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Antioxidant and Antimicrobial Assessment of Licorice Supercritical Extracts

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Abstract

Licorice (*Glycyrrhiza glabra* L.) is a plant used widely in herbal medicines due to their several biological potentials. The supercritical extraction of licorice roots was investigated to assess the antioxidant and antimicrobial activity of the extracts. Extraction conditions were pressures from 15 to 40 MPa, 313.15 and 333.15 K, and ethanol cosolvent in the range of 0 to 20% mass. In the case of high-pressure extractions using pure carbon dioxide (CO_2) fractionation of the supercritical extract was accomplished in a two-cell decompression system. Fractionation was carried out with the aim to examine the potential separation of the antioxidant and antimicrobial licorice compounds and thus increase the bioactive properties of the fractions obtained in each separation cell. Main licorice bioactive compounds, liquiritin, liquiritigenin, glycyrrhizin, isoliquiritigenin and glabridin, were identified by HPLC and quantified using standards. Extracts obtained with supercritical CO_2 and ethanol cosolvent contain the higher amounts of phenolic compounds and also the higher antioxidant activity but exhibit low or even no antimicrobial activity. Using pure CO_2 at high pressure coupled with the on-line fractionation of the extract, two samples were obtained which showed, respectively, lower phenolic compounds content and good antimicrobial capacity (first fraction) and higher phenolic compounds content and antioxidant capacity (second

fraction). Thus, the advantages of supercritical on-line fractionation are demonstrated in the extraction of Licorice roots.

Keywords: supercritical carbon dioxide, bioactive compounds, *Glycyrrhiza glabra*, licorice, antioxidant, antibacterial.

1. Introduction

Licorice is a ligneous perennial shrub typical of the Mediterranean region, Asia Minor and Middle East and also widely cultivated in southern Russia and Iran (Ody, 2000). The roots and rhizomes of licorice are used extensively in herbal medicines due to their, among others, emollient, detoxification, anti-ulcer, anti-inflammatory, gastro-protective and anti-allergenic properties (Mukhopadhyay and Panja, 2008). Licorice contains a variety of bioactive compounds, different sugars, phenolic compounds, isoflavones, coumarins, stilbenoids and saponins such as glycyrrhizin (Qiao et al., 2015), with positive pharmaceutical functions, such as anti-inflammatory, anti-viral, anti-carcinogenic (Fukai et al., 2004; Hatano et al., 2000), antifungal properties (Fatima et al., 2009), as well as antioxidant and antimicrobial activities (Thakur et al., 2016). Different extraction procedures have been investigated to obtain bioactive extracts from licorice roots, being water, ethanol and methanol, the main solvents studied.

Visavadiya et al. (2009) and Gupta et al. (2016) reported the antioxidant activity of extracts obtained by soxhlet extraction with ethanol and water. Hejazi et al. (2017) used the same extraction technique, but with methanol solvent. Methanolic extracts were subsequently fractionated with water and several organic solvents covering different polarities, in order to evaluate the fractions for the *in vitro* antioxidant capacity and apoptotic effects in cell systems. Cheel et al. (2010) and Tohma and Gulçin (2010) studied the radical scavenging activity of extracts obtained by infusion using ethanol and water. Karami et al. (2015) evaluated the antioxidant capacity of extracts obtained by microwave assisted extraction (MAE) with water and 80% ethanol and methanol. From these works, polar solvents were generally more effective to obtain extracts containing high concentrations of phenolic compounds and better antioxidant capacities, specially water, ethanol and their mixtures.

Furthermore, the antioxidant activity of licorice extracts was strong related with the presence of glycyrrhizin, a compound with an antioxidant mechanism studied and reported in the literature (Beskina et al., 2006).

Recently, several studies aiming to expand the knowledge about the biological properties of licorice extracts in regard to antimicrobial activity have been reported. Several studies have shown the ability of licorice aqueous, ethanol and methanol extracts, obtained by different extraction processes, to inhibit the growth of Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella spp*. (Chandra and Gunasekaran, 2917, García-Ruiz et al., 2015, Astaf eva and Sukhenko, 2014, Escisli et al., 2008). Based on the above inhibitory activities against bacteria, it has been suggested that licorice may have a therapeutic and/or preventive capacity for oral infections (Gafner et al., 2011).

Supercritical fluid extraction (SFE) is an extraction technology also applied to obtain antioxidant and antimicrobial extracts from natural sources (Fornari et al., 2012). Supercritical fluids allow high extraction rates due to their high solvation power (liquid-like density), low viscosities and high diffusion coefficients. Supercritical CO_2 ($SCCO_2$) is the preferred supercritical fluid, due to its low critical temperature (304 K) and moderate critical pressure (7.4 MPa), which prevents or minimizes the degradation of bioactive compounds. Moreover, CO_2 is inexpensive, inert, non-toxic, non-flammable and allows obtaining solvent-solvent free products. Despite these interesting properties, only a few studies have been reported for the SFE of licorice roots and their objective was the extraction of glycyrrhizic acid. Kim et al. (2004) examined the extraction behavior of this compound at different pressures (11-50 MPa), temperatures (313-393 K) and using water and 70% aqueous methanol as cosolvent. The best result (98% glycyrrhizic acid recovery) was obtained at 30

77 MPa, 343 K and 15% of aqueous methanol cosolvent. Likewise, Hedayati and Ghoreishi
78 (2015) studied glycyrrhizic acid extraction by a combined static-dynamic procedure at
79 several pressures (10-34 MPa), temperatures (318-358 K), dynamic extraction times (40-120
80 min), CO_2 flow rates (0.8-2 mL/min) and using different methanol/water mixtures as
81 cosolvent. The highest extraction (54% compound recovery) was reached at 30 MPa, 341 K,
82 extraction time of 108 min and 46.5% methanol (v/v). In a further contribution, Hedayati and
83 Ghoreishi (2016) studied the same extraction parameters but using water as cosolvent, and
84 determined the optimal conditions simulating the experimental results by an artificial neural
85 network model.

86 Based on this background, this work presents a study of the SFE of licorice roots using CO_2
87 with and without the addition of ethanol as a cosolvent and, for the first time, the *in vitro*
88 evaluation of the antioxidant and antimicrobial activity of the supercritical fractions obtained.

89 ## 2. Materials and methods

90 ### 2.1. Chemicals

91 Ethanol (99.5% purity) and Sodium Carbonate anhydrous (99.5% purity) were purchased
92 from Panreac (Barcelona, Spain). CO_2 (N38) was supplied from Carburos Metálicos (Madrid,
93 Spain). Gallic acid standard (> 98% purity), 2,2-Diphenyl-1-pycrilhydrazyl (DPPH, 95%
94 purity), 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥
95 95% purity), (±)-6-Hydroxy-2,5,7,8-tetramethyllchromane-2-carboxylic acid (Trolox, 97%
96 purity), Folin-Ciocalteu´s reagent, chloramphenicol (≥ 98% purity), liquiritin, glabridin,
97 liquiritigenin, glycyrrhizic acid ammonium salt and isoliquiritigenin were purchased from
98 Sigma–Aldrich (St. Louis, MO, USA). Difco Wilkins-Chalgren Agar and BBL Mueller
99 Hinton II Broth was purchased from Becton, Dickinson and Company (France).

2.2. Sample preparation

Root of licorice harvested in Spain was obtained from Murciana herbalist's (Murcia, Spain) and the water content was 9.90% wt. The sample was ground using a Premill 250 hammer mill (Lleal S.A., Granollers, Spain) and the mean particle size was 100 µm (all particles were lower than 500 µm). All samples were stored in polyethylene bags under vacuum and kept at 4 °C until extraction.

2.3. Supercritical fluid extraction

A pilot-extractor (model SF2000; Thar Technology, Pittsburgh, PA, USA) was used for the SFE assays. The extractor comprises a cylinder cell (0.273 L) and two separator-cells (S1 and S2) (0.5 L capacity each), with independent control of temperature and pressure. A detailed description of the equipment used can be found elsewhere (Villanueva-Bermejo et al., 2017). The experimental conditions are listed in Table 1. The mass of licorice root was 0.160 kg of licorice root in all the experiments, being the apparent density of the packed bed 586 kg/m^3 (estimated porosity of 0.7). Different extractions were accomplished at 15-40 MPa, 313.15 and 333.15 K, and two CO_2 flow rates, 50 and 70 g/min, during 3 h in dynamic extraction (no static extraction was applied), representing a CO_2/plant ratio of 56 and 79 kg/kg, respectively. Ethanol was used as a cosolvent, being the concentration of ethanol in the supercritical extractive solvent in the range 0 to 20% wt (CO_2/ethanol ratio of 4 kg/kg). CO_2 and the cosolvent were mixed in the desire ratio previous to be pumped into the extraction cell.

The extracts were obtained by reducing pressure in the separator-cells. In the case of runs 1 to 6 (Table1) both separators were maintained at the system recirculation pressure (5 MPa) and the extracted material was recovered from S1 and S2 and mixed in a single fraction. Moreover, runs 7 to 10 were carried out at higher pressures and the extract was fractionated in the decompression cascade system comprising two separators. For this purpose, the first

separator vessel (S1) was kept at 15 MPa and the second one was maintained at the recirculation pressure (5 MPa). Thus, two fractions (S1 and S2) were collected in each of the runs 7 to 10.

2.4. HPLC analysis

HPLC analysis was performed as previously described by the authors Wei et al. (2015). A Prominence-i LC-2030C 3D Plus (Shimadzu) equipped with a quaternary solvent delivery system, an autosampler and DAD detector and RP-C18 (250 × 4.6 mm; 3 μm) was used. The column temperature was set at 25 ºC. The mobile phase consists of acetonitrile (A) and 0.026% aqueous H_3PO_4 (v/v), applying the following gradient elution: at 0-20 min, 20%-25% A, 20-30 min, 25% -34% A, 30-50 min, 34%-50% A, 50-60 min, 50% -60% A and 60-80 min, 60% A. After 5 min, the initial conditions were achieved. The flow rate was 0.7 ml/min and was kept constant during analysis. Injection volume was 20 μl and detection was accomplished at 254, 280 and 370 nm. Calibration curves with the standards were used to determine the content of these bioactive compounds in the different extracts.

2.5. Total phenolic compounds (TPC) determination

The total phenolic content in licorice extracts was determined using the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 50 μl of extract were mixed with 3 mL of milliQ water and 250 μl of Folin Ciocalteu reagent. The content was thoroughly mixed and after 3 min, 750 μl of sodium carbonate solution (20% mass) and 950 μl of milliQ water were added to the mixture. After 2 h at room temperature and remained in darkness, the absorbance was measured at 760 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Fischer Scientific Inc., MA, USA). The results were expressed as GAE (mg of gallic acid equivalents/g of extract). All analyses were done in triplicate.

2.6. Antioxidant capacity

2.6.1. ABTS assay

The antioxidant capacity of the extracts was determined by the $ABTS^{\cdot+}$ radical scavenging assay following the method described by Re et al. (1999). $ABTS^{\cdot+}$ radical cation was generated by mixing $ABTS^{\cdot+}$ stock solution (7 mM) with 2.45 mM potassium persulfate after incubation of the mixture at room temperature for 16 h under darkness. Once the $ABTS^{\cdot+}$ radical was formed, the solution absorbance was adjusted to 0.700 ± 0.02 at 734 nm by ethanol in a Genesys 10S UV-Vis spectrophotometer (Thermo Fischer Scientific Inc., MA, USA). Afterwards, 990 µL of $ABTS^{\cdot+}$ solution was added to 10 µl of sample and the reaction mixture was allowed to stand at room temperature and under darkness, until the absorbance reached a plateau. The absorbance was recorded at 734 nm and the results were expressed as IC_{50} value (Inhibitory concentration: concentration of extract necessary to inhibit the initial concentration of radical by 50%), as well as Trolox equivalents (TEAC) (µmol Trolox/g extract), which were calculated taking into account the Trolox standard and sample concentrations that produce the scavenging of 50% of $ABTS^{\cdot+}$ radical. All the analyses were carried out in triplicate.

2.6.2. DPPH assay

The ability of extracts to scavenge DPPH free radicals was determined according to the method described by Brand-Williams et al. (1995). Licorice extract were added to 975 µL of DPPH radical in ethanol ($6.1 \cdot 10^{-5}$), which was daily prepared. The reaction took place at room temperature in the dark until it reached a plateau. Then, the absorbance was measured at 515 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo Fischer scientific, MA, USA). A calibration curve (linear regression) was used to determine the DPPH concentration

in the reaction medium. A control sample (i.e. a sample comprising the same volume of solvent instead of extract) was used to measure the maximum DPPH absorbance. Trolox was used as reference standard, so results were expressed as TEAC values (µmol Trolox/g extract), as well as IC_{50} value (µg/mL). All analyses were done in triplicate.

2.7. Antibacterial activity assay

The extracts and fractions collected were individually tested against a Gram-positive bacteria, *Staphylococcus aureus* American Type Culture Collection-ATCC 25923 and a Gram-negative bacteria, *Escherichia coli* ATCC 25922. A broth microdilution method was used, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1999), for determination of the minimum inhibitory concentration (MIC). All tests were performed in Mueller–Hinton broth supplemented with 0.5% tween 20. The inocula of bacterial strains were prepared from overnight Mueller–Hinton broth cultures at 37 ºC. Test strains were suspended in Muller–Hinton (bacteria) broth to give a final density 10^7 cfu/mL. The extract and fractions were diluted in ethanol ranging from 50 to 1 mg/mL.

The 96-microwell plates were prepared by dispensing into each well 185 µl of culture broth, 10 µl of the different extracts dilutions, antibiotic solution (chloramphenicol as positive control) or solvent (ethanol as negative control), and 5 µl of the inoculums. In addition, blanks were prepared adding 190 µL of broth medium to the solvent or extracts wells. The final volume of each well was 200 µl. After dispensing the inoculum, the plates were read in an Infinite 200 PRO plate reader (TECAN, Trading AG, Switzerland) spectrophotometer at 620 nm for T0 (Zero Time). Then, the plates were incubated at 37 °C for 24 h and the absorbance was read for TF (Final Time). Each test was performed in triplicate and repeated twice.

193 The inhibition percentage was calculated following the method described by Cueva et al.
194 (2010) as (Eq. 1):

$$\%\ \text{Inhibition} = \left(1 - \frac{\left(\text{TF}_{\text{Sample}} - \text{T0}_{\text{Sample}}\right) - \left(\text{TF}_{\text{Blank of sample}} - \text{T0}_{\text{Blank of sample}}\right)}{\left(\text{TF}_{\text{Growth}} - \text{TO}_{\text{Growth}}\right) - \left(\text{TF}_{\text{Blank}} - \text{T0}_{\text{Blank}}\right)}\right) \times 100 \quad (1)$$

195 Where $\text{TF}_{\text{Sample}}$ and $\text{T0}_{\text{Sample}}$ corresponded to the absorbance at 620 nm of the strain growth
196 in the presence of the licorice extracts after and before incubation, respectively;
197 $\text{TF}_{\text{Blank of sample}}$ and $\text{T0}_{\text{Blank of sample}}$ corresponded to the broth medium with extracts after
198 and before incubation, respectively; $\text{TF}_{\text{Growth}}$ and $\text{T0}_{\text{Growth}}$ correspond to the strain growth
199 in the presence of the solvents after and before incubation; and TF_{Blank} and T0_{Blank}
200 corresponded to the broth medium with solvent after and before incubation.

201 For active extracts, the survival parameter IC_{50} value was defined as the concentration
202 required to obtain 50% inhibition of growth after 24 hours of incubation at 37 ºC and was
203 estimated by nonlinear regression using the sigmoidal dose-response (with variable slope)
204 equation (2):

$$\text{Y} = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{\left(1 + 10^{(\log \text{IC}_{50} - \text{X}) \times \text{Slope})}\right)} \quad (2)$$

205 where, X represents the logarithm of concentration, Y is the % Inhibition which starts at the
206 Bottom and goes to the Top with a sigmoid shape, Log IC_{50} is the logarithmic of IC_{50}, and
207 Slope represents the slope parameter. The PRISM program (GraphPad Software, Inc.) was
208 used for the approximation of the four parameters. For each data set, comparison of the fit to
209 the previous sigmoidal dose response model (4 parameters) was carried out using PRISM,

and also the fit to the same model with the Bottom and Top parameters constrained, respectively, to 0 and 100% was possible.

3. Results and Discussion

3.1. Supercritical Fluid Extraction

The experimental conditions applied for the SFE of licorice roots, as well as extraction yield obtained (mass of extract/mass of licorice) are reported in Table 1. As can be observed, the extraction yields ranged from 0.66% (run 1) to 3.07 % (run 6).

With regard to the effect of pressure (Table 1, runs 1 and 7), a rise in this parameter considerably increased the extraction yield. In this sense, when no cosolvent is used, an extraction yields 2.7-fold higher was reached at 30 MPa (1.76%) in comparison with yield at 15 MPa (0.66%). Temperature effect seem to exhibit crossover behavior, since at 40 MPa a temperature rise produced a yield increase (runs 8 and 10), but at 30 MPa the same temperature rise resulted in a yield decrease (runs 7 and 9).

Indeed, the use of ethanol had a considerably influence over the amount of extract obtained. In this respect, the addition of 10% cosolvent at 15 MPa and 70 g/min (run 2) implied an extraction yield 3.3-fold higher than the obtained without ethanol (run 1). This effect is clearly represented in the Figure 1. As it is showed, the extraction yields linearly increased (r^2= 0.960) with the addition of cosolvent in the range studied. Maximum yield is obtained in run 6 (3.07%) when the maximum amount of ethanol (20%) is used. These results are in agreement with the analysis reported by Hedayati and Ghoreishi (2015) concerning the yield and recovery of glycyrrhizin (no total yields were reported) in the supercritical extractions carried out using methanol: water mixtures as modifier, in percentages up 5% (v/v).

232 Furthermore, Kim et al. (2004) also highlighted the relevant effect of methanol: water as CO_2
233 modifier to extract the same compound, glycyrrhizin, from licorice root.

234 The influence of the amount of cosolvent over yield is especially relevant if it is compared to
235 the yields obtained at higher pressure. Despite the increase in the solvation power as a
236 consequence of the higher density of CO_2 when pressure rises from 15 to 30 MPa at a
237 constant temperature, the extraction yield obtained at 15 MPa, 70 g/min and 10% cosolvent
238 (2.19%) was -1.25-fold higher than the obtained at 30 MPa at the same flow rate but without
239 cosolvent (1.76%).

240 The yields obtained in the fractions S1 and S2 collected in runs 7 to 10 are given in Table 2.
241 In general, most of extract was recovered in the second separator, with yields up to 8 times
242 higher than the yield obtained in the first separator.

243 **3.2. Total phenolic compounds (TPC) and antioxidant capacity**

244 Table 3 shows the amount of TPC (mg GAE/g extract) in the extracts obtained at the different
245 experimental conditions and their antioxidant capacity expressed as IC_{50} (µg extract/mL) and
246 TEAC value (µmol trolox/g extract) as determined by the ABTS and DPPH assays.

247 The TPC content varied from 48.47 (run 8-S1) to 180.06 mg GAE/g extract (run 6). The
248 lower concentrations were obtained by using pure CO_2 (run 1) and particularly in the first
249 fraction (S1) of runs 7 to 10. The higher concentrations of TPC were obtained when ethanol
250 was used and, at constant pressure of 30 MPa, 313 K of temperature and 50 g/min of CO_2
251 flow rate, TPC increased linearly (r^2= 0.979) as the amount of ethanol did (Figure 2). The
252 concentrations of TPC obtained in the SFE licorice extracts were higher than those reported
253 in other studies. The TPC extracted in run 6 (180.06 mg GAE/g extract) was almost 4-fold
254 higher than the obtained with 80% ethanol by MAE (Karami et al., 2015, 2013), and 7.7, 3.8

and 2.3-fold higher than the achieved using, respectively, water (Gupta et al., 2016), ethanol (Visavadiya et al., 2009) and methanol (Hejazi et al., 2017) by Soxhlet extraction.

The effect of supercritical solvent flow on TPC can be analysed by comparison of the values obtained for runs 2 and 4, which were carried out at 15 MPa, 313 K, 10% ethanol cosolvent and, respectively, 70 and 50 g/min CO_2 (total supercritical solvent flow of 77.8 and 55.6 g/min). The increase of flow resulted in a decrease of TPC from 163.03 to 141.18 mg GAE /g extract probably due to the co-extraction of substances other than phenolics, as can be inferred from the higher yield obtained in run 2 (almost 10% higher).

Regarding the effect of pressure and temperature on TPC, Figure 3 show the total amount of TPC extracted (S1+S2 samples) in runs 7 to 10 as a function of pressure, together with the total yield obtained in these runs. As can be observed in the figure, the TPC values show the same crossover behaviour described previously concerning extraction yield. Then, it can be concluded that at 30 MPa the increase of temperature decrease total yield and TPC content, while at 40 MPa an increase of temperature increase total yield and TPC content.

Regarding the antioxidant capacity, the ABTS and DPPH assays show the same tendency, as it can be deduced from the relation between the IC_{50} values depicted in Figure 4. As in the case of TPC, the antioxidant capacity increased with the amount of ethanol, so the highest values were reached with 20% cosolvent (556 and 760 μmol/g for the ABTS and DPPH assay). Nevertheless, despite the linear tendency TPC-%ethanol (Figure 2), no linear correlation between the amount of ethanol and the antioxidant capacity was observed, with a significant increase in the antioxidant capacity of the extract obtained with 20% ethanol (run 6) assessed by both the ABTS and DPPH assay. Moreover, the TEAC values exhibit a general trend to increase with increasing TPC (Figure 5), but TEAC and TPC values could not be linearly correlated for both the ABTS and DPPH assay. Several studies related a strong

279 and positive correlation between TPC and antioxidant activity (Casagrande et al., 2018;
280 Skotti et al., 2014). Nevertheless, this trend is not always satisfied and maybe related to the
281 presence of antioxidants other than phenolic compounds, such as carotenoids, ascorbic acid,
282 among others (Millao and Uquiche, 2016; Rufino et al., 2010). In the case of supercritical
283 licorice root extracts, other no-phenolic compounds may be present (although were not
284 analyzed in this work), in particularly those comprising licorice essential oil, which may also
285 contribute to the antioxidant activity (e.g. estragole, eugenol and anethol (Fenwick et al.,
286 1990) and thus, no linear relation between TPC and TEAC values was obtained.

287 Taking into account that lower IC_{50} values indicates a stronger radical scavenging activity,
288 the extract obtained in run 6, at 15 MPa, 50 g/min CO_2 and 20% cosolvent (IC_{50} 7.74 and
289 18.59 µg/mL, respectively, in ABTS and DPPH assays) showed a considerably higher
290 antioxidant capacity than extracts which were obtained from licorice using water or organic
291 solvents and other extraction techniques. In this regard, the IC_{50} of run 6 extract was around
292 75-fold lower than the obtained by Gupta et al. (2008) with ethanolic extracts (575 µg/mL
293 and 1424 µg/mL in ABTS and DPPH assay, respectively), as well as 7.4 (ABTS assay) and
294 1.5 (DPPH assay) fold lower than the obtained by Visavadiya et al. (2009) with the same
295 solvent. Hejazi et al. (2017) obtained DPPH IC_{50} values of 71.93 and 77.86 µg/mL in
296 methanol and chloroform fractions, respectively, so the IC_{50} of run 6 extract was 3.9 and 4.2
297 times lower than the obtained with these two organic solvents. In the case of the water extract
298 obtained by Thakur et al. (2016) by means of sonication (IC_{50} value of 189.9 and 334.7
299 µg/mL for DPPH and ABTS assay), our run 6 supercritical extract showed an efficacy to
300 scavenge these free radicals between 10.2 (DPPH assay) and 18.0 (ABTS assay) fold higher.
301 Furthermore, extracts with similar antioxidant capacity were obtained using pure CO_2 at high
302 pressure (30 and 40 MPa) and 333.15 K by fractionation of the extract at 15 MPa (S2
303 fractions of runs 9 and 10).

3.3. Antibacterial activity

Antimicrobial activity of licorice extracts against *E. coli* and *S. aureus*, as representative of Gram-negative and Gram-positive bacteria, was evaluated as the inhibition of the bacterial growth, and expressed as the IC_{50} parameter. The antimicrobial assays showed that licorice extracts can inhibit the growth of the evaluated bacteria. The IC_{50} values (mg/ml) corresponding to the inhibition of *E. coli* (ATCC 25299) and *S. aureus* (ATCC 25923) are given in Table 4.

In general, results of antimicrobial activity showed that *S. aureus* ATCC 25923 (Gram-positive) was more resistant (higher IC_{50} values) than *E. coli* ATCC 25299 (Gram-negative) to the antimicrobial effect of all extracts tested. These results are in agreement with the data presented by Chandra and Gunasekaran (2017) in their study of licorice extracts obtained by solid-liquid extraction with chloroform solvent. A greater susceptibility of *E. coli* than *S. aureus* to other antimicrobial materials was also reported by other authors (García-Ruiz et al., 2015). The structures of cell envelope (cytoplasmic membrane and cell wall component), which differ between Gram-positive and Gram-negative bacteria, may influence these observed results. Antimicrobial agents commonly contact the cell envelope first and thus, the structural differences produce a key role in the antimicrobial susceptibility. In particular, the extracts obtained using ethanol (higher contents of phenolic compounds, runs 2 to 6), exhibit lower and even no antimicrobial activity in comparison with the extract produced with pure CO_2 (run 1). On the other hand, fractions obtained in the first separator (lower content of phenolic compounds) exhibited the best antimicrobial activity for both bacteria. These findings indicate that licorice antimicrobial compounds are soluble in CO_2 at pressures higher than 15 MPa and can be concentrated by cascade decompression of high pressure SFE. It

327 suggests that antimicrobial activity of licorice extracts seems to be dependent on the type of
328 phenolic structure rather than the total content of phenolic compounds.

329 **3.4. HPLC analysis**

330 The major bioactive constituents of licorice are glycyrrhizin and several flavonoids, such as
331 liquiritin, isoliquiritin and their aglycones. These compounds are supposed as the active
332 principles responsible for its pharmacological efficacy (Zhang and Ye, 2009). The bioactive
333 compounds identified in this work by HPLC analysis were five compounds: liquiritin (RT:
334 12.80 min), liquiritigenin (RT: 35.06 min), glycyrrhizic acid ammonium salt (glycyrrhizin)
335 (RT: 48.21), isoliquiritigenin (RT: 49.73 min) and glabridin (RT: 69.15 min). The results of
336 the HPLC analysis are presented in Table 5. In the case of experiments accomplished with
337 on-line fractionation (runs 7 to 10), the compound concentrations obtained in the total extract
338 (S1+S2 fractions) are also reported in Table 5.

339 The most abundant compound found in all the extracts obtained in this work is glabridin, a
340 compounds well-characterized in the literature due to several important biological activities
341 such as antioxidant, anti-inflammatory and anti-atherogenic (Asl and Hosseinzadeh, 2008;
342 Simmler et al., 2013). Extract obtained using pure CO_2 in run 1 contains 49.50 ± 0.14 mg
343 glabridin per g extract. Without cosolvent, the concentration of glabridin in the extract (mg
344 glabridin / g extract) increases with pressure, as is represented in Figure 6 for runs 1, 7 and 8
345 (313.15 K, 70 g/min of CO_2 without cosolvent). Nevertheless, glabridin recovery (mg
346 glabridin / g root) seem to attain a maximum around 30 MPa. Significantly higher glabridin
347 concentration and recovery were obtained when ethanol is used as cosolvent. Moreover, an
348 increase of ethanol cosolvent produces a decrease in glabridin concentration (runs 3 to 6 in
349 Tables 5) probably due to the simultaneous extraction of other compounds (and according
350 with the increase of extraction yield observed). In relation with the samples obtained by

supercritical fractionation, temperature seems to affect significantly glabridin fractionation since fractions obtained in S1 at 313.15 K present lowest concentration of glabridin than the corresponding S2 fraction (runs 7 and 8) while the opposite behavior was found at 333.15 K (runs 9 and 10).

Another metabolite identified in all samples was liquiritin, which is a flavone observed in the sweetening agent licorice and has been associate with anti-inflammatory effects (Gao et al., 2017). Liquiritin content slightly increase with the percentage of ethanol and considerably lower amounts of this compound were quantified in all samples (0.2-1.2 mg/g) in comparison with glabridin (26-198 mg/g).

Among phenolic compounds in licorice are typically isoliquiritigenin and liquiritigenin (Kondo et al., 2007). These were identified and quantified in the extracts of runs 2, 3, 4, 5 and 6, observing an increase of their concentration with the increase of the percentage of ethanol cosolvent. Thus, these compounds could not be quantified in the supercritical fractionation assays (runs 7 to 10) since no ethanol was used as cosolvent and despite the higher extraction pressures applied. On the contrary, the content of triterpenoid glycyrrhizin was found to decrease with increasing ethanol cosolvent and thus, it was not possible to quantify this compound in runs 4, 5 and 6 while it was identified and quantified in all samples obtained in runs 7 to 10. Glycyrrhizin is an important ingredient in various medicines, such as antimicrobial, anti-ulcer, anti-hepatotoxic and antivirus formulas (Cinatl et al., 2003; Dehpour et al., 1995). Yet, plant extracts use to be much more effective than isolated compounds, as highlighted Cheel et al. (2010) for the case of licorice aqueous extracts.

Figure 6 shows the effect of pressure on glycyrrhizin concentration in the extract and recovery per gram of root extracted. The figure refers to runs 1, 7 and 8 (313.15 K, 70 g/min of CO_2 without cosolvent). Glycyrrhizin concentration show a slight decrease with increasing

pressure, but the recovery attains a maximum close to 30 MPa, similarly to glabridin. Yet, this maximum recovery is lower than 1.5 mg /g root, considering the content of glycyrrhizin in licorice root reported by Kim et al. (2004) (138 mg glycyrrhizin / g root). Nevertheless, Kim et al. (2004) could not extract glycyrrhizin with pure supercritical CO_2 even applying pressures of 50 MPa. Hedayati and Ghoreishi (2015) reported a maximum glycyrrhizin recovery of 54% (74 mg glycyrrhizin / g root) when using methanol: water as CO_2 cosolvent, which is a value significantly higher than the maximum recovery obtained in this work using ethanol cosolvent (4.6 mg glycyrrhizin/g root).

Concerning the relation between the compounds detected and the biological activities observed (antioxidant and antibacterial) in supercritical extracts, it can be stated that the content of liquiritigenin and isoliquiritigenin do not affect the antibacterial activity, since these compounds were not detected in the most active extracts (S1 fractions and extract of run 1). Furthermore, the content of glabridin seems not to affect the antibacterial activity of these most active extracts (Figure 7). On the contrary, regarding the antioxidant activity a dependence of the antioxidant capacity on glabridin content is observed, as depicted in Figure 8 for all licorice supercritical extracts obtained in this work.

4. Conclusions

Supercritical licorice roots extracts were obtained using different extraction conditions to investigate the viability of producing antioxidant and/or antimicrobial fractions. Two strategic approaches were investigated: the effect of using ethanol as a polar cosolvent and the effect of supercritical fractionation using a two-cell cascade decompression system.

In general, licorice supercritical extracts obtained in this work present considerably higher antioxidant activity in comparison with extracts produced using liquid solvents (methanol, chloroform, ethanol, water) as reported in the literature. As expected, the higher contents of

phenolic compounds were obtained in the extracts produced with supercritical CO_2 and ethanol cosolvent, which in turn exhibit the higher antioxidant activity. The extraction yield and total phenolic compounds content varies linearly with the amount of ethanol used. Nevertheless, this linear correlation was not observed in regards the antioxidant activity. Furthermore, the increase of the content of glabridin favors the antioxidant activity of the extract.

On the other hand, the extracts produced with supercritical CO_2 and ethanol cosolvent show low or even no antimicrobial activity against *E. coli* ATCC 25299 and *S. aureus* ATCC 25923. In this respect, high pressure extraction (30-40 MPa) using pure CO_2 and on-line fractionation resulted in a first fraction (at 15 MPa) with a better antimicrobial effect although with lower content of phenolic compounds.

The study concludes and highlights the capability of supercritical fractionation applied to licorice roots to selective separate substances and to obtain antimicrobial and antioxidant fractions.

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582 **Table 1.** Experimental conditions and extraction yield (mass of extract/mass of raw material)
583 obtained in the supercritical extraction of licorice roots. Extraction time: 3 h. (PS1) pressure
584 in first separator, pressure in second separator was maintained at $SCCO_2$ recirculation
585 pressure. Run 1 – 8 at 313.15 K and 9 – 10 at 333.15 K.

Run	Pressure (MPa)	CO_2 flow (g/min)	Ethanol (% mass)	P S1 (MPa)	Extraction yield (%)
1	15	70	0	5	0.66
2	15	70	10	5	2.19
3	15	50	5	5	1.64
4	15	50	10	5	2.01
5	15	50	15	5	2.48
6	15	50	20	5	3.07
7	30	70	0	15	1.76*
8	40	70	0	15	0.56*
9	30	70	0	15	0.74*
10	40	70	0	10	0.89*

586 *Extraction yield expressed as the sum of the mass collected from both separator vessels
587 (S1and S2).

588 **Table 2.** Extraction yield (mass of extract/mass of raw material) obtained in the S1 and S2
589 fractions of licorice roots extracts. Extraction time: 3 h. Pressure in first separator was 15
590 MPa and pressure in second separator was maintained at $SCCO_2$ recirculation pressure.

Run	Extraction yield in separators (%mass)	
	S1	**S2**
7	0.24	1.52
8	0.06	0.50
9	0.09	0.65
10	0.27	0.62

591

Table 3. Mean values and standard deviations of total phenolic compounds (TPC) and antioxidant capacity (ABTS and DPPH assay) of licorice root supercritical extracts. S1: first separator fraction, S2: second separator fraction.

Run	TPC	ABTS		DPPH	
	(mg GAE/g extract)	IC_{50} (µg/mL)	TEAC (µmol Trolox/g extract)	IC_{50} (µg/mL)	TEAC (µmol Trolox/g extract)
1	76.2 ± 1.5	42.1 ± 3.2	102.2 ± 7.8	126.0 ± 0.5	112.5 ± 0.4
2	141.2 ± 1.1	28.4 ± 3.6	147.4 ± 13.7	32.4 ± 2.5	442.1 ± 34.2
3	153.0 ± 1.1	13.7 ± 0.1	312.5 ± 0.7	42.6 ± 0.9	331.4 ± 8.6
4	163.0 ± 1.3	13.0 ± 0.8	329.6 ± 21.1	37.8 ± 04	418.7 ± 4.4
5	174.9 ± 1.9	12.0 ± 0.1	358.6 ± 3.9	32.3 ± 0.4	440.8 ± 5.2
6	180.1 ± 0.9	7.7 ± 0.6	555.7 ± 45.7	18.6 ± 0.4	759.6 ± 16.2
7-S1	69.05 ± 0.9	105.3 ± 1.7	40.7 ± 0.7	248.1 ± 4.1	57.1 ± 0.9
7-S2	159.3 ± 1.2	33.3 ± 1.0	128.6 ± 4.0	42.8 ± 2.5	330.6 ± 9.0
8-S1	48.5 ± 3.6	44.7 ± 0.1	95.8 ± 0.3	185.8 ± 0.9	76.0 ± 0.4
8-S2	128.3 ± 3.4	11.84 ± 0.1	361.7 ± 3.0	21.9 ± 0.1	647.9 ± 3.1
9-S1	62.5 ± 2.5	38.8 ± 2.8	110.6 ± 7.9	149.5 ± 0.8	94.4 ± 0.5
9-S2	140.5 ± 7.7	10.3 ± 0.2	416.0 ± 8.9	54.4 ± 3.6	259.9 ± 17.3
10-S1	95.2 ± 8.7	15.7 ± 0.1	272.5 ± 0.2	74.0 ± 0.4	190.9 ± 0.6
10-S2	138.0 ± 8.9	7.7 ± 0.1	554.0 ± 6.6	59.2 ± 0.2	238.7 ± 0.7

DPPH: 2,2-Diphenyl-1-pycrilhydrazyl
ABTS: 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
GAE: Gallic acid equivalent
IC_{50}: Concentration of extract necessary to inhibit the initial concentration of radical by 50%
TEAC: Trolox equivalent antioxidant capacity

Table 4. Antimicrobial activity of licorice root supercritical extracts.

Sample	*E. coli* IC_{50} (mg/ml)	*S. aureus* IC_{50} (mg/ml)
1	1.17	1.41
2	1.50	2.26
3	1.60	1.60
4	2.19	--
5	--	--
6	--	--
7-S1	0.76	1.49
7-S2	2.16	2.20
8-S1	1.86	2.00
8-S2	1.62	2.21
9-S1	1.30	1.49
9-S2	1.61	1.77
10-S1	1.02	1.36
10-S2	1.21	1.50
Chloramphenicol	0.08	0.09

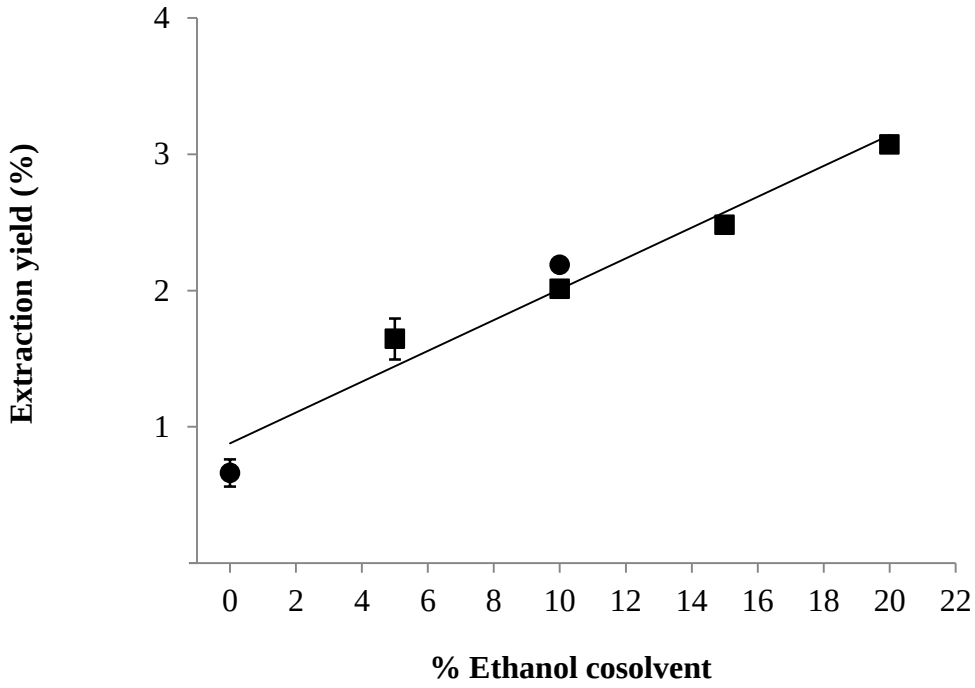
IC_{50}: Concentration of extract required to obtain 50% inhibition of bacterial growth

602 **Table 5.** Concentration of bioactive compounds of supercritical extracts of licorice under
603 different conditions.

Run	Liquiritin (mg/g)	Liquiritigenin (mg/g)	Glycyrrhizin (mg/g)	Isoliquiritigenin (mg/g)	Glabridin (mg/g)
1	0.40± 0.01	ND	0.80 ± 0.01	ND	49.50 ± 0.14
2	0.60± 0.01	0.80 ± 0.01	2.10 ± 1.27	1.40 ± 0.85	168.60 ± 1.70
3	0.80± 0.01	LLQ	1.20 ± 0.01	0.40 ± 0.01	198.10 ± 0.71
4	0.80± 0.01	1.60 ± 0.01	UDL	2.40 ± 0.01	192.10 ± 0.99
5	0.80± 0.01	1.80 ± 0.01	UDL	0.90 ± 0.14	151.50 ± 0.71
6	1.20± 0.01	2.60 ± 0.01	UDL	2.30 ± 1.27	147.40 ± 0.28
Concentration in samples S1 and S2					
7-S1	UDL	ND	0.20 ± 0.01	ND	26.60 ± 1.41
7-S2	UDL	ND	0.70 ± 0.14	ND	140.80 ± 7.35
8-S1	UDL	ND	0.40 ± 0.01	ND	47.40 ± 7.07
8-S2	UDL	UDL	0.60 ± 0.01	UDL	143.50 ± 6.65
9-S1	0.20 ± 0.01	ND	0.70 ± 0.14	UDL	140.50 ± 0.99
9-S2	0.20 ± 0.01	UDL	0.60 ± 0.01	ND	74.40 ± 1.98
10-S1	UDL	UDL	0.40 ± 0.01	ND	118.30 ± 0.14
10-S2	0.80± 0.01	ND	0.80 ± 0.01	UDL	113.80 ± 0.01
Concentration in total extract (S1 + S2 samples)					
7	UDL	ND	0.732± 0.14	ND	125.23 ± 6.54
8	UDL	UDL	0.579± 0.01	UDL	133.20 ± 6.70
9	0.20 ± 0.01	UDL	0.612± 0.03	UDL	82.44 ± 1.86
10	0.56 ± 0.01	UDL	0.679± 0.01	UDL	115.17 ± 0.04

604 ND: no detected

605 UDL: under detection limit



606

607 **Figure 1.** Extraction yield obtained in the supercritical fluid extractions of licorice root as a
608 function of the amount of cosolvent (%mass). (●, ■) 15 MPa. Squares and circles
609 correspond, respectively, to 50 and 70 g/min CO_2. The standard deviations obtained in
610 duplicate experiments carried out in run 1 (pure CO_2) and run 3 (5% ethanol) are depicted in
611 the figure.

612

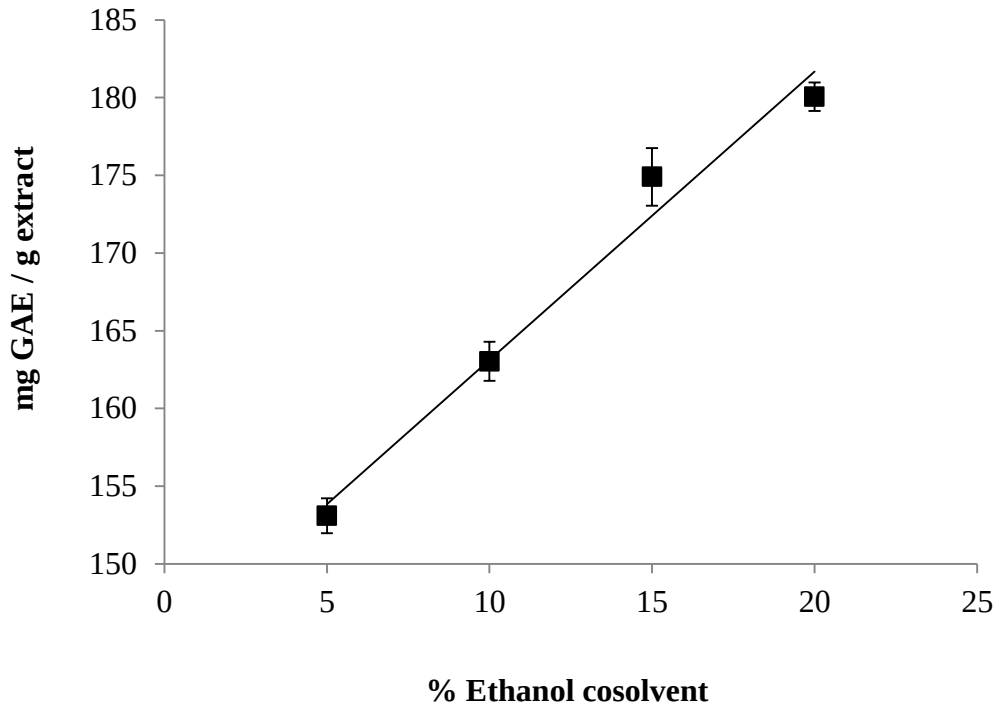


Figure 2. Supercritical fluids extractions of licorice roots at 15 MPa, 313.15 K and 50 g/min CO_2 flow: Effect of ethanol cosolvent addition in the extraction of phenolic compounds expressed as mg of gallic acid equivalent (GAE)/ g of extract.

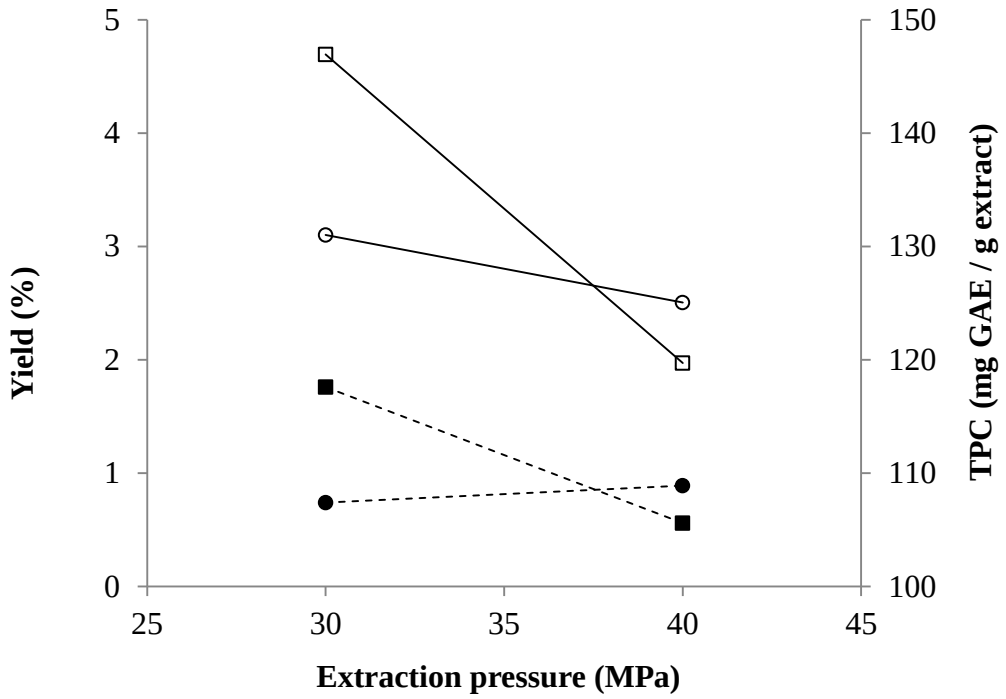
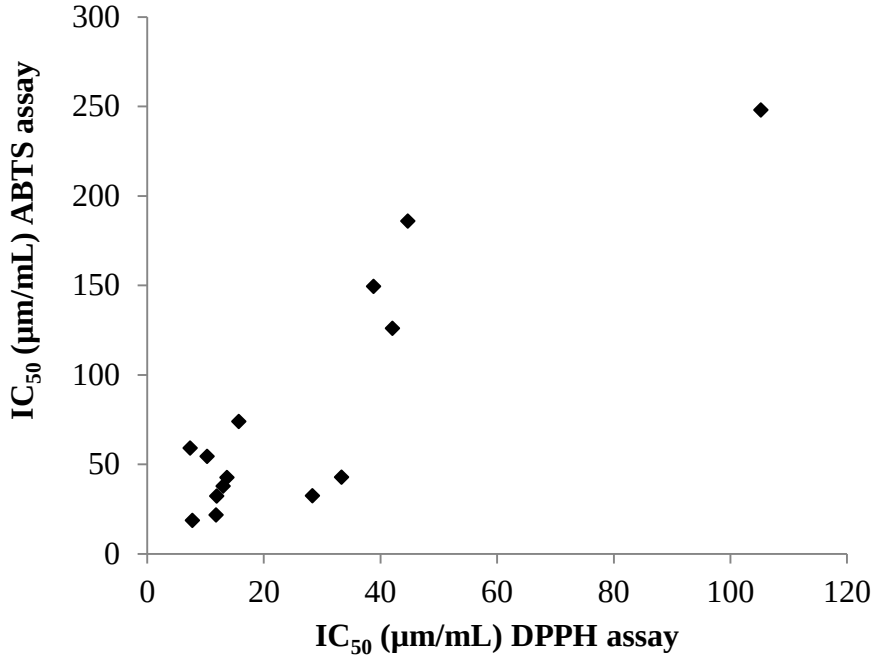
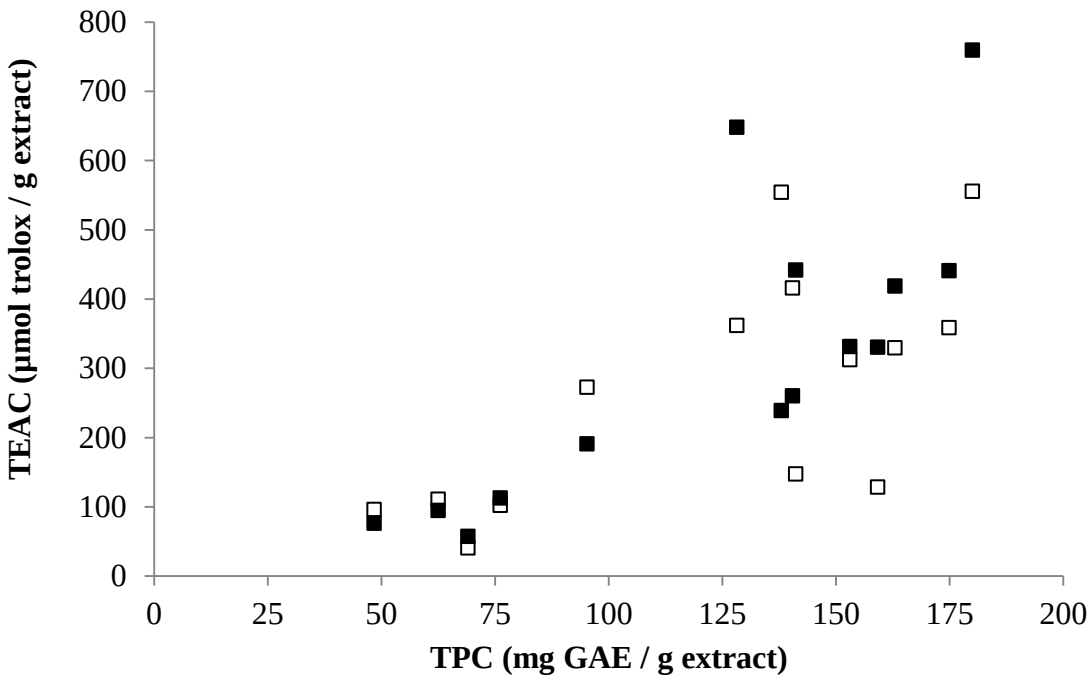


Figure 3. Total yield and total phenolic compounds (TPC) extracted (S1+S2 samples) obtained in runs 7 to 10. (■, □) 313.15 K; (●, ○) 333.15 K. Full symbols represent extraction yield and empty symbols represent TPC content.



621

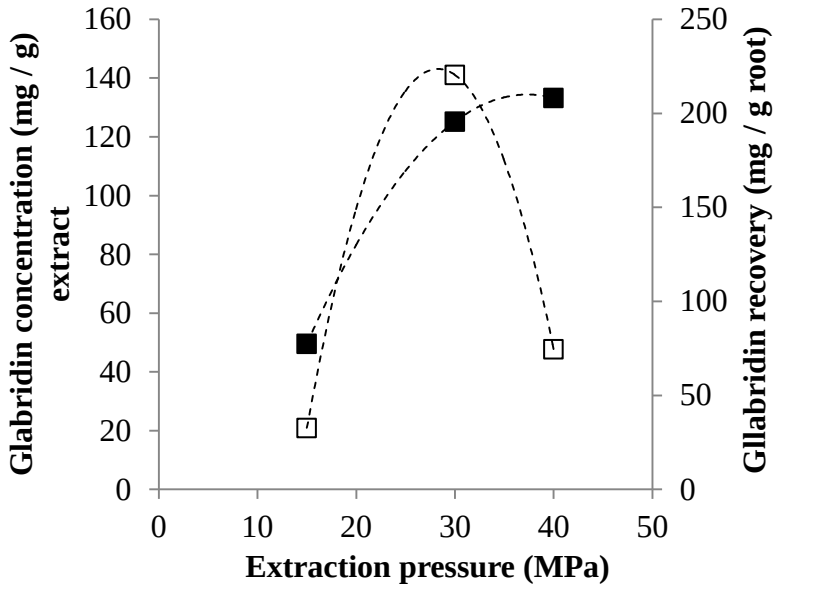
622 **Figure 4.** Analysis of the relation between the IC_{50} (Concentration of extract necessary to
623 inhibit the initial concentration of radical by 50%) values of licorice supercritical extracts
624 calculated with the ABTS and DPPH assay. (ABTS: 2,2´-azino-bis (3-ethylbenzothiazoline-
625 6-sulfonic acid) diammonium salt; DPPH: 2,2-Diphenyl-1-pycrilhydrazyl).



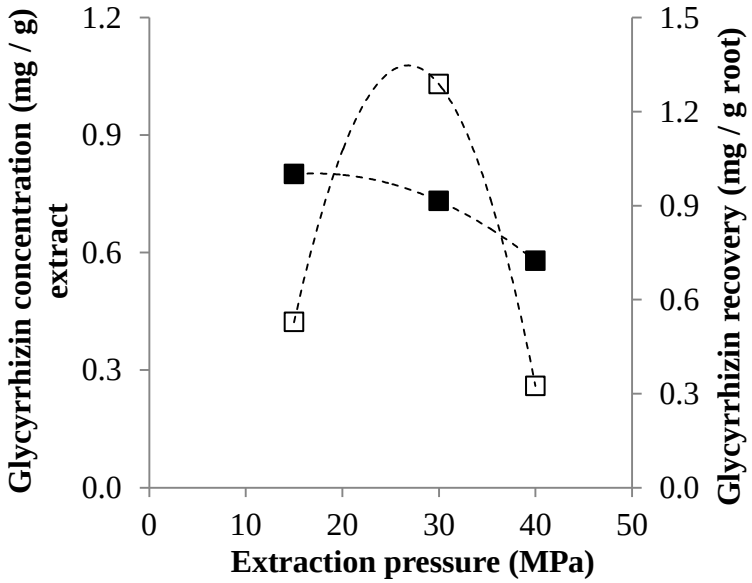
626

627 **Figure 5.** Trolox equivalent antioxidant capacity (TEAC) vs. total phenolic compounds
628 (TPC) values of licorice root supercritical extracts: (□) ABTS and (■) DPPH assays.
629 (ABTS: 2.2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH:
630 2,2-Diphenyl-1-pycrilhydrazyl).

631

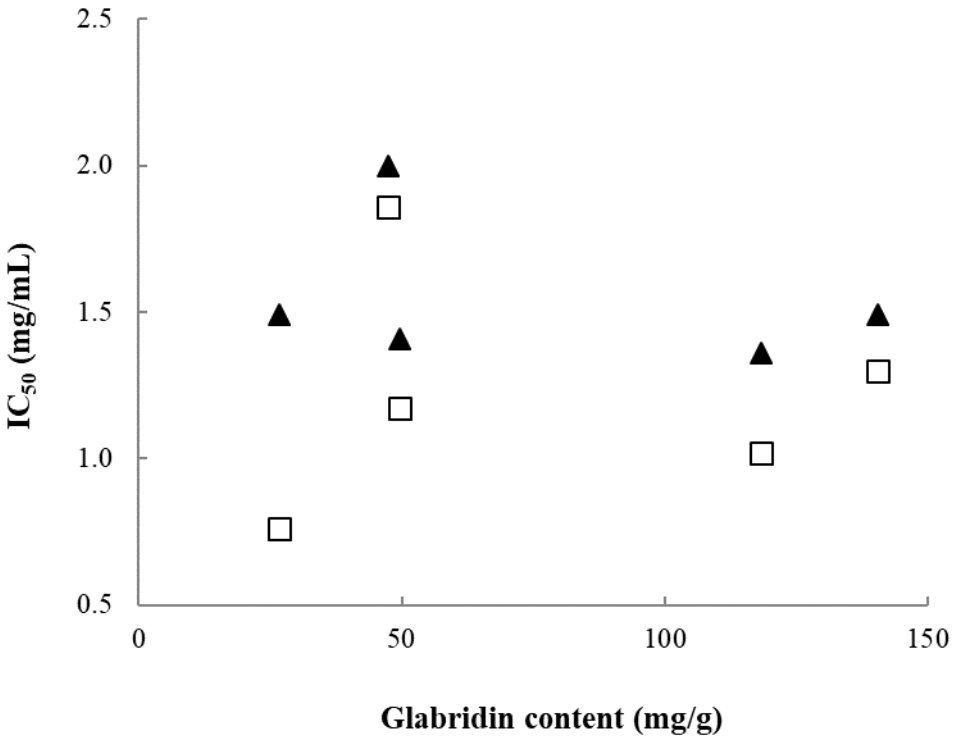


(a)



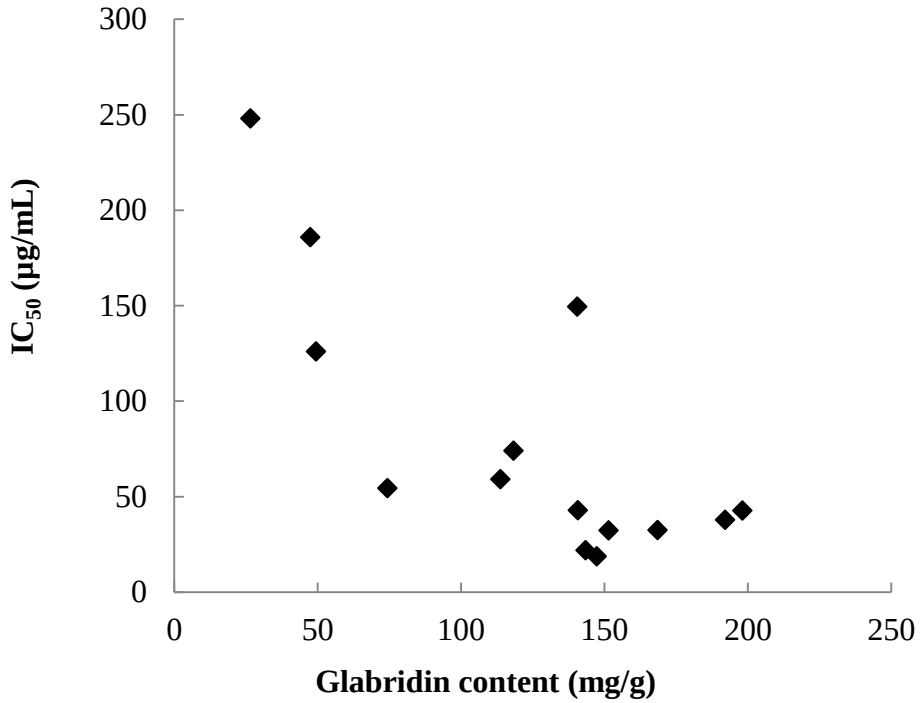
(b)

632 **Figure 6.** Effect of pressure ((313.15 K, 70 g/min of CO_2 without cosolvent)) on the
633 concentration and recovery of (a) glabridin and (b) glycyrrhizin: (■) concentration; (□)
634 recovery.



635

636 **Figure 7.** Antibacterial activity of the most active samples (S1 fractions and extract of run 1):
637 IC_{50} (Concentration of extract required to obtain 50% inhibition of bacterial growth) values
638 obtained for (□) *E. coli* and (▲) *S. aureus* as a function of the glabridin content**.**



639

640 **Figure 8.** Antioxidant activity: IC_{50} (Concentration of extract necessary to inhibit the initial
641 concentration of radical by 50%) value obtained with the DPPH assay as a function of the
642 glabridin content.