds can interact with the membrane
331 proteins of bacteria by means of hydrogen bonding through their hydroxyl groups which can
332 result in changes in membrane permeability and cause cell destruction. They can also
333 penetrate bacterial cells and coagulate cell content (Tian et al., 2009).

334 *3.4. Antimicrobial effects of different combinations of CE, EA and PE*

335 The combination effects of CE, EA and PE with an SCMD were assessed. The 2D
336 contour surface plots of the responses (zone inhibition diameter) are depicted in Fig.4, for *B.*
337 *subtilis* and *S. aureus.*

338 Satisfactory values for the determination coefficients (R^2 = 0.97 and R^2 = 0.92) were
339 obtained for *B. subtilis* and *S. aureus* respectively, indicating a high degree of correlation
340 between the observed (Table 2) and predicted values (Fig. 4). The data of DIZ were analysed
341 using ANOVA and shown in Eq. 4 and 5.

342 $Y_{S.aureus} = 19.5X_1 + 21.8\,X_2 + 16.1X_3\ \ 6\ 0.6\,X_1X_2 + 6.4\,X_1X_3 + 3X_2X_3 + 30.6X_1X_2X_3$ (Eq. 4)

343 $Y_{B.subtilus} = 19.5\,X_1 + 18.1\,X_2 + 15.72\,X_3 + 3.2\,X_1X_2 - 6.5\,X_1X_3 - 7.6\,X_2X_3 + 3\,X_1X_2X_3$ (Eq.5)

344 From the regression equations, it can be observed that the dependent variables (CE, X_1,
345 EA, X_2 and PE, X_3) have a significant (P<0.01) and highly linear effect on DIZ for *S. aureus*
346 and *B. subtilis*, (Y) within the experimental range. The DIZ for *S. aureus* and *B. subtilis* were
347 affected more significantly by EA at $p < 0.01$ (p = 0.0021 and 0.0007, respectively), the DIZ
348 against these strains tended to expand as the amount of EA increased. This means that
349 inhibition increases as the concentration of the compounds contained in the EA in the mixture
350 rises, while the opposite is observed when the contents of the PE increased. The DIZ values
351 were not significantly affected (P>0.05) by the cross product. The calculated *t*-values of X_1X_2,
352 X_1X_3, X_2X_3 and $X_1X_2X_3$ in the case of *S. aureus* were p= 0.91, 0.32, 0.6 and 0.37, respectively
353 and p= 0.34, 0.09, 0.12 and 0.84, respectively, against *B. subtilis.* However, neither of the
354 two statistical models are not sufficiently significant (being *P*- value = 0.075 for DIZ *B.*
355 *subtilus*, and *p*-value= 0.22 for DIZ *S. aureus*).
356
357 The combination effects of the dependent variables on DIZ against *S. aureus* and *B. subtilis*
358 can also be seen in the contour plot shown in Fig. 4. The combination of the three samples

against *B. subtilis* (Table 2) shows that the PE has a negative influence on the effect of the CE and EA samples (antagonist effect). A non-significant synergistic effect was recorded between the CE and the EA. These last two extracts may have different modes of action, and their combination with different ratios could be of interest in order to seek a synergistic or additive effect. According to Koech (2013), the combination of two agents exhibits significant potential or synergism only if the test organism is resistant to at least one of the agents. In contrast, Delaquis, Stanich, Girard, and Mazza (2002) noted that mixed fractions may produce additive, synergistic or antagonistic effects against individual test microorganisms.

Conclusion

Extraction of phenolic compounds from S. holoschoenus was optimised using a simplex centroid design which showed that the 70% acetone was most effective than ethanol, methanol and their combinations. The extract was found to have antibacterial and antioxidant activities. The fractionation enriched the ethyl acetate fraction on tannins and gave them higher efficiency, while the petroleum ether fraction decreased the antibacterial effect of crude extract and ethyl acetate fraction. Therefore, further phytochemical investigation needs to be done on these extracts to isolate and identify active constituents.

References

Abdel-Mogib, M., Basaif, S. A., & Sobahi, T. R. (2001). Stilbenes and a New Acetophenone Derivative from *Scirpus holoschoenus*. *Molecules*, 1420-3049.

Abozed, S. S., El-kalyoubi, M., Abdelrashid, A., & Salama, M. F. (2014). Total phenolic contents and antioxidant activities of various solvent extracts from whole wheat and bran. *Annals of Agricultural Sciences, 59*(1), 63-67.

Bahorun, T., Gressier, B., Trotin, F., Brunet, C., Dine, T., Luyckx, M., Pinkas, M. (1996). Oxygen species scavenging activity of phenolic extracts from hawthorn fresh plant organs and pharmaceutical preparations. *Arzneimittelforschung, 46* .(11), 1086-1089.

Bento, T. S., Torres, L. M., Fialho, M. B., & Bononi, V. L. (2013). Growth inhibition and antioxidative response of wood decay fungi exposed to plant extracts of Casearia species. *Letters in Applied Microbiology, 10*, 1111-12159.

Bhalodia, N. R., & Shukla, V. J. (2011). Antibacterial and antifungal activities from leaf extracts of Cassia fistula: An ethnomedicinal plant. *Journal Advanced Pharmaceutical Technology & Research, 2*(2), 104-109.

Biyiti, L. F., Meko, D. J. L., & Amvam Zollo, P. H. (2004). Recherche de l'activité antibactérienne de quatre plantes médicinales Camerounaises. *Pharmacologie et Medecine Traditionelle en Afrique, 13*, 11-20.

Bohr, G. E., Meier, B., & Sticher, O. (2000). Analysis of Procyanidins. In Atta-ur_Rahman (Ed.), *Bioactive Natural Products*, 497-570.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods. *International Journal of Food Microbiology, 94*(3), 223-253.

Chan, S. W., Lee, C. Y., Yap, C. F., Wan Aida, W. M., & and Ho, C. W. (2009). Optimisation of extraction conditions for phenolic compounds from limau purut (Citrus hystrix) peels. *International Food Research Journal, 16*, 203-213.

Chirinos, R., Rogez, H., Campos, D., Pedreschi, R., & Larondelle, Y. (2007). Optimization of extraction conditions of antioxidant phenolic compounds from mashua (Tropaeolum tuberosum Ruíz & Pavón) tubers. *Separation and Purification Technology, 55*(2), 217-225.

Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. *American Society for Microbiology, 12*(4), 564–582.

Dai, J., & Mumper, R. J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*(10), 7313-7352.

Dangles, O. (2012). Antioxidant activity of plant phenols: chemical mechanisms and biological significance *Current Organic Chemistry, 16*(6), 692-714.

Delaquis, P. J., Stanich, K., Girard, B., & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology, 74*, 101- 109.

Djeridane, A., Yousfi, M., Nadjemi, B.,, Boutassouna, D., Stocker, P., & Vidal, N. (2006). Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry, 97*, 654-660.

Doughari, J. H., El-mahmood, A.M., Tyoyina, I., (2008). Antimicrobial activity of leaf extracts of Senna obtusifolia (L.) *African Journal of Pharmacy and Pharmacology, 2*, 007-013.

Gil, M. I., Toms-Barbern, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant Activity of Pomegranate Juice and Its Relationship with Phenolic Composition and Processing. *Journal Agricultural and Food Chemistry, 48*, 4581-4589.

Gulcin, W. (2004). Comparison of antioxidant activity of clove (Eugenia caryophylata Thunb) buds and lavender (Lavandula stoechas L.). *Food Chemistry, 87*(3), 393-400.

Guzman, J. D. (2014). Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobial activity. *Molecules, 19*(12), 19292-19349.

Hagerman, A. E., & Butler, L. G. (1978). Protein precipitation method for the quantitative determination of tannin. *Journal Agricultural and Food Chemistry, 26*(4), 809-812.

Jayaprakasha, G. K., & Patil, B. S. (2007). In vitro evaluation of the antioxidant activities in fruit extracts from citron and blood orange. *Food Chemistry, 101*, 410 - 418.

Koech, K. R., Wachira, F.N., Ngure, R.M., Wanyoko, J.K., Bii C., Karori S.M. (2013). Antibacterial and Synergistic Activity of Different Tea Crude Extracts against Antibiotic Resistant S. Aureus, E. Coli and a Clinical Isolate of S. Typhi. *Science Journal of Microbiology*, 2276-2626.

438 Leopoldini, M., Russo, N., & Toscano, M. (2011). The molecular basis of working
439 mechanism of natural polyphenolic antioxidants. *Food Chemistry 125*(2), 288-306.
440 Li, G., Wang, X., Xu, Y., Zhang, B., & Xia, X. (2013). Antimicrobial effect and mode of
441 action of chlorogenic acid on *Staphylococcus aureus*. *European Food Research and*
442 *Technology, 238*(4), 589-596.
443 Morales, R., Pardo-De-Santayana, M., & Tardio, T. (2006). The perception of plants in the
444 complete works of Cervantes, particularly "Don Quijote". *Proceedings of the IVth*
445 *International Congress of Ethnobotany (ICEB 2005)*, 451-459.
446 Moussi, K., Nayak, B., Perkins, L. B., Dahmoune, F., Madani, K., & Chibane, M. (2015).
447 HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves extract
448 of Rhamnus alaternus L. *Industrial Crops and Products, 74*, 858-866.
449 Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of*
450 *Chromatography. 1054*(1), 95-111.
451 NCCLS. (2001). Development of in vitro susceptibility testing and quality control parameters.
452 Approved guideline, 2nd ed. NCCLS document M23-A2. NCCLS, Wayne, Pa.
453 Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., & Pulkrabek, J. (2009).
454 Antimicrobial properties of selected essential oils in vapour phase against foodborne
455 bacteria. *Food Control, 20*, 157-160.
456 Owen, R. J., & Palombo, E. A. (2007). Anti-listerial activity of ethanolic extracts of medicinal
457 plants Eremophila alternifolia and Eremophila duttonii in food homogenates and milk.
458 *Food Control*, 18, 387-390.
459 Popescu, A., Negreanu-Pirjol, T., Rosca, C., Arcus, M., Bucur, L., and Istudor, V. (2011).
460 HPLC analysis of polyphenols and antioxidant capacity determination of *Scirpus*
461 *holoschoenus* L. rhizome. Ovidius University Annals of Chemistry, *22*(1), 62-66.
462 Shahidi, F., & Naczk, M. (2011). Analysis of polyphenols in food. In S. Otles (Ed.), *Methods*
463 *of Analysis of Food Components and Additives, Second Edition*, 253-308.
464 Shirwaikar, A., Shirwaikar, A., Rajendran, K., & Punitha, I. S. R. (2006). In Vitro
465 Antioxidant Studies on the Benzyl Tetra Isoquinoline Alkaloid Berberine. *Biological*
466 *and Pharmceutical Bulletin, 29* (9), 1906-1910.
467 Su, Y., Ma, L., Wen, Y., Wang, H., & Zhang, S. (2014). Studies of the in vitro antibacterial
468 activities of several polyphenols against clinical isolates of methicillin-resistant
469 Staphylococcus aureus. *Molecules, 19*(8), 12630-12639.
470 Taguri, T., Tanaka, T., & Kouno, I. (2006). Antibacterial spectrum of plant polyphenols and
471 extracts depending upon hydroxyphenyl structure. *Biological and Pharmaceutical*
472 *Bulletin, 29*, 2226-2235.
473 Taguri, T., Tanaka, T. & Kouno I. (2004). Antimicrobial Activity of 10 Different Plant
474 Polyphenols against Bacteria Causing Food-Borne Disease. *Biological and*
475 *Pharmceutical Bulletin, 27*(12), 1965-1969.
476 Tian, F., Li, B., Ji, B., Yang, J., Zhang, G., Chen, Y., & Luo, Y. (2009). Antioxidant and
477 antimicrobial activities of consecutive extracts from Gallachinensis: The polarity
478 affects the bioactivities. *Food Chemistry, 113*, 173-179.
479 Uma, D. B., Ho, C. W., & Aida, W. M. W. (2010). Optimization of Extraction Parameters of
480 Total Phenolic Compounds from Henna (Lawsonia inermis) Leaves. *Sains Malaysiana*
481 *39*(1), 119-128.
482 Vaquero, M. J. R., Alberto, M. R., & de Nadra, M. C. M. (2007). Antibacterial effect of
483 phenolic compounds from different wines. *Food Control, 18*(2), 93-101.
484 Viswanath, V., Urooj, A., & Malleshi, N. G. (2009). Evaluation of antioxidant and
485 antimicrobial properties of finger millet polyphenols. *Food Chemistry, 114*, 340-346.

Watson, R. R. (2014). Determination of polyphenols, flavonoids, and antioxidant capacity in dry seeds. In R. R., Watson (Ed.), *Polyphenols in Plants: Isolation, Purification and Extract Preparation*, 305-324.

Figure captions

Fig. 1: Schematic diagram of preparation and fractionation of *S. holoschoenus* extract.

Fig. 2: Response-surface contour plots of the quadratic model for TPC as a function of the composition of methanol 70%, ethanol 70%, and acetone 70%. Ac: 70% acetone; Et: 70% ethanol; Me: 70% methanol.

Fig. 3: The free radical scavenging activity percentage of crude extract and fractions of *S. holoschoenus* evaluated by DPPH assay. a-f denotes the different tested concentrations, 1- 4 is the statistical comparison of the values obtained with each concentration.

Fig. 4: Response-surface contour plots for the effect of different combinations of the studied extract and its fractions on zone inhibition diameter values against *S. aureus* (a) and *B. subtilis* (b).

Table 1

Experimental data and the observed responses value of total phenolic compounds (TPC) referred to dry weight (DE) of each extract. GAE. gallic acid equivalents.

Run	time (h)	acetone 70%	ethanol 70%	methanol 70%	TPC (mg GAE /g DE)	
					Experimental values	Predicted values
1	24	1	0	0	182.29±0.22^{a}	186.11
2	24	0	1	0	122.99±0.59^{b}	126.15
3	24	0	0	1	178.55±0.97^{c}	175.38
4	24	0	0.5	0.5	138.95±2.90^{d}	101.03
5	24	0.5	0	0.5	100.38±2.32^{e}	180.81
6	24	0.5	0.5	0	98.32±2.63g	105.30
7	24	0.33	0.33	0.33	100.384±2.32^{ef}	111.84
8	24	0.66	0.16	0.16	131.92 ±1.55^{f}	116.63
9	24	0.16	0.66	0.16	136.56 ±2.23^{f}	123.24
10	24	0.16	0.16	0.66	132.55 ±5.62^{ef}	138.22
11*	1	1	0	0	133.07±2.90^{C}	
12*	2	1	0	0	138.36±3.39^{C}	
13*	3	1	0	0	149.09±0.89^{B}	
14*	3x1	1	0	0	241.47±1.16^{A}	

* time optimization for the selected solvent (70% acetone)

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 design matrix and experimental responses (inhibition zone diameter (mm)) obtained for tested bacteria at concentration of 90.2 mg/mL for CE, EA and PE.

Run	**Extracts**			Responses inhibition zone diameter (mm)	
	Crude extract	Ethylacetate fraction	Petroleum ether fraction	*S. aureus*	*B. subtilis*
01	100%	00%	00%	19.50 ± 0.50^{ab}	18.10 ± 0.50^{a}
02	00%	100%	00%	21.80 ± 0.60^{a}	19.50 ± 0.20^{b}
03	00%	0%	100%	16.10 ± 0.70^{c}	15.70 ± 0.30^{d}
04	50%	50%	00%	20.50 ± 1.34^{ab}	19.70 ± 0.10^{b}
05	50%	00%	50%	19.40 ± 1.60^{b}	15.96 ± 0.05^{dc}
06	00%	50%	50%	19.70 ± 1.03^{ab}	15.80 ± 0.20^{d}
07	33%	33%	33%	21.00 ± 1.00^{ab}	16.66 ± 0.52^{cd}
08	33%	33%	33%	22.00 ± 0.80^{ab}	16.25 ± 0.20^{ac}
09	33%	33%	33%	20.00 ± 1.00^{ab}	17.25 ± 0.43^{cd}

Table 3

Total phenolics, total flavonoids and total tannins contents of *S. holoschoenus* rhizome extract and fractions.

Fraction	Total phenolics (mg GAE/gDE*)	Total flavonoids (mgQE/gDE*)	Total tannins (mg TE/gDE*)
Ethyle acetate (EA)	253.47 ± 18.35	6.62 ± 0.04	156.33 ± 10.41
Petroleium ether (PE)	170.00 ± 1.73	12.89 ± 0.37	58.00 ± 2.64
Crude extract (CE)	236.02 ± 1.24	1.96 ± 0.08	97.67 ± 1.53

* Values were referred to dry weight (DE) of EA, PE and CE.

Table 4

The inhibitory effect of crude extract and fractions of *S. holoschoenus* at different concentrations against the tested bacteria.

		Concentration of *S. holoschoenus* extracts (mg Dry Extract/ mL)								
Strains	Extracts	90.20	45.10	22.55	11.27	5.63	2.82	2.42	2.02	1.62
		Inhibition zone (mm)								
S. aureus	CE	19.5 ± 0.50[1]	18 ± 0.00[1]	17 ± 0.28[12]	15.3±0.25[1]	15.2±1.25[1]	14.9±0.10[1]	12.8±0.25[1]	11.5±0.50[1]	12.5±0.25[1]
	EA	21.8 ±1.67[2]	21.3 ±0.57[2]	19.5±0.50[1]	17.2±1.73[2]	15±00[1]	15.3±1.23[1]	13.8±2.75[2]	13.2±0.28[2]	13.2±0.28[2]
	PE	16.1±0.7[3]	15.2±1.52[3]	14±1.73[2]	15±0.00[12]	13.7±1.89[1]	13.3±1.15[2]	11.7±0.70[1]	10.7±0.57[1]	10.3±0.70[3]
B. subtilis	CE	18.2±0.28[1]	15.3±0.57[1]	14.3±0.57[1]	12.8±0.76[1]	11.7±0.57[1]	10.3±0.57[1]	07.7±2.88[1]	07.3±1.52[1]	07.3±1.15[1]
	EA	19.5±0.50[2]	18.3±0.28[2]	16.3±0.57[2]	15±0.50[2]	13.3±0.57[1]	11.2±0.28[1]	10.5±0.50[12]	09.5±0.50[12]	09±1.00[1]
	PE	15.8±0.28[3]	13±1.0[1]	12.5±0.5[3]	11.7±0.57[3]	10.8±1.60[1]	12.2±1.00[1]	12 .2±0.28[3]	11±1.00[2]	10.7±1.52[2]

CE: Crude extract, EA: Ethyl acetate fraction, PE: Petroleum ether fraction, 1-3 is the comparison of the values obtained for each concentration of *S. holoschoenus* , same number indicates similar level of statistical differences.

Table 5

Minimal Inhibitory Concentration (MIC. µg/mL) and Minimal Bactericidal Concentration (MBC, µg/mL) of rhizome extract and fractions from *S. holoschoenus*

Microorganisms	Extracts								
	Crude extract			Ethyl acetate fraction			Ether petroleum fraction		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
B. subtilis	800	800	1	400	800	2	800	> 2000	113
S. aureus	800	800	1	600	> 2000	75	1400	> 2000	64

Fig 1.

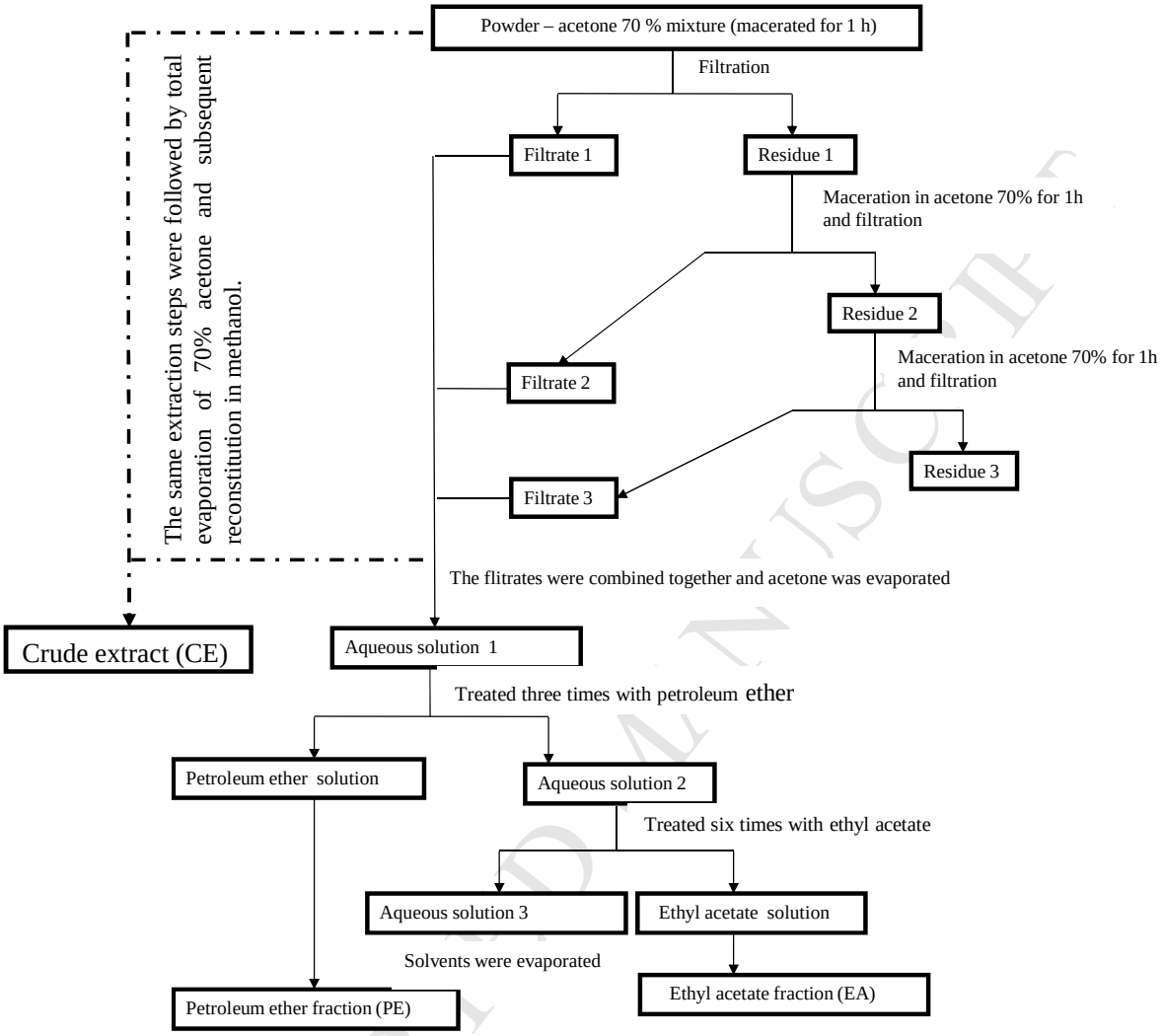


Fig 2.

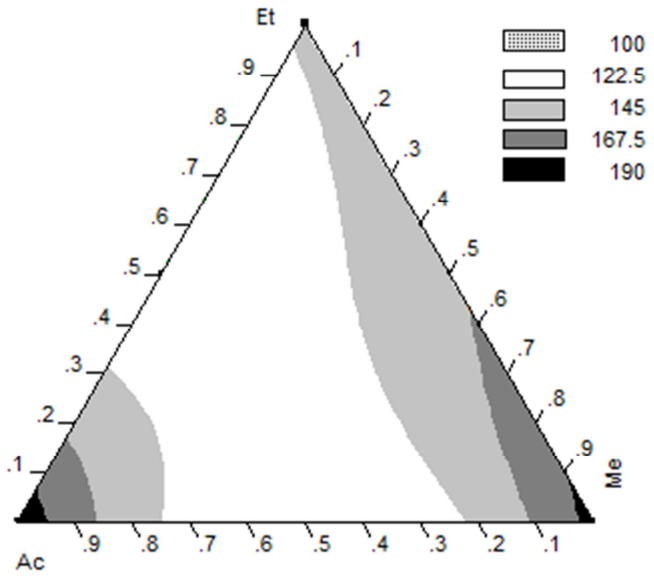


Fig 3.

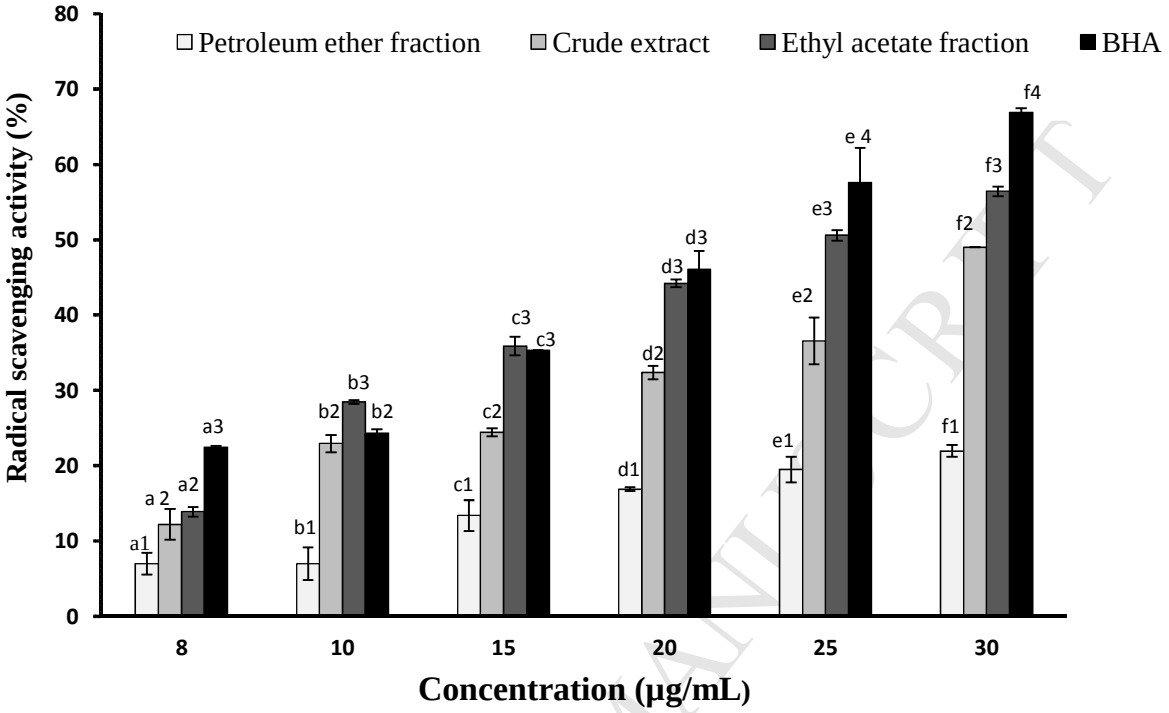
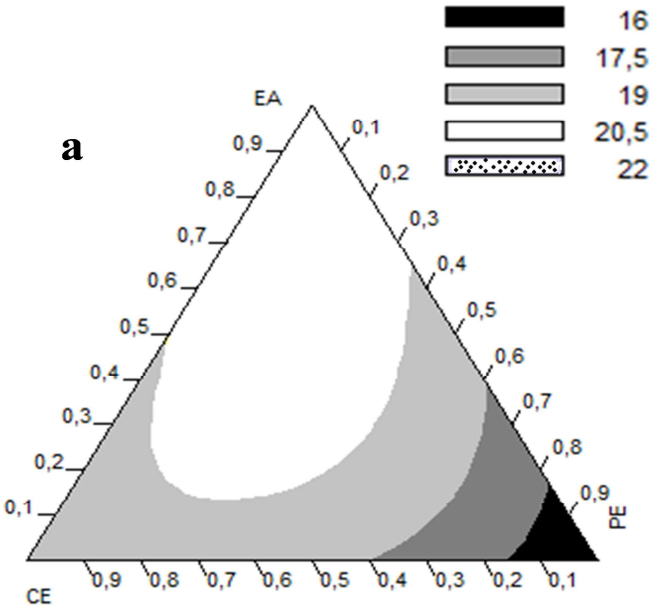
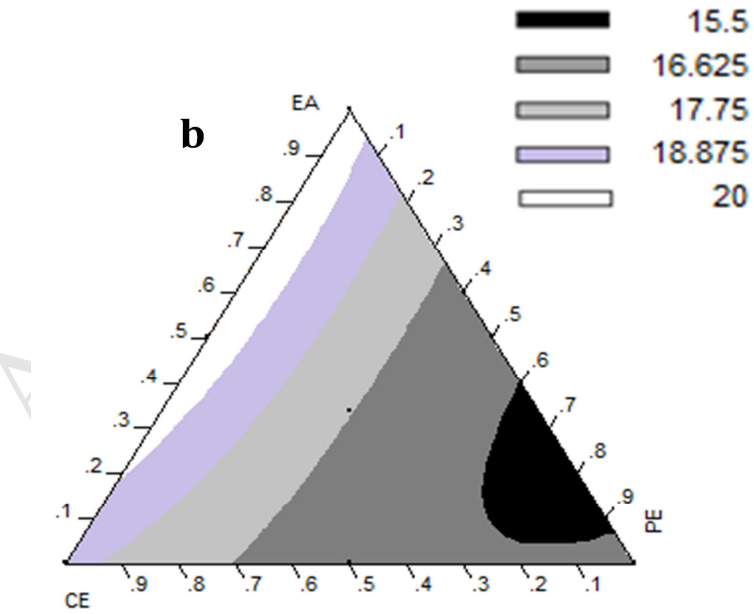


Fig 4.


a
16
17,5
19
20,5
22
EA
CE
PE
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9


b
15.5
16.625
17.75
18.875
20
EA
CE
PE
.1
.2
.3
.4
.5
.6
.7
.8
.9

Highlights

Highest total phenolic content (TPC) of *S. holoschoenus* was extracted with 70% acetone

Three cycles of 1 hr extraction procedure was more effective in TPC extraction.

TPC, tannins and flovoinoids were measured in crude extract and its fractions.

Ethyl acetate fraction showed the highest antioxidant activity.

Petroleum ether fraction affected negatively the antibacterial activity of samples.