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Abstract

Phenolic compounds, naturally present in plants, are particularly relevant as nutraceutics for their antioxidant activities. Here, choline chloride-based natural deep eutectic solvents (NaDESs) were used for efficient extraction of total phenolic compounds (TPC) from leaves, pods and roots of *Rhamnus alaternus* plant. In each extract, the polyphenols content and their antioxidant activities were evaluated. More, their antibacterial activity was carried out against Gram-positive and Gram-negative bacteria. The investigated extracts presented antioxidant and antimicrobial activities. The leaves, pods and roots extracts obtained using the choline chloride-glycerol (ChCl-Gly) mixture exhibited a high value of TPC comprised within 437.7 and 317.0 mg GAE/100 g d.w. and showed the best antioxidant properties with ABTS radical scavenging capacity (IC_{50}: from 44.55 to 53.56 µg/mL), DPPH assay (IC_{50}: from 26.36 to 118.23 µg/mL), reducing activity (EC_{50}: from 77.53 to 85.05 µg/mL) and iron chelation activity (EC_{50}: from 91.73 to 108.7 µg/mL). In contrast, choline chloride-urea (ChCl-Ur) extracts showed a low antioxidant capacity. Lastly, the best bioactive extracts were characterized using HPLC-MS/MS to determine their bioactive compounds. The present study suggests that the DES-based method developed was selective, efficient and sustainable for extraction of polyphenols.

Keywords: *Rhamnus alaternus*, natural deep eutectic solvents, green extraction, sustainable process, bioactive compounds, antioxidant, antimicrobial activity.

1. Introduction

Recently, the green extraction techniques have become of utmost importance in research related to biomolecules production. Therefore, food, cosmetic and pharmaceutical industries operated a transition from conventional extraction to the green extraction processes, using alternative cost-effective and earth-friendly solvents (Nagarajan et al., 2019; Palos-Hernández et al., 2022). Considering environmental protection and collective awareness, consumers are more and more demanding for highly valued bio-based products (Bugge et al., 2016; Catone et al., 2021). Plants are an important source of various bioactive molecules notably polyphenolic compounds (Si et al., 2016), known for their pharmacological properties including antioxidant, antibacterial, anti-inflammatory and anticancer effects (Zhang et al., 2011).

Rhamnus alaternus, popularly called "Imlilesse", belongs to the *Rhamnaceae* family and the *Reynosia* Genus. Widely distributed around the Mediterranean region, this plant has been widely consumed as infusion for thousands of years, notably for treating dermatological complications and for its hypotensive, laxative, purgative effect (Ben Ammar et al., 2007). Recent investigations have demonstrated the health benefits and high functional activities of bioactive compounds present in *R. alaternus* including antioxidant, antibacterial, antihyperlipidemic, antigenotoxic and antimutagenic activities (Bhouri et al., 2011; Tacherfiout et al., 2018). The main bioactive substances identified in various parts of *R. alaternus* are polyphenols, flavonoids, anthraquinones, coumarin and anthocyanins (Longo et al., 2005; Moussi et al., 2015). Polyphenols are the most active constituents in secondary metabolites of plants with high biological activities including antioxidant capacity (Quideau et al., 2011).

To date, the extraction of bioactive compounds from *R. alaternus* is largely carried out using conventional solid-liquid extraction methods involving organic solvents such as ethanol, acetone and ethyl acetate (Boussahel et al., 2015). However, the consumption of such solvents may contribute to environmental pollution due to their toxicity, volatility and flammability. In this regard, at the beginning of the 2000's, Natural Deep Eutectic Solvents (**NaDESs**) emerged as alternative for green chemistry. NaDESs were simply prepared by mixing hydrogen bond donors (**HBD**) and hydrogen bond acceptors (**HBA**) (Trivedi et al., 2016). Characterized by their low toxicity, biodegradability, high solubility, biocompatibility, low vapor pressure and being eco-friendly, NaDESs are appropriate for substituting conventional solvents (Jeong et al., 2015). Furthermore, NaDESs were successfully applied for the extraction of bioactive compounds such as polyphenols, flavonoids and saponins from natural resources (Dai et al., 2013; Sillero et al., 2021).

To the author's knowledge, the extraction of bioactive compounds from *R. alaternus* has never been explored using Natural Deep Eutectic Solvents. Therefore, this study aimed to evaluate the efficiency of three NaDESs, in order to develop green and sustainable process for the extraction of high-value phenolic compounds, from various parts of *R. alaternus* (*i.e.,* leaves, pods and roots) and to investigate the bioactive properties of collected extracts. To reach this aim, three NaDESs were investigated and constituted of choline chloride as HBA and of glycerol, ethylene glycol or urea, as HBD. Compared to conventional ethanol extraction, each extract was evaluated for its total polyphenol content (TPC) and its related bioactivities, notably its antioxidant and antimicrobial activities. Finally, the most promising NaDES extract in terms of TPC was characterized with LC-MS/MS analysis in order to identify its major constituents.

2. Material and methods

2.1. Plant materials, reagents, equipment and consumable

R. alaternus was collected from Beni Ourthilane region - Algeria (36°19'57"N,5°5'19"E) in July 2019. A voucher specimen (Ra-19-001) was kept in the laboratory of pharmacognosy, pharmacy department, University Salah Boubnider - Constantine 3, Algeria. Dried at room temperature under shadow, the leaves, pods and roots of *R. alaternus* were ground using a cutting mill (SM100 RETSCH, Retsch, Haan, Germany) to obtain a fine powder, stored in controlled atmosphere until use.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl) and Trolox® were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium phosphate dibasic dihydrate and sodium phosphate monobasic dihydrate were provided from VWR Chemicals (Leuven, Belgium). The ascorbic acid, potassium persulfate, ferrozine (3-(2-pycril)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt), iron (II) chloride, EDTA, Folin-Ciocalteu's phenol reagent (2N), gallic acid, potassium ferricyanide (III), iron (III) chloride, iron (III) ferrocyanide, trichloroacetic acid, sodium carbonate, ethylene glycol, choline chloride, urea and glycerol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and ethanol (Sigma-Aldrich) were of analytical grade. Water was purified by Arum® 611 water purification system (Sartorius, Göttingen, Germany). For the homogeneity of the unities, all concentrations in the manuscript were presented in mass/volume. CAS number and purity of each chemical used are given in the Supplementary data (Table S1). For antioxidant tests, all spectrophotometry experiments were carried out using Thermo Scientific Multiskan™ spectrophotometer and 96 wells-microplates (Thermo Scientific™, Roskild, Denmark).

2.2. Solid-liquid extraction

2.2.1. Natural deep eutectic solvents preparation

Three natural deep eutectic solvents (NaDESs) were selected for the extraction of phenolic compounds from *R. alaternus's* leaves, pods and roots: choline chloride-glycerol (**ChCl-Gly**), choline-chloride-ethylene glycol (**ChCl-EG**) and choline chloride-urea (**ChCl-Ur**). These NaDESs were prepared as previously described (Tang & Row, 2020; Wan Mahmood et al., 2019). Briefly, the hydrogen bond acceptor (**HBA**) *i.e.,* choline chloride (**ChCl**) was mixed either with the hydrogen bond donor (**HBD**) *i.e.,* ethylene glycol (**EG**), glycerol (**Gly**) or with urea (**Ur**) at a molar ratio 1:2. These solvents were prepared at 80 °C under stirring using a magnetic agitator (2mag MIX 15; Merck, Darmstadt, Germany) until the formation of a transparent and homogenous liquid (3 - 4 h).

The prepared NaDESs were mixed with water (8:2 v/v) to reduce their viscosity and change their polarity in order to increase the extraction efficiency by improving the solubility of TPC in the corresponding solvents (Chanioti & Tzia, 2018). Indeed, the high viscosity of NaDESs used for extraction process can reduce the mass transport efficiency (Dai et al., 2013; Wei et al., 2015). Yet, the water content mixed to NaDESs must not be too high since it could break the hydrogen bonds within the NaDESs constituents (Vilková et al., 2020).

2.2.2. Extraction process

The phenolic compounds from various part of *R. alaternus* (*i.e.,* leaves, pods and roots) were separated by solid-liquid extraction (**SLE**). Briefly, 1 g of powder obtained from each part of the plant was dissolved in 10 mL of solvent, constituted of one NaDES mixed with water in a 8:2

(v/v) ratio. Each SLE experiment was led for 24 h at 30 °C using a thermostated waterbath, and under stirring using a multipoint magnetic stirrer (2mag MIX 15, Merck, Darmstadt, Germany) set at 350 rpm. After extraction, each sample was centrifuged (Hettich universal 320, Sérézin du Rhône, France; 1008 g, 10 min) to remove any solid impurities. The collected supernatants were then filtered through 0.45 µm filter (Millex® Syringe Filter, PTFE, Merck KGaA, Darmstadt, Germany) in order to eliminate any remaining particles before further analysis. Each extract was named by its part of the plant (leaves, pods or roots) followed by the nature of the solvent used for SLE extraction (ChCl-Gly, ChCl-EG, ChCl-Ur, or Ethanol). After extraction, each extract was diluted by successive tenfold dilution in ultrapure water until the final dilution factor of 1:1000 (v/v) and was used as initial extract for further analysis (polyphenols identification, antioxidant and antimicrobial activity, and HPLC/MS/MS analysis).

2.3. Determination of the total phenolic content

For all investigated extracts, the total phenolic content (**TPC**) was determined by Folin-Ciocalteu method adapted to a microplate assay (Singleton & Rossi, 1965). Then, 220 µL of ultrapure water, 5 µL of each diluted extract, 15 µL of Folin-Ciocalteu reagent (10 % v/v) and 60 µL of aqueous sodium carbonate (7.5 % w/v) were mixed in each well. Finally, the microplate was stored in the dark for 60 min at ambient temperature, and analysed at 765 nm by spectrophotometry. The absorbance of each sample was measured five times.

The total phenolic compound was determined using a calibration curve performed with gallic acid as standard, in a concentration range varying from 0 to 35 µg/mL (R^2 = 0.998). The results were expressed as mg gallic acid equivalent (mg **GAE**) per 100 grams of plant powder (d.w.).

2.4. Determination of antioxidant capacity of the extracts

2.4.1. Radical scavenging activity

ABTS radical scavenging activity. To evaluate the ability of various extracts from *R. alaternus* to scavenge the $ABTS^{\circ+}$ radical, the method described by Re et al. (1999) was followed. Initially, 16.55 mg potassium persulfate was added to 25 mL of the ABTS solution (7 mM) prepared in ultrapure water, allowing the mixture to stand for 12 to 16 h in the dark, at ambient temperature. The concentrated $ABTS^{\circ+}$ solution was then diluted with 4 mM phosphate buffer pH 7.4 to reach an absorbance value of 0.7 ± 0.02 at 734 nm. Then, 150 µL of $ABTS^{\circ+}$ diluted solution was added to 150 µL of extracts, previously prepared at various concentrations (0 - 120 µg/mL) in the phosphate buffer. After 10 min of incubation, the absorbance was measured at 734 nm. Each concentration was repeated five times. The Trolox® was investigated as reference from 0 to 25 µg/mL in the 4 mM phosphate buffer. From the absorbance measurement, the radical scavenging activity (%) was calculated as follows (**Equation 1**) in order to plot a calibration curve (Radical scavenging activity (%) vs. Trolox® concentration):

$$Radical\ scavenging\ activity\ (\%) = \frac{A_{734\ (control)} - A_{734\ (sample)}}{A_{734\ (control)}} \times 100 \qquad (1)$$

Where $A_{734\ (control)}$ is the absorbance of the control (initial $ABTS^{\circ+}$ radical solution; the sample volume was replaced by ultrapure water), and $A_{734\ (sample)}$ is the absorbance of the remaining radical in the presence of biological extracts or Trolox®.

DPPH radical scavenging activity. The ability of various extracts from *R. alaternus* to scavenge the 2,2-diphenyl-1 picrylhydrazyl free radical was evaluated by the DPPH *in vitro* assay similarly to Brand-Williams et al. (1995), with slight modifications. After extraction, each extract was first diluted in ultrapure water. Briefly, 150 µL of each diluted extract after solid-liquid extraction

(final investigated concentration: 0 to 100 µg/mL) was added to 150 µL of the methanolic DPPH° solution (40 µg/mL) daily prepared. Each investigated concentration was repeated 5 times. The mixture was homogenized vigorously using a micropipette for 1 min and stored in the dark at ambient temperature for 30 min. Then, the sample absorbance was read at 517 nm by spectrophotometry. Ascorbic acid (150 µL) was investigated from 5 to 100 µg/mL as standard for plotting the calibration curve. The radical scavenging activity (%) of each extract or standard solution was calculated as follows (**Equation 2**):

$$Radical\ scavenging\ activity\ (\%) = \frac{A_{517\ (control)} - A_{517\ (sample)}}{A_{517\ (control)}} \times 100 \qquad (2)$$

Where $A_{517\ (control)}$ was the absorbance of the blank DPPH° reagent in the presence of methanol and $A_{517\ (sample)}$ the absorbance of the DPPH° reagent solution mixed either with the ascorbic acid or the plant extracts.

The Inhibitory Concentration 50 (**IC_{50}**) – the concentration that scavenges 50 % of DPPH° free radicals (µg/mL) - was calculated for all *R. alaternus* extracts and compared with the one of ascorbic acid.

2.4.2. Reducing power

The reducing capacity of each biological extract was investigated by the method described by Oyaizu. (1986), with slight modifications. Briefly, 70 µL of various samples extracted and diluted in microplate from (0 to 250 µg/mL) were prepared in phosphate buffer (0.2 M, pH 6.6) and then added with 35 µL of potassium ferricyanide (K3FeCN6; 1 % w/v). After 20 min of incubation at 50 °C, 135 µL of ultrapure water was added in each well, followed by 33 µL of trichloroacetic acid (10 % w/v) and 27 µL of iron (III) chloride ($FeCl_3$; 0.1 % w/v). After 10 min of incubation at 25 °C, the absorbance was measured at 700 nm. Ascorbic acid (70 µL) was

197 investigated as positive control from 5 to 70 µg/mL. Each concentration was repeated 5 times.

198 The reducing capacity of the various extracts was evaluated as follows:

199 $$Reducing\ capacity\ (\%) = 100 - \left(\frac{A_{700\ (control)} - A_{700\ (sample)}}{A_{700\ (conrol)}} \times 100\right) \quad (3)$$

200 Where: $A_{700\ (control)}$ is the absorbance of a 66 µM-Prussian blue solution in the absence of ascorbic

201 acid, which was experimentally determined at 0.83 absorbance value at 700 nm,

202 And $A_{700\ (sample)}$ is the sample or ascorbic acid absorbance at 700 nm.

203 **2.4.3. Iron (II) chelating activity**

204 The ability of various extracts from *R. alaternus* to chelate Fe^{2+} was evaluated using the method

205 described by Canabady-Rochelle et al. (2015) adapted from Decker & Welch. (1990). Briefly, 7.5

206 µL of ferrous chloride solution ($FeCl_2$, 253.4 µg/mL) was mixed with 277.5 µL of extract diluted

207 in order to investigate various calculated concentrations (from 0 to 300 µg/mL) in ultrapure

208 water. After 3 min of incubation at 25 °C, 15 µL of ferrozine solution (5 mM) was added in each

209 well. The microplate was shaken using the spectrophotometer agitator, and then incubated for

210 10 min at 25 °C before absorbance measurement at 562 nm. EDTA was investigated as

211 reference from 0 to 10 µg/mL. Each sample and standard were repeated (n = 5). The iron-

212 chelating capacity (%) was calculated as follows (**Equation 4**):

$$Iron - chelation\ capacity\ (\%) = \frac{A_{562\ (control)} - A_{562\ (sample)}}{A_{562\ (control)}} \times 100 \quad (4)$$

213 Where the blank $A_{562\ (control)}$ and $A_{562\ (sample)}$ correspond to the absorbance of ultrapure water in

214 the absence of sample and to the absorbance of sample, both mixed with all reagents.

215

2.5. Antimicrobial activity of the plant extracts

2.5.1. Antimicrobial test

To evaluate the antimicrobial activity of the samples and NaDESs, extracts of *R. alaternus* and solvents alone were tested against two Gram+ bacteria (*i.e., Staphylococcus aureus* ATCC 29213 (**Sa**) and *Enterococcus faecalis* ATCC 29212 (**Ef**)) and three Gram- bacteria strains (*i.e., Pseudomonas aeruginosa* ATCC 27853 (**Pa**), *Escherichia coli* ATCC 25922 (**Ec**) and *Klebsiella pneumoniae* ATCC 700603 (**Kp**)). All strains were obtained from the L2CM laboratory (UMR 7053 CNRS-UL, France). The minimum inhibitory concentrations (**MIC**) were determined (**Section 2.5.3.**).

2.5.2. Preparation of the inoculum

Bacteria cells were grown 18 ± 2 h at 35 °C in Mueller-Hinton broth, cation-adjusted (**MHB-CA**). They were then centrifugated (3000 g, 5 min) and collected pellets were diluted in 10 mL NaCl 0.85 % (w/v). The absorbance was recorded at 540 nm with a microplate reader (Thermo Multiskan FC). Bacteria cells were diluted in MHB-CA to 0.5 McFarland turbidity and further diluted to 1:100 to reach a final concentration of 1 [0.4 - 1.6].10^6 CFU/mL.

2.5.3. MIC determination

The MIC- defined as the lowest concentration of extract with no visible bacterial growth – was determined using the microdilution broth method in 96-well microplates (Eloff, 1998) following the standard (ISO 20776-1, 2019) guidelines with some adaptations.

First, solvents (Ethanol and NaDESs) or extracts were diluted in MHB-CA (final volume: 50 µL). Then, 50 µL of bacterial inoculum were added to each well for a total volume of 100 µL per

237 microplate well (final bacterial concentration: 5 [2 – 8].10^5 CFU/mL). Growth controls (*i.e.,*
238 bacteria + MHB-CA), sterility control (medium without bacteria), and product control (medium
239 without bacteria with solvents (*i.e.,* Ethanol, ChCl-Gly, ChCl-EG, ChCl-Ur) or extracts) were
240 performed. Each test was repeated 8 times on the microplate. Plates were then incubated 18 ±
241 2 h at 35 °C. The MIC was expressed here as the % of extract (v/v) in the wells.

242 First, solvents were investigated alone at 25, 20, 15, 10, 5, 2.5, 1.25, 0.6 and 0.3 % to determine
243 the maximal concentration to use without affecting the cell growth. Then, the extracts were
244 studied from 1 % to 0.004 % (two-fold serial dilution) in a first set of experiments (n = 8). Last,
245 the remaining extracts were investigated at 10, 5, 1, 0.5 and 0.25 % (n = 2 only) in a second
246 series of experiments.

247 **2.6. Characterization of ChCl-Gly extracts by HPLC-MS/MS**

248 The *R. alaternus* leaves, pods and roots extracts using ChCl-Gly were analyzed by mass
249 spectrometry (Bruker Daltonics – micrOTOF – Q LCMS/MS, Bremen, Germany) system. The
250 separation was performed using Hypersil gold C18 column (100 mm × 2.1 mm, 3 µm) at 40 °C.
251 The mobile phase was composed of water containing 0.1% formic acid (solvent A) and
252 acetonitrile 100 % (solvent B). The elution program was set as follows: 95 % A/ 5 % B between 0
253 and 5 min, 1% A/ 99 % B between 5 and 40 min, stay at 99 % B between 40 and 45 min and 95
254 % A/ 5 % B between 45 and 55 min to reach the baseline. Before injection, all extracts were
255 filtered through 0.45 µm membrane filter (J.T. Baker®, Strasbourg, France). Then, 2 µL of each
256 one were injected at 200 µL/min flow rate during the whole experiment.

The MS analysis was performed with negative and positive ion modes, scanned m/z from 100 to 1000 for the determination of phenolic compounds. Each data was calibrated with Sodium formate at 1 mM in water/isopropanol 1/1. The ESI source conditions were as follows: drying gas at 5 L/min, the nebulizer pressure at 4 bar, the capillary voltage at 4500 V, and the capillary temperature was set at 190 °C. Data acquisitions were executed in autoMSMS mode at 20 eV collision energy.

2.7. Statistical analysis

Data were collected and expressed as the mean ± standard deviation of three independent experiments and analysed for statistical significance from control, using the Dunnett test SPSS 11.5 Statistics Software (SPSS, Chicago, IL, USA). The criterion for significance was set at $p < 0.05$. The IC_{50} values, from the in vitro data, were calculated by regression analysis.

3. Results and discussion

3.1. Determination of total phenolic content

In this study, three different NaDESs (*i.e.,* ChCl-Gly, ChCl-EG, and ChCl-Ur) were investigated for the extraction of TPC from leaves, pods and roots of *R. alaternus* plant and compared to ethanol as conventional solvent and used as reference (Fig. 1).

Fig. 1 shows the TPC for various *R. alaternus* extracts according to the nature of the NaDES used in comparison to ethanol. Whatever the plant part (*i.e.,* leaves, pods and roots), the extracts obtained using NaDESs contains significantly more polyphenols (from 437.7 to 279.8 mg GAE/100 g d.w.) than those obtained using ethanol (from 250 to 222.6 mg GAE/100 g d.w.). Furthermore, the TPC are significantly more concentrated in leaves samples than in pods and

finally roots, whatever the solvents studied in this work. Such trend is the highest for ChCl-Gly and the lowest for ethanol. Comparing the solvents efficiency on extraction process, the ChCl-Gly is more efficient than other solvents studied in this work. In ChCl-Gly, the highest TPC values are determined at 437.7, 361.3 and 317.0 mg GAE/100 g d.w. for leaves, pods and roots, respectively. The ChCl-Gly is followed by ChCl-EG (TPC varying from 344.2 to 304.8 mg GAE/100 g d.w.) and ChCl-Ur (TPC varying from 346.2 to 279.8 mg GAE/100 g d.w.), for leaves, pods and roots, respectively. The lowest amounts of polyphenols determined for the ethanol extracts are of 250.0, 237.8 and 222.6 mg GAE/100 g d.w. for leaves, pods and roots, respectively. In general, this highest extraction efficiency of TPC with NaDESs comparing with ethanol is related to the H-bonding interactions between the polar compounds (polyphenols) and NaDESs as previously reported (Duan et al., 2016). Duan and co-workers extracted polyphenols from Chinese herbal using various deep eutectic solvents. Also, our results are in agreement with a recent study reported in the literature by Islamčević Razboršek et al. (2020) in which the extraction of phenolic compounds from *Aronia melanocarpa* plant using NaDESs is studied. This work found higher extraction yield of phenolic compounds with NaDESs than those observed with ethanol.

[Fig. 1]

3.2. Determination of antioxidant capacity of the extracts

3.2.1. Radical scavenging activity

ABTS radical scavenging activity. The capacity of extracts to scavenge ABTS free radicals was evaluated in this study to determine extracts with high radical scavenging capacity (lowest IC_{50} values). As shown in Fig. 2A, the NaDESs are more efficient than the ethanol, since they show

300 the lowest IC_{50} values. The highest radical scavenging activity is detected with ChCl-Gly extracts
301 followed by ChCl-EG extracts > ChCl-Ur extracts > Ethanol extracts, respectively. When solvents
302 are compared, extracts obtained using ChCl-Gly are more active with an IC_{50} values ranging
303 from 44.55 to 53.56 µg/mL, followed by ChCl-EG with IC_{50} values varying from 55.47 to 58.71
304 µg/mL, and finally ChCl-Ur extracts with IC_{50} ranging from 60.22 to 63.72 µg/mL. In comparison
305 to NaDESs solvents, ethanol extracts present the lowest efficiency in terms of radical
306 scavenging activity with an IC_{50} values comprised within 66.28 and 69.56 µg/mL. Whatever the
307 plant's part (*i.e.*, leaves, pods or roots), the values of IC_{50} and The Trolox Equivalent Antioxidant
308 Capacity (TEAC) values are close in the three parts (Fig. 2A, Table S2). These results confirm that
309 the phenolic compounds extracted from different parts of *R. alaternus* using NaDESs present an
310 excellent antioxidant activity.

311 **[Fig 2.]**

312 ***DPPH radical scavenging activity.*** The DPPH radical scavenging capacity was evaluated for the
313 different solvent extracts and for various parts of plant (leaves, pods and roots; Fig. 2B). The
314 IC_{50} values range from 26.36 µg/mL for leaves extracted by ChCl-Gly (the highest activity) to
315 118.23 µg/mL for pods extracted using ChCl-Ur (the lowest activity) (Table S3). For ChCl-EG
316 extracts, the IC_{50} values range from 51.36 to 68.81 µg/mL, with DPPH radical scavenging activity
317 rather similar to the ethanol extracts. On the whole, the IC_{50} values increase in the following
318 order: ChCl-Gly extracts < ChCl-EG extracts < Ethanol extracts < ChCl-Ur extracts (Fig. 2B),
319 meaning the highest and the lowest DPPH radical scavenging activity are determined for the
320 ChCl-Gly and the ChCl-Ur extracts, respectively. All extracts obtained by using NaDESs present
321 stronger $DPPH^{\circ}$ radical scavenging capacity than those obtained with ethanol, except ChCl-Ur.

Our results are similar to the study of Oliveira and co-workers (Oliveira et al., 2021) on the radical scavenging capacity of *Curcuma longa* L. plant. Former authors showed that *Curcuma longa* L. leaves extracts were better hydroxyl radical scavengers after deep eutectic solvents extraction notably for choline chloride / lactic acid (1:2) or choline chloride / acetic acid (1:2) in comparison to ethanol, used as reference solvent.

3.2.2. Reducing power

The reducing power of a biological extract indicates its ability to reduce the Fe^{3+}/ferricyanide complex to the Fe^{2+} form, which is checked by measuring the formation of Perl's Prussian blue at 700 nm (Pan et al., 2010). The various NaDESs extracts from *R. alaternus* (*i.e.,* leaves, pods and roots) were evaluated to determine the lowest EC_{50} values (Fig. 3, panel A). The nature of solvent and the part of the plant also influence significantly the reducing capacity. The EC_{50} values of the investigated extracts increases which mean the decreases of reducing power in the following order: ChCl-Gly extracts > Ethanol extracts > ChCl-EG extracts > ChCl-Ur extracts. Compared to other solvent extracts, the ChCl-Gly extracts give the highest reducing power, corresponding to the lowest EC_{50} concentrations ranging from 77.53 to 85.05 µg/mL. The lowest reducing power was obtained in ChCl-Ur extracts, with EC_{50} values ranging from 395.97 to 542.71 µg/mL (Table S4). This analysis suggests that ChCl-Gly extracts are the most efficient ones in terms of reducing power. While, according to the study of Ozturk and co-authors were studied the extraction of polyphenols from orange peel waste using various DESs, this may be related to a more efficient extraction of polyphenol compounds with this former NaDES (Ozturk et al., 2018). Indeed, the reducing power of *R. alaternus* may be due to their TPC presence,

343 which may contribute to the antioxidant capacity by different reaction mechanisms (Duh et al.,
344 2001).

345 **[Fig. 3]**

346 **3.2.3. Iron (II) chelating activity**

347 Plant extracts enriched in polyphenols and other bioactive compounds may also present metal
348 chelation properties including iron chelating capacity; this latter activity plays a central role in
349 antioxidative mechanisms while inhibiting the formation of free radicals (Halliwell, 2007;
350 Yoshino & Murakami, 1998).

351 The capacity of *R. alaternus* extracts to chelate Fe^{2+} enabled the calculation of EC_{50} value, which
352 is the maximal effective concentration of extracts to obtain 50 % of iron chelating activity (Table
353 S5). The decrease of EC_{50} values meaning an increase the iron chelating activity. Hence, the
354 iron-chelation properties of the various extracts (Fig. 3, panel B) evolve in the following order:
355 ChCl-Gly extracts > Ethanol extracts > ChCl-EG extracts > ChCl-Ur extracts. The ChCl-Gly extracts
356 have the highest iron-chelating activity (smallest EC_{50}) with values ranging from 91.73 to 108.7
357 µg/mL. Besides, the ChCl-Ur exhibits the lowest iron-chelating properties with EC_{50} values
358 varying from 138.20 to 215.70 µg/mL. Furthermore, the Ethanol extracts chelate Fe^{2+} more
359 efficiently than ChCl-EG and ChCl-Ur ones. Hence, the ability of *R. alaternus* extracts to complex
360 iron is attributed to the presence of polyphenols, as previously reported in other plant study
361 (Oliveira et al., 2021). Indeed, extracts from various parts of *Curcuma longa* L. (*i.e.,* leaves,
362 rhizome and flowers) present chelating properties after solid-liquid extraction carried out using
363 DESs choline chloride-based solvents or ethanol as reference. In accordance with $ABTS^{\bullet+}$, DPPH

radical scavenging capacity and reducing power tests, ChCl-Ur is the less efficient solvent for producing extracts endowed with the radical scavenging capacity and reducing capacity.

3.3. Antimicrobial activity

First, the antimicrobial activity of the solvents itself (Ethanol or NaDESs) was evaluated alone against five bacterial strains of clinical interest. Although few MICs were obtained, in most cases, the bacterial growth was affected by the solvents, even without reaching a MIC (Table S6). Then, the microplates wells were gently homogenized and the OD540 was recorded. We arbitrarily defined that a bacterial growth showing an optical density OD ≥ 75 % of the OD of the growth control indicates few or no effect of the solvent. In some case, 1.3 % of the solvent must be used as maximum value to avoid a negative effect on the bacterial growth (Table S7). Our results also show the solvent negative effect on the bacterial growth in agreement with the literature (Percevault et al., 2021). Former authors demonstrated that DES (choline chloride / ethylene glycol (1:2) and betaine / citric acid (2:3)) are appropriate for the storage of bioactive compounds to avoid microbial contamination, which could be promising for food and pharmaceutical applications.

Therefore, the MIC of the extracts was determined against the bacteria with at most 1 % of extract, in order to avoid any interference with the solvent activity. Only the extract of Roots-ChCl-Gly showed a MIC at 0.5 % against *E. faecalis*. All other combinations showed no visible effect. Hence, in a second series of experiments, higher concentrations of extracts (10, 5, 1, 0.5, 0.25 %) were investigated with care of the solvent potential effect (Table 1).

Among the Gram- bacteria, only the Leaves-ChCl-Ur extract has an effect on *E. coli* (MIC reached at 10 % without solvent interference). In three cases (*i.e.*, Roots-ChCl-Gly vs. *P. aeruginosa*, Leaves-ChCl-Ur and Pods-ChCl-Ur against *K. pneumoniae*), a MIC value of 10 % is obtained, but at such concentration, the solvent fragilizes the cells (< 75 % of the growth control determined for the solvent alone) and is thus, a result of impaired bacteria.

On the other hand, the two Gram+ bacteria are more sensitive to the extracts. The Leaves-ChCl-Ur and Pods-ChCl-Gly extracts vs. *S. aureus*, and the Pods-Ethanol and Pods-ChCl-Gly extracts vs. *E. faecalis* show a MIC at 10 %, yet with solvent interference. Only the extracts Leaves-Ethanol, Leaves-ChCl-Gly and Leaves-ChCl-EG show no effect on *E. faecalis*. While, on all other combinations, MICs are defined between 0.5 and 10 %, without solvent interference on this strain.

Roots extracts (whatever the solvent) seems to have the best antimicrobial activity, with MICs on both Gram+ bacteria (*i.e., Staphylococcus aureus* and *Enterococcus faecalis*) without solvent interference. This antimicrobial activity towards Gram+ bacteria may be related to their absence of outer membrane, which could render them less resistant against extracts rich in bioactive molecules compared to Gram- bacteria (Brul & Coote, 1999). Our results are in agreement with Zhao and colleagues (Zhao et al., 2015), who found that extracts from *Sophora japonica* using DESs choline chloride-based (*i.e.,* choline chloride / ethylene glycol (1:2), choline chloride / glycerol (1:2) and choline chloride / urea (1:2)) presented antimicrobial properties against gram+ bacteria (*Staphylococcus aureus*).

Table 1. Minimum inhibitory concentrations of the extracts against the five bacterial strains.

Extract	Gram+		Gram-		
	Staphylococcus aureus (Sa)	*Enterococcus faecalis* (Ef)	*Pseudomonas aeruginosa* (Pa)	*Escherichia coli* (Ec)	*Klebsiella pneumoniae* (Kp)
Leaves-Ethanol	5%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %	≥ 10 %
Leaves-ChCl-Gly	5%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %	≥ 10 %
Leaves-ChCl-EG	5%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %	≥ 10 %
Leaves-ChCl-Ur	10%$^{++}$	10%$^{+}$	≥ 10 %	10%$^{+}$	10%$^{++}$
Pods-Ethanol	10%$^{+}$	10%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %
Pods-ChCl-Gly	10%$^{++}$	10%$^{++}$	≥ 10 %	≥ 10 %	≥ 10 %
Pods-ChCl-EG	5%$^{+}$	10%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %
Pods-ChCl-Ur	5%$^{+}$	5%$^{+}$	≥ 10 %	≥ 10 %	10%$^{++}$
Roots-Ethanol	5%$^{+}$	5%$^{+}$	10 %*	≥ 10 %	≥ 10 %
Roots-ChCl-Gly	5%$^{+}$	0,5%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %
Roots-ChCl-EG	5%$^{+}$	5%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %
Roots-ChCl-Ur	5%$^{+}$	10%$^{+}$	≥ 10 %	≥ 10 %	≥ 10 %

(+): weak or no effect of the solvent; (++): combined effect of the solvent; (*): strong effect of the solvent; ≥ 10 %: no effect.

3.4. Characterization of ChCl-Gly extracts by HPLC-MS/MS

The qualitative determination of bioactive compounds extracted from three different extracts of *R. alaternus* (*i.e.,* leaves, pods and roots) with ChCl-Gly - corresponding to the best extract in term of biological activities - were analyzed by HPLC-MS/MS (Table 2). Twenty-one compounds

were determined in the extracts. Among them, twelve molecules were newly identified in this plant comparing to the literature (Nekkaa et al., 2021), including tyrosine (2.5 min), dihydroquercetine pentoside glucoside (9.1 min), tryptophane (9.8 min), aloesin (11.3 min), aloesol 7-glucoside (11.5 min), tectoridin (12.8 min), glucuronic acid (12.6/13.7 min), 7-hydroxy-3-(2-hydroxypropyl)-5-methylisochromen-1-one (15.3 min), aloesol (15.4 min), piperonylidene acetone (16.4 min), (R)-linalyl beta-vicianoside (18.1 min), neryl rhamnosyl-glucoside (18.3 min). Furthermore, six compounds *i.e.,* aloesin (11.3 min), aloesol glucoside (11.6 min), aloesol 7-glucoside (11.5 min), 7-hydroxy-3-(2 hydroxypropyl)-5-methylisochromen-1-one (15.3 min), aloesol (15.4 min) and emodin (25.1 min) were common to the three extracts collected from the various part of plant. Based on Table 2, the leaves provide the richest part of *R. alaternus* in term of number of bioactive compounds identified in this extraction comparing to other parts, and is followed by pods than roots. On the other hand, these finding evidences that the *R. alaternus* plant presents total phenolic compounds with high antioxidant effect (*i.e.,* dihydroquercetine pentoside glucoside, kaempferol, dihydroquercetin 3-O-glucoside, kaempferide 3-glucoside and tectoridin), and organic acids (*i.e.,* malic acid, citric acid and glucuronic acid), in addition to amino acids including tyrosine and tryptophan.

The ChCl-Gly (1:2) may have a better affinity towards bioactive compounds through hydrogen bonding interactions, which would favour the antioxidant effects of extracts.

4. Conclusion and Perspectives

For the first time, three choline chloride-based NaDESs (choline chloride-glycerol (ChCl-Gly), choline-chloride-ethylene glycol (ChCl-EG) and choline chloride-urea (ChCl-Ur)) were used for

437 the extraction of bioactive compounds from various parts of *R. alaternus* plant (*i.e*., leaves,
438 pods and roots). Among all the studied extracts, the ChCl-Gly extracts from *R. alaternus'* leaves
439 presents the best content of total phenolic compounds and provides the highest antioxidant
440 activity compared to other investigated solvents such as NaDESs and ethanol, used in
441 reference. All NaDESs extracts show interesting antimicrobial activity with significant inhibitory
442 potential against Gram+ strains. Furthermore, the leaves, roots and pods extract carried out
443 using ChCl-Gly were characterized and quantified by HPLC-MS/MS, which confirms that the
444 phenolic compounds and flavonoids are the main antioxidant compounds. The *in vitro*
445 biological analysis of extracts suggests that ChCl-Gly is an efficient solvent for the extraction of
446 secondary metabolite from leaves, pods and roots of *R. alaternus*. These bio-based sustainable
447 and green processes present a great potential for pharmaceutical, cosmetic and food
448 applications.

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Table 2. Identification of bioactive compounds present in leaves, pods and roots of *R. alaternus* extracted using NaDES (ChCl-Gly) analysed by LC-MS/MS.

N°	Compound name	Molecular formula	tr (min)	$[M + H]^-$			$[M + H]^+$			plant part
				Measured *m/z* (Da) $[M + H]^-$	Error (ppm)	MS/MS Fragmentation	Measured *m/z* (Da) $[M + H]^+$	Error (ppm)	MS/MS Fragmentation	
1	Dihydroquercetine pentoside glucoside	$C_{26}H_{30}O_{16}$	9.1	597.151	8.5	417.085 $[M\text{-hexose-H}]^-$ 285.038 $[M\text{-hexose - pentosyl - H}]^-$	599.162	1.6	n.d	leaves
2	Kaempferol	$C_{15}H_{10}O_6$	19.3	285.039	3.5	239.032 $[M\text{-HCOOH-H}]^-$ 229.049 $[M\text{-CO-CO-H}]^-$ 185.058 $[M\text{-CO-CO-}CO_2\text{+H}]^+$	287.056	3.5	285.052 $[M\text{-CHO+H}]^+$ 241.051 $[M\text{-HCOOH+H}]^+$ 231.072 $[M\text{-CO-CO+H}]^+$ 213.050 $[M\text{-CO-CO-}H_2O\text{+H}]^+$ 185.058 $[M\text{-CO-CO-}H_2O\text{-CO+H}]^+$	Pods
3	Dihydroquercetin 3-O-glucoside	$C_{21}H_{22}O_{12}$	9.5	465.108	8.5	285.041 $[M\text{-hexose-2H}]^-$	467.116	8.5	305.065 $[M\text{-sucrose+H}]^+$ 181.014 $[M\text{-sucrose-}C_6H_4CH]^+$ 181.014 $[C_8H_5O_5]^+$	leaves
4	Kaempferide 3-glucoside	$C_{22}H_{22}O_{11}$	14.6	n.d	n.d	n.d	463.125	4.3	301.069 $[M\text{-hexosyl+H}]^+$	Leaves, pods
5	Tectoridin	$C_{22}H_{22}O_{11}$	12.8	461.118	19.5	n.d	463.125	4.3	283.057 $[M\text{-hexose}]^+$ 255.063 $[M\text{-hexose-}CO_2]^+$	Leaves, pods
6	Emodin	$C_{15}H_{10}O_5$	25.1	269.045	3.7	241.047 $[M\text{-CO-H}]^-$ 255.053 $[M\text{-}CO_2\text{-H}]^-$ 210.029 $[M\text{-}CO_2\text{-}CH_3\text{-H}]^-$	271.058	7.3	229.046 $[M\text{-}CH_2CO\text{+H}]^+$ 225.051 $[M\text{-}H_2O\text{-CO+H}]^+$ 1978.059 $[M\text{-}H_2O\text{-2CO+H}]^+$	Leaves, pods, roots
7	Malic acid	$C_4H_6O_5$	1.8	133.015	7.5	114.004 $[M\text{-}H_2O\text{-H}]^-$ 71.021 $[M\text{-H2O-}CO_2\text{-H}]^-$	n.d	n.d	n.d	Leaves, pods
8	Citric acid	$C_6H_8O_7$	2.1	191.015	10.5	129.014 $[M\text{-}H_2O\text{-}CO_2\text{-H}]^-$ 111.007 $[M\text{-}2H_2O\text{-}CO_2\text{-H}]^-$ 87,011 $[M\text{-}H_2O\text{-}CO_2\text{-}CH_2CO\text{-H}]^-$	193.033	5.2	n.d	Leaves, roots
9	Tyrosine	$C_9H_{11}NO_3$	2.5	n.d	n.d	n.d	182.080	5.5	165.083 $[M\text{-OH+H}]^+$ 135.077 $[M\text{-HCOOH+H}]^+$ 119.057 $[M\text{-HCOOH-}NH_3\text{+H}]^+$	pods
10	Tryptophan	$C_{11}H_{12}N_2O_2$	9.8	203.078	2.4	142.062 $[M\text{-}CO_2\text{-}NH_3\text{-H}]^-$ 116,050 $[M\text{-}CO_2\text{-}NH_3\text{-CN-H}]^-$	205.095	9.7	188.078 $[M\text{-}NH_3\text{+H}]^+$ 144.080 $[M\text{-}NH_3\text{-}CO_2\text{+H}]^+$ 118.068 $[M\text{-}NH_3\text{-}CO_2\text{-CN+H}]^+$	Leaves, pods
11	Aloesol 7-glucoside	$C_{19}H_{24}O_9$	11.5	395.135	0.0	351.103 $[M\text{-}C_2H_4O\text{-H}]^-$ 231.063 $[M\text{-glycosyl-H}]^-$ 203.064 $[M\text{-glycosyl-CO-H}]^-$	397.149	0.0	233.077 $[M\text{-glycosyl+H}]^+$ 215.066 $[M\text{-glycosyl-}H_2O\text{ + H}]^+$ 203.066 $[M\text{-glycosyl-}CH_2O\text{ + H}]^+$	Leaves, pods, roots

12	7-hydroxy-3-(2-hydroxypropyl)-5-methylisochromen-1-one	$C_{13}H_{14}O_4$	15.3	233.078	17	189.051 $[M\text{-}CH_3COH\text{-}H]^-$ 159.036 $[M\text{-}CH_3COH\text{-}CH_2O\text{-}H]^-$	235.095	4.2	217.077 $[M\text{-}H_2O+H]^+$ 191.068 $[M\text{-}CH_3COH+H]^+$ 176.046 $[M\text{-}CH_3COH\text{-}CH_3+H]^+$	Leaves, pods, roots
13	Piperonylidene acetone	$C_{11}H_{10}O_3$	16.4	189.051	26	174.030 $[M\text{-}CH_3\text{-}H]^-$ 159,042 $[M\text{-}CH_2COH\text{-}H]^-$ 146.034 $[M\text{-}CH_2COH\text{-}CH_3\text{-}H]^-$	191.069	5.2	176.047 $[M\text{-}CH_3+H]^+$ 151.037 $[M\text{-}H_2O\text{-}CH_2CO+H]^+$ 148.050 $[M\text{-}CH_3\text{-}CO+H]^+$	Leaves, pods
14	(R)-Linalyl beta-vicianoside	$C_{21}H_{36}O_{10}$	18.1	449.233	9.1	447.231 $[M\text{-}H]^-$ 315.179 [M-pentosyl-H]$^-$ 161.042 $[M\text{-pentosyl-}C_{10}H_{17}OH\text{-}H]^-$	471.216	8.5	333.079 $[M\text{-}C_{10}H_{18}+Na]^+$	leaves
15	Neryl rhamnosyl-glucoside	$C_{22}H_{38}O_{10}$	18.3	507.251	16	461.246 $[M\text{-}H]^-$ 315.178 [M-Dehydrohexosyl-H]$^-$ 141.042 $[M\text{-Dehydrohexosyl-}C_{10}H_{17}OH\text{-}H]^-$	485.236	0.0	347.093 $[M\text{-}C_{10}H_{18}+Na]^+$ 204.040 $[M\text{-}C_{10}H_{18}$-dehydrohexosyl+Na$]^+$	leaves
16	Glucuronic acid	$C_{16}H_{20}O_{10}$	12.6/13.7	371.098	0.2	249.062 [M-benzoic acid-H]$^-$ 175.019 [M-benzoic acid-glycerol-H]$^-$ 121.029 [benzoic acid-H]$^-$	373.111	5.3	n.d	leaves
17	Aloesol di glycoside	$C_{25}H_{34}O_{14}$	10.3	n.d	n.d	n.d	559.202	0.0	397.151 [M-Hexosyl+H]$^+$ 233.074 [M-2Hexosyl+H]$^+$	Leaves, pods
18	Aloesin–glycoside	$C_{25}H_{32}O_{14}$	10.1	555.180	13	n.d	557.189	3.6	395.139 [M-Hexosyl+H]$^+$ 233.078 [M-2Hexosy+H]$^+$	pods
19	Aloesin	$C_{19}H_{22}O_9$	11.3	393.121	5.1	273.079 $[M\text{-}HOC_6H_3CO\text{-}H]^-$ 231.061 [M-glycosyl - H]$^-$ 203.068 [M-glycosyl-CO - H]$^-$	395.135	3.5	233.077 [M-glycosyl + H]$^+$ 215.067 [M-glycosyl-H_2O + H]$^+$ 203.065 [M-glycosyl-CH_2O + H]$^+$	Leaves, pods, roots
20	Aloesol-glucoside	$C_{19}H_{24}O_9$	11.6	395.135	2.5	275.089 $[M\text{-}HOC_6H_3CO\text{-}H]^-$ 231.066 [M-glycosyl - H]$^-$ 203.068 [M-glycosyl-CO - H]$^-$	397.151	6.0	233.077 [M-glycosyl + H]$^+$ 215.067 [M-glycosyl-H_2O + H]$^+$ 203.066 [M-glycosyl-CH_2O + H]$^+$	Leaves, pods, roots
21	Aloesol	$C_{13}H_{14}O_4$	15.4	233.078	60	189.051 [M-C_2H_4O - H]$^-$	235.094	8.5	217.077 $[M\text{-}H_2O+H]^+$ 191.068 [M-C_2H_4O + H]$^+$ 176.047 [M-C_2H_4O-CH_3 + H]$^+$	Leaves, pods, roots

n.d: not determined

Acknowledgements

This research work was supported by PHC-Tassili (Code 20MDU917). Besides, the authors acknowledge the support of the “Laboratoire Réactions et Génie des Procédés” by the “Impact Biomolecules” project of the “Lorraine Université d’Excellence” (Investissements d’avenir – ANR project number 15-004).

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Figure captions

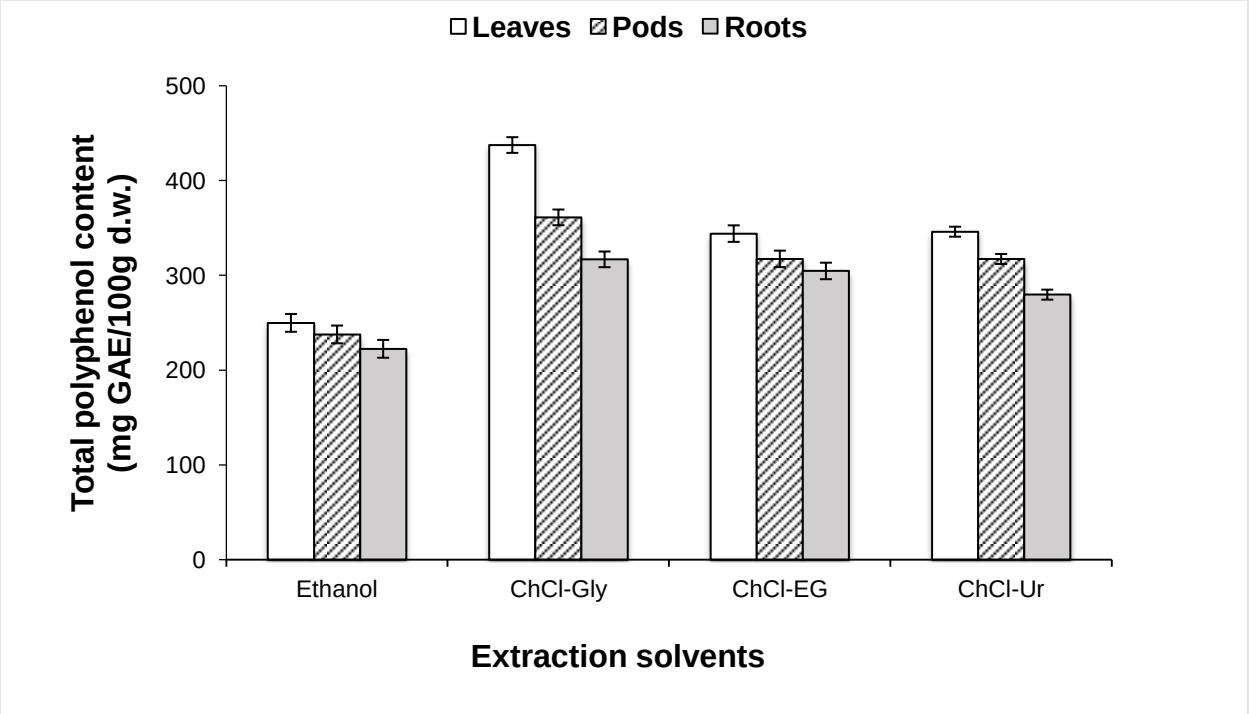
Fig. 1. Total phenolic content (TPC) of *R. alaternus's* leaves, pods and roots extracted with natural deep eutectic solvents (NaDESs: ChCl-Gly, ChCl-EG, ChCl-Ur) and compared to Ethanol as reference.

Fig. 2. Radical scavenging activity of different parts from *R. alaternus* extracted using natural deep eutectic solvents (NaDESs): ChCl-Gly, ChCl-EG, ChCl-Ur in reference to Ethanol. (A) ABTS test and (B) DPPH test.

Fig. 3. Reducing activity (A) and Iron chelating capacity (B) of different parts from *R. alaternus* extracted using natural deep eutectic solvents (NaDESs); ChCl-Gly, ChCl-EG, ChCl-Ur and Ethanol.

Figure graphics

Fig. 1



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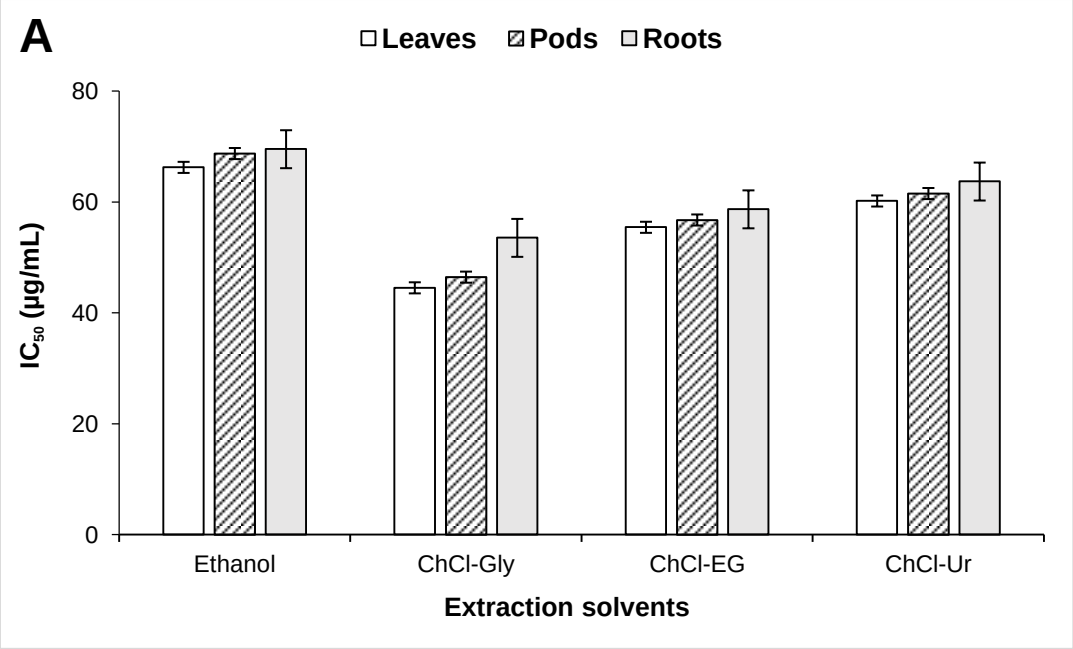
647 **Fig. 2**



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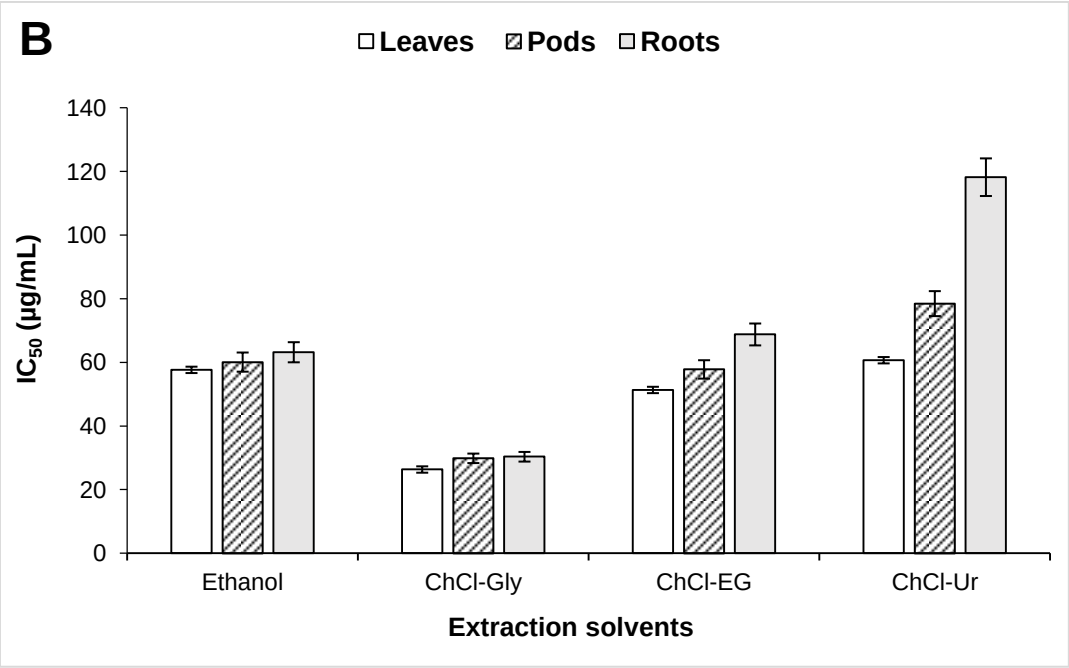

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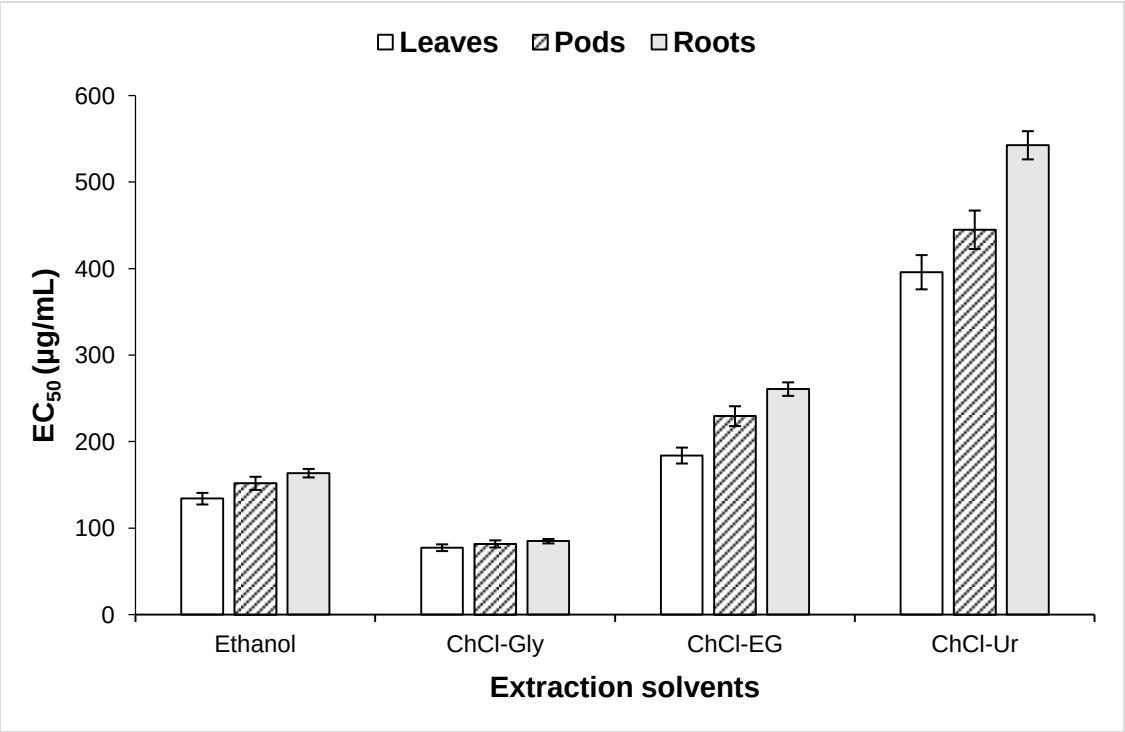
652 **Fig. 3**

A


Leaves
Pods
Roots
600
500
400
300
200
100
0
EC50 (µg/mL)
Ethanol
ChCl-Gly
ChCl-EG
ChCl-Ur
Extraction solvents

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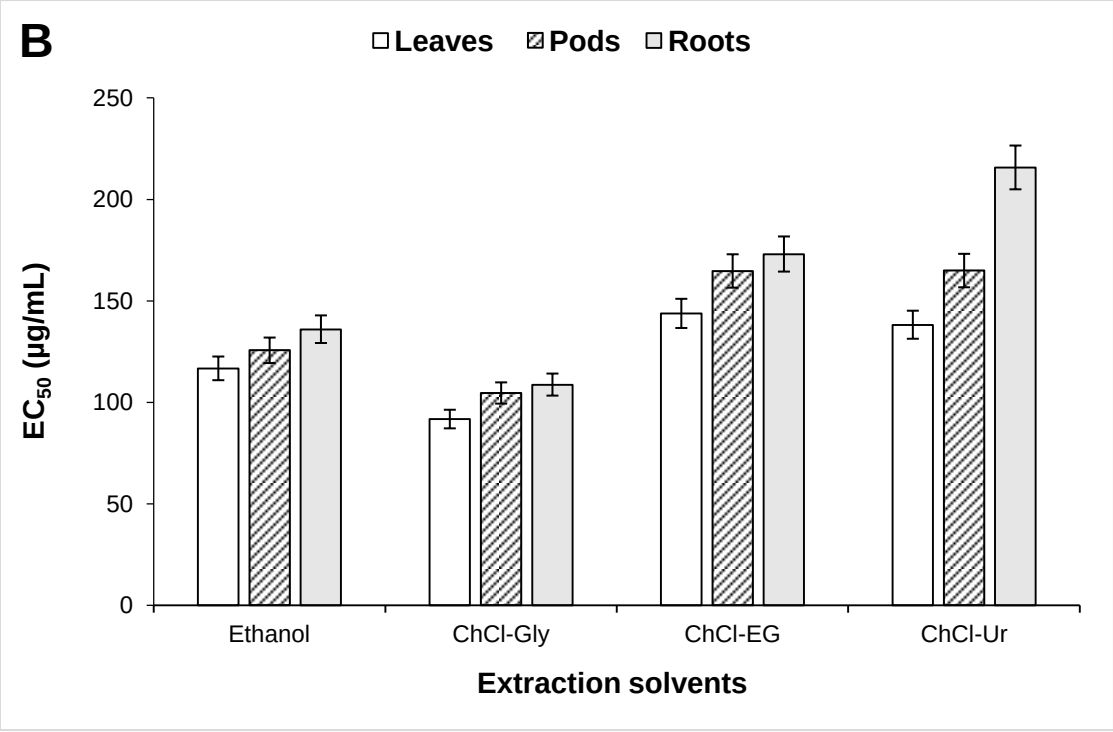
B
Leaves
Pods
Roots
250
200
150
100
50
0
EC50 (µg/mL)
Ethanol
ChCl-Gly
ChCl-EG
ChCl-Ur
Extraction solvents

654

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