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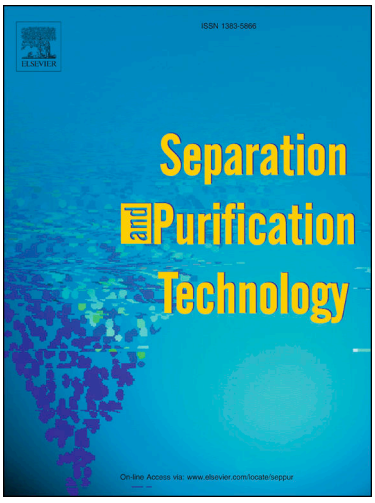

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Extraction of Phenolic Compounds from Rosemary using Choline Chloride – based Deep Eutectic Solvents

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Abstract

Deep eutectic solvents (DES) have been explored as an alternative to hazardous solvents to extract natural bioactive compounds. In this work, choline chloride-based DES (neat and hydrated) were assessed in the extraction of phenolics from rosemary leaves. The DES studied on an initial screening showed a total phenolic content (TPC) up to 220 % higher than the control. The solubilities of the main rosemary biocompounds, estimated by COSMO-RS, showed a positive correlation to TPC. Choline chloride:1,2-propanediol (CPH) was selected for further optimization. At the optimal conditions (65 ºC, liquid: solid ratio of 40:1 and 50 %wt of water), the antioxidant activity and TPC were 80 mg Trolox equivalent/g and 78 mg gallic acid equivalent/g, respectively. The antimicrobial activity of the optimized extract revealed inhibition of 39-51% to all bacteria tested. In summary, an extract with good antioxidant and antimicrobial activities was obtained from rosemary leaves using CPH as solvent.

Keywords: Solubility; carnosic acid; carnosol; rosmarinic acid; COSMO-RS.

Abbreviations

DES	Deep Eutectic Solvents
[Ch]Cl	Choline Chloride
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
COSMO-RS	Conductor-like Screening Model for Realistic Solvatation
TPC	Total Phenolic Content
L:S ratio	Liquid: solid ratio
DPPH	2,2 – diphenyl-1-picrylhyldrazyl radical
TE	Trolox equivalent
GAE	Gallic acid equivalent
abs	Absorbance
RSM	Response Surface Methodology
X_1	Extraction temperature
X_2	Liquid: solid ratio
X_3	Water content
IE	Inhibition effect

1. Introduction

Rosmarinus officinalis L. (rosemary) is a Mediterranean herb that has been used since antiquity for medicinal and culinary purposes, due to its health benefits and aromatic characteristics [1,2]. Its biological activities are related to bioactive molecules, mainly phenolic compounds, responsible for the antioxidant, antibacterial, and anti-inflammatory properties [3]. Moreover, rosemary extracts exhibit a special role in preventing a range of diseases due to their ability to inhibit or delay cellular oxidative processes [4,5].

The food oxidation and damage caused by microorganisms are critically related to food spoilage. Rosemary has been proposed as a food additive in short shelf-life products, acting as antioxidant and antimicrobial agent, against *Staphylococcus aureus, Bacillus cereus, Salmonella poona, Escherichia coli, Listeria monocytogenes* and others [1,6–9]. The acceptance of rosemary extracts as a food additive by Food and Agriculture Organization of the United Nations (FAO) [10] and European Food Safety Authority (EFSA) [11] can enhance industrial application in the fields of foods and beverages, personal care, nutrition, and health [12]. For this purpose, efficient and safe processes that use biocompatible and biodegradable solvents need to be developed.

It is known that organic solvents, *e.g*., ethanol, methanol, and chloroform, are commonly applied to obtain bioactive compounds from plants, mainly in conventional solid-liquid extractions [13,14]. However, the extraction performance should take into account the solvents biocompatibility and environmental criteria under the green chemistry concepts [3,15].

Firstly reported by Abbott and co-workers, deep eutectic solvents (DES) are a low melting point mixture, comprised of two or more components forming strong hydrogen bonding complexes, where the eutectic point temperature is lower than that of an ideal

solution [16,17]. Choline chloride ([Ch]Cl), an abundant and natural quaternary ammonium salt, is the most used hydrogen bond acceptor (HBA), while organic acids, sugars, and polyols are widely used as hydrogen bond donors (HBD) [3]. Moreover, DES have been used for the extraction of bioactive compounds from plant resources, such as phenolics from *Moringa oleifera* L. leaves [18], flavonoids from *Ginkgo biloba* leaves [19], triterpenes from *Cynomorium songaricum* [20], anthocyanins and pectin from *Myrciaria cauliflora* fruit by-product [21].

The composition of the DES affects their properties and chemical characteristics and, consequently, their performance in the extraction of biomolecules of interest. The evaluation of their properties, such as polarity and solubility, can thus be useful in screening tests to select promisor solvents, as well in the interpretation of the results obtained. For this purpose, thermodynamic models can be used to predict these properties for bioactive compounds, in particular in less common solvents [22]. Among them, COSMO-RS (Conductor-like Screening Model for Realistic Solvatation), a quantum chemical model of unrestricted applicability, is a fully predictive model *i.e.*, independent of experimental data [23,24]. One of the applications of COSMO-RS is the prediction of the solubility of a solute in a wide range of solvents [25]. Several COSMO-RS works on this subject are available, such as, the simulation of the solvent action of eutectic mixtures in the extraction of hydroxytyrosol from olive leaves [26], the prediction of relative solubility of biomolecules from rosemary in different ethanol: water mixtures [27], the design of liquid drug-based formulations, using choline-chloride based eutectic systems [28], the solubility prediction of ginger bioactive compounds in water [29], anthocyanin and pectin in DES [21] and diterpenes from rosemary in more than one thousand DES [30].

The extraction of phenolic compounds from rosemary was evaluated in studies such as de Oliveira *et.al* [14], using organic solvents, and Barbieri *et.al* [15] in which the use of DES was evaluated. We recently proposed the use of the COSMO-RS model as an alternative to the experimental screening traditionally used in the previous selection of solvents [30].

In this work, it was evaluated the performance of choline chloride-based DES regarding the extraction of rosemary biocompounds. The selection of the most promisor DES was supported by COSMO-RS, trough the relative solubility analysis of the main rosemary phenolics. Besides that, for the most suitable solvent, its water content, extraction temperature, and liquid: solid ratio (L:S ratio, weight of solvent per weight of dried rosemary powder) were optimized by experimental design, to maximize total phenolic content (TPC) and antioxidant activity. Finally, the antibacterial activity of the rosemary extract obtained at optimal conditions was evaluated.

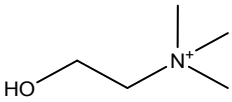
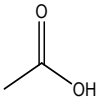
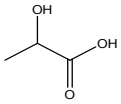
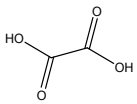
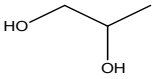
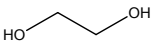
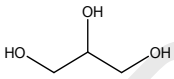
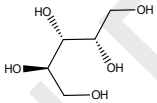
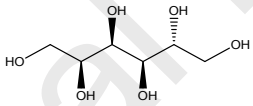
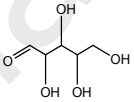
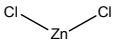
2. Materials and methods

2.1 Materials

Rosemary leaves were collected from UFPR's Canguiri Experimental Farm (25°23'12.3"S 49°07'33.3"W). The material was hand-selected, dried in an oven at 40°C with air circulation until constant weight. The dried leaves were ground in a laboratory mill (Requipal, MR 320, São Paulo, Brazil), sieved to obtain particle sizes < 0.2 mm, vacuum packed, and frozen for further application at -10°C.

The reagents and their chemical structure, molecular formula, molar mass, purity, and source are shown in Table 1.

Table 1. Chemical structure, molecular formula, molecular weight, purity and source of the chemicals used in this study

Chemical Name	Chemical Structure	Molecular Formula	Molar Mass	Purity (%)	Source
Choline chloride		$[N_{111(2OH)}]Cl$	56.6	98	Inlab
Acetic acid		$C_2H_4O_2$	60.05	≥ 99	Sigma-Aldrich
Lactic acid		$C_3H_6O_3$	90.08	≥ 99	Sigma-Aldrich
Oxalic acid		$C_2H_2O_4$	90.03	≥ 99	Sigma-Aldrich
1,2-propanediol		$C_3H_8O_2$	76.09	≥ 99	Sigma-Aldrich
Ethylene glycol		$C_2H_6O_2$	62.07	99	Neon
Glycerol		$C_3H_8O_3$	92.09	≥ 99	Sigma-Aldrich
Xylitol		$C_5H_{12}O_5$	152.12	98	Dinâmica
Sorbitol		$C_6H_{14}O_6$	182.14	97	PanReac
Xylose		$C_5H_{10}O_5$	150.13	98	Êxodo
Zinc chloride		$ZnCl_2$	136.29	≥ 99	Sigma-Aldrich

2.2 Methods

Figure 1 shows the experimental setup with the main steps applied to develop this work.

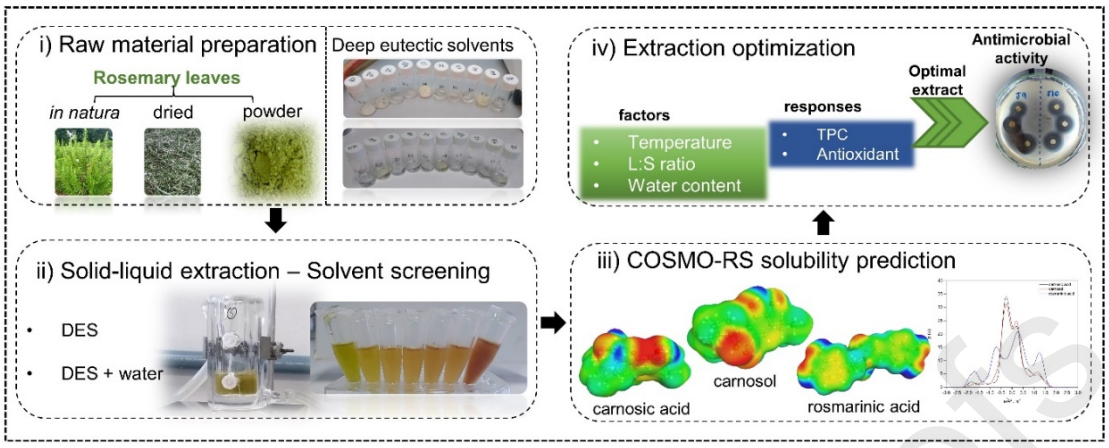


Fig. 1 – Experimental setup with the four main steps applied in this work.

2.2.1 Preparation of the deep eutectic solvents

The different DES were prepared by heating the HBA: HBD mixture, according to Table 2, at 70°C with constant stirring until obtaining a homogeneous transparent liquid.

Table 2. Deep eutectic solvents and its proportion used in this study

HBA	HBD	HBA: HBD ratio	Acronyms
Choline chloride	Acetic acid	1:2	CAA
	Latic acid	1:2	CLA
	Oxalic acid	1:1	COA
	1,2-propanediol	1:2	CPH
	Ethylene glycol	1:2	CEH
	Glycerol	1:2	CGH
	Xylitol	2:1	CXH
	Sorbitol	2:1	CSH
	Xylose	2:1	CXY
	Zinc chloride	1:2	CZC

2.2.2 Solvent screening and optimization of extraction conditions

For the solvent screening, solid-liquid extractions were carried out using different DES, neat (Table 2) and hydrated (30 wt %). The temperature was kept at 30 ºC, L:S ratio

at 20:1, and constant stirring at 600 rpm for 150 min. For comparison purposes, ethanol (pure and in aqueous solution, 70 wt %) was used as a control for the extraction. In the sequence, the supernatant was recovered by centrifugation at 4000 rpm ($1500 \times g$) for 20 minutes using a Microcentrifuge (Thermo Scientific, Heraeus Fresco 21 Microcentrifuge, UK), and filtered using a 0.20 μm syringe filter.

After the solvent screening, for the most promisor DES the Response Surface Methodology (RSM) was applied to optimize the extraction conditions, according to Table 3. In a 2^k RSM there are k different factors that can contribute to a response y, according to this polynomial Equation (1):

$$y = \beta_0 + \sum \beta_i X_i + \sum_{i<j} \beta_i \beta_j X_i X_j \quad Eq.\,(1)$$

where β_0, β_i, and β_{ij} are adjusted coefficients for the intercept, linear coefficients, and interaction terms, respectively; X_i and X_j are the independent variables. In this study, extraction temperature (ºC), L:S ratio and water addition (% wt) were submitted to a 2^3 factorial design, to optimize the antioxidant activity and phenolic content, in solid-liquid extractions during 150 min.

Eleven experiments were developed in triplicate and the mathematical models were subjected to analysis of variance (ANOVA) and regression analysis using Statistica 7.0 software (StatSoft, Tulsa, OK, USA). The antioxidant activity and total phenolic content graphs were generated from adjusted models.

Table 3. Experimental design factors and levels of experiments at 150 minutes

Independent variable	Experimental value		
	-1	0	+1
Extraction temperature (ºC)	25	45	65
L:S ratio	5:1	20:1	35:1
Water addition (% wt)	5	20	35

2.2.2 Total phenolic content (TPC) and bioactive assays

The total phenolic content (TPC) was determined by colorimetric analysis with Folin-Ciocalteu reagent (FC), according to [31]. In test tubes, 1.68 mL of distilled water, 20µL of sample, and 100 µL of FC were mixed. After 3 min, an aliquot of 100 µL of 20% sodium carbonate was added into each tube, followed by stirring in a vortex (Gomixer, MX-S). After 60 min of reaction at room temperature in the darkness, the absorbance was measure using a UV-VIS spectrophotometer (Shimadzu, UV-1800, Tokyo, Japan) at 760 nm. The data were compared to a standard curve of gallic acid (TPC = 1.1213×abs_{760nm} – 0.0068; R^2 = 0.99), and the results expressed as mg gallic acid equivalent per g of rosemary (mg GAE/g).

The antioxidant activities (AA) of the extracts were evaluated using the 2,2 – diphenyl-1-picrylhyldrazyl radical (DPPH•) [32]. The color change of the reagent solution (125µmol/L) from purple to yellow was monitored by visible spectroscopy at 517 nm. The percentage of DPPH• reduction (%AA) was obtained through Equation 1, and antioxidant activity was calculated using a standard curve (Antioxidant activity = 4.8975 × %AA – 3.75888; R^2 = 0.99).

$$\%AA = \frac{(ABS_b - ABS_s)}{ABS_b}.100 \quad Eq.\,(1)$$

where, ABS_b and ABS_s is the blank and sample absorbance values, respectively, at 517 nm, after 30 min of reaction in the darkness, and at room temperature. Results were converted and expressed in mg of Trolox equivalent per g of dry rosemary leaves (mg TE/g).

2.2.3 HPLC-DAD

The quantification of rosmarinic acid and carnosic acid was made using a HPLC-DAD (Shimadzu, model PROMINENCE). The analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), Kinetex 5 μm C18 100 Å, from Phenomenex. The separation was conducted in a gradient system of 0.1% of acetic acid-methanol (phase A) and 0.1% of acetic acid-ultra-pure water, at least in duplicate, according to Wojeicchowski and co-workers [30]. For this, the following conditions were applied: 0 min of phase A; 7 min of phase A; 11 min 80% of phase A; 23 min 80 % of phase A; 24 min 90 % of phase A; 28 min 40 % of phase A; 40 min 40 % of phase. Rosmarinic acid and carnosic acid displayed a retention time of 14 and 32.2 min, and DAD was set at 330 nm and 280 nm, respectively. The amount of biomolecules extracted were expressed in mg of extracted compound per g dry weight of rosemary leaves (mg/g).

2.2.4 Antibacterial activity

The disk diffusion sensitivity method was used to evaluate antibacterial activity of the extract [7,33]. *Staphylococcus auereus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Clostridium perfringens* (ATCC 13124), *Listeria monocytogenes* (ATCC 7644) and *Salmonella* spp. (ATCC 13076) were tested. These cultures were regenerated in

Mueller-Hinton broth, and so plated in Mueller-Hinton agar (MHA), at 37°C for 24 h. Subsequently to the incubation, the bacteria suspensions were standardized to 10^8 CFU/mL, according to McFarland scale (0.5). The MHA plates were inoculated with the standardized suspensions and spread on the plate. Sterile and white discs of 5-mm diameter (Laborclin©), with the extract or controls samples (20 µL), were laid on the plate surface in triplicate. Chloramphenicol 30µg and ethanol were uses as positive and negative control, respectively. After 24 h at 37°C, clear inhibition zones surrounding the disks were observed and measured as antibacterial activity in millimeters, using a digital caliper. The halos were labeled as S = susceptible > 18 mm; I = intermediate 13 to 17 mm and R = resistant < 12mm, according to the minimum inhibition ranges [34]. Finally, the inhibition effect (IE %) of rosemary extract was estimated for all evaluated bacteria and compared to their respective antibiotic inhibition zones.

2.2.5 In silico solubility predictions using COSMO-RS

COSMO-RS is a known thermodynamic predictive model, developed by Klamt and co-workers [24] based on quantum chemistry (COSMO) and statistical thermodynamics (RS) [35]. COSMO-RS prediction involves two steps, a microscopic and macroscopic. The first one consists of quantum chemistry simulation of a virtual conductor environment in which a specific molecule, under study, is embedded. The macroscopic step determines the σ-profile, which means the probability distribution of a molecular surface segment with a specific charge density (σ) [35]. The σ-profiles provides detailed information about the molecular polarity and can be considered a finger-print of the molecule [25,35].

The relative solubility of the principal rosemary extract compounds (rosmarinic acid, carnosol and carnosic acid) on the studied DES was calculated using COSMOthermX, at 30 °C. In this approach, for each solute, the logarithm of the relative solubility in the solvent with the highest solubility value it was set to 0 and all other solvents were given relative to the best solvent [27,36]. With this approach, a fast-qualitative evaluation of solutes` solubility can be obtained, without any information about thermodynamics properties such as, enthalpy of fusion and melting point temperature, required to the absolute solubility predictions (quantitative data) [37].

For this purpose, each molecule was optimized using the COSMO-BP-TZVP of the TmoleX software package [38], which includes a def-TZVP basis set, DFT with the B-P83 functional level of theory and the COSMO solvation model. COSMO-RS relative solubility predictions were carried on COSMOthermX (version 19.0.1) [39] using the parametrization, BP_TZVP_C30_19.ctd.

3. Results and discussion

3.1 Screening of deep eutectic solvents

Initially, ten different neat DES (Table 2) (without water) were applied in the extraction of phenolic compounds from rosemary leaves at 30°C, 150 min, 20:1 (L:S ratio), and constant stirring. All the DES tested were based on the same HBA, *i. e.* choline chloride ([Ch]Cl). This quaternary ammonium salt possess important properties and benefits, such as non-toxicity, biodegradability and moderate cost due to large production [40,41], making it the most widespread component to form DES [42].

However, only four choline chloride-based neat DES allowed the successful extraction of bioactive compounds (green bars), namely those composed by acetic acid, lactic acid, ethylene glycol and 1,2-propanediol as HBD, Fig.2. The other neat DES hindered the extraction due to their high viscosity, indicated in Fig.2 as "not observed." The high viscosity of DES results from the extensive hydrogen-bonding network between HBA and HBD, which reduces the diffusion rate and mass transfer [43,44]. To overcome it, an amount of water can be used to reduce the DES viscosity, and thus, improve the extraction efficiency.

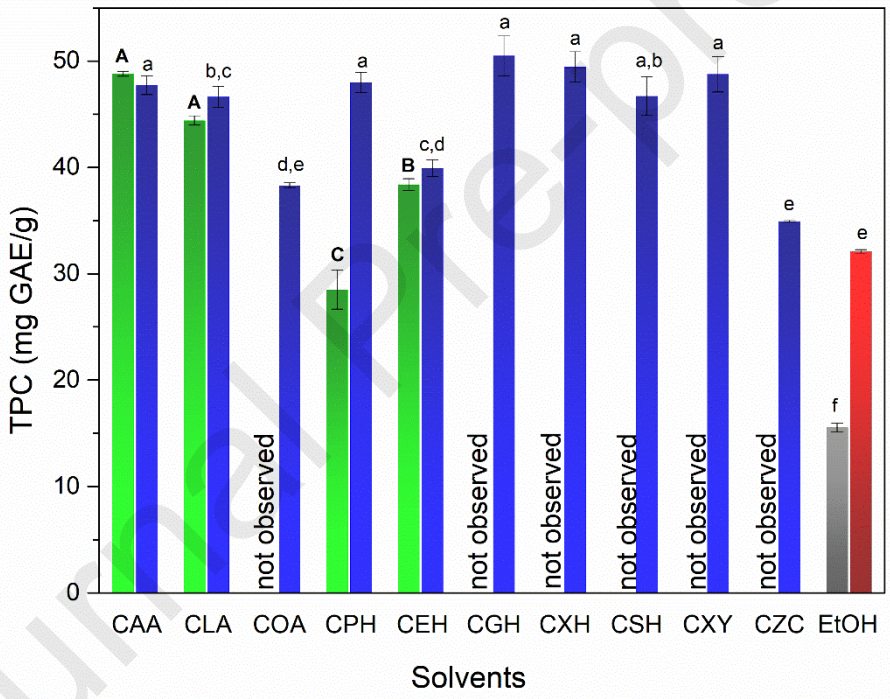


Fig. 2 – Total phenolic content of extracts (mg GAE/g) obtained from different solvents: pure DES (■), and with 30 % wt of water addition (■); pure ethanol (■) and ethanol 70 % wt (■) as controls, at 30 ºC, 2.5 h, and 20:1 (L:S ratio). Absent green bars, indicated as "not observed", means unsuccessful extraction of that DES without water addition. Different small letters on the top of blue bars and different capital letters on green bars represent statistical difference, ($p < 0.05$) determined by ANOVA and Fisher LSD test.

For this purpose, 30 wt% of water was added to the ten DES, and the extractions were carried out at the same condition as before. It is worth noting that the addition of water allowed the use of all DES. Furthermore, except for CAA, an increase in the TPC when compared to DES without water was observed, as indicated by the blue bars in Fig.2. The most pronounced effect of adding water was observed for CPH, in which the TPC is 68% higher than the neat DES without water.

CAA, CPH, CGH, CXH, and CHY exhibited the highest TPC values, about 50 mg GAE/g, without significant difference among these samples ($p > 0.05$). All the extracts obtained by hydrated DES were richer in phenolic compounds (220 to 320 %) than absolute ethanol (gray bar, Fig.2), indicating better ability to extract rosemary biomolecules, as found by others [15,27].

3.2 COSMO-RS relative solubility prediction

Despite the hydrophobicity of the main biomolecules present in the rosemary extract, it is clear the positive effect of water addition to the DES. For a better understanding of this effect the relative solubility of the main rosemary bioactive compounds (carnosic acid, carnosol and rosmarinic acid) were evaluated using the COSMO-RS and compared to the results from section 3.1.

The relative solubilities of each compound in the various DES (with 30 wt% of water) were estimated at 30 ºC. For each compound, the logarithm of the highest relative solubility was set to 0, and all other solvents were ranked relatively to the best one [27,36]. Those which presents log (x_{solute}) data close to 0 are the best solvents [45]. The same procedure was carried out for the neat DES, and results are available in the Table S1 (Supplementary Material).

The phenolic content and predicted relative solubility of the main rosemary bioactive compounds in all evaluated DES were plotted in Fig.3, where it is possible to observe a direct correlation between the variables. This correspondence was statistically verified by Pearson's coefficient (r), where zero indicates the absence of relationship and 1, perfect linear correlation. A r-value greater than 0.5 indicates a high level of correlation [46]. In this work, the correlation was statistically verified by a Pearson's coefficient of 0.73, 0.77 and 0.80 for rosmarinic acid, carnosol and carnosic acid, respectively. COSMO-RS prediction showed a drop in the relative solubility of evaluated biomolecules for COA and CZC, which explains the lowest TPC values obtained using these solvents. These samples exhibited the lowest content of carnosic acid and rosmarinic acid, *e.g.* 4.12 and 6.21 mg of carnosic acid and rosmarinic acid, respectively for CZC (Table S2, Supplementary Material). On the other hand, CXY achieved one of the highest TPC, as well carnosic acid content (10.15 mg /g) and the maximum relative solubility for all the biomolecules. These results show that the higher the solubility of a compound in a solvent, the greater extraction efficiency it achieves, as suggested by Bi et al. (2013). From there, we can suggest for other experiments related to solid-liquid extraction, the use of this approach to assist in the selection of solvents, instead of the trial and error methodology.

Moreover, Fig.3 indicates that the relative solubilities of carnosic and carnosol are close to each other, due to molecular similarity as described by their sigma-profiles (Fig. S1, Supplementary Material). Since these compounds represent more than 90 % of the antioxidant activity of rosemary extract [48], a suitable solvent should be able to solubilize them.

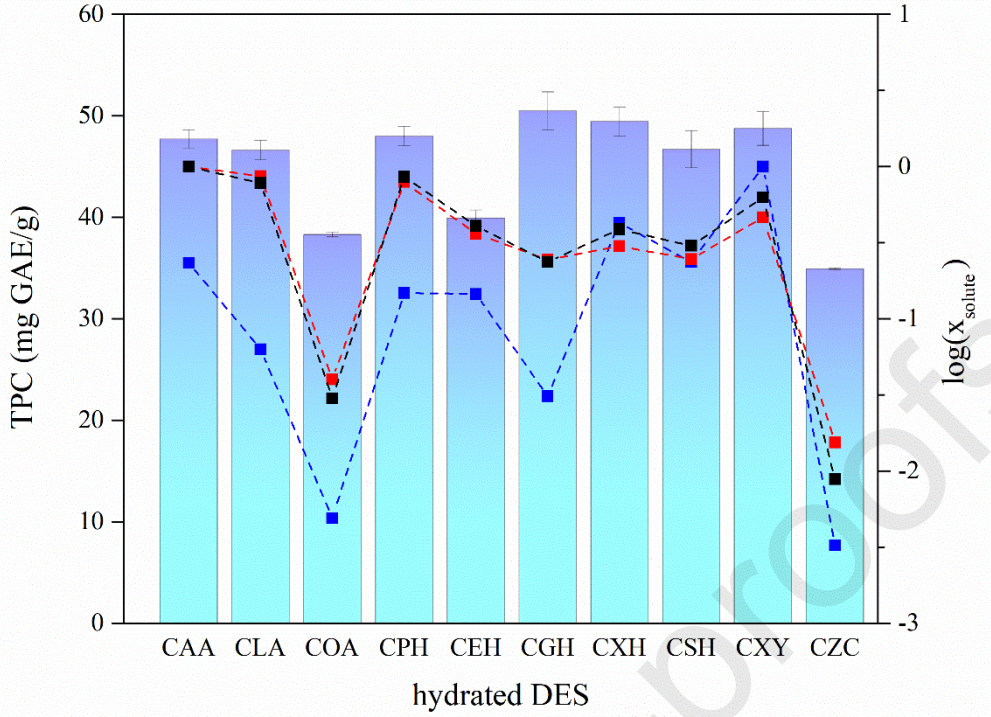


Fig.3 – Relative solubility of carnosic acid (■), carnosol (■), and rosmarinic acid (■) into different DES (30 % of water, 30 ºC), and their phenolic content in mg GAE/g (bars ■) obtained by these solvents. Dashed lines are a guide for visual evaluation.

According to COSMO-RS predictions, the relative solubilities of the evaluated biomolecules were lower after water addition, probably due to the high level of biomolecules' hydrophobicity. Through Fig.S1 it is clear that carnosic acid and carnosol have a large surface between -1.0 and +1.0 $e/\dot{A}^2$, indicating high hydrophobicity. Rosmarinic acid, instead, has a lower apolar zone, with peaks in the polar region (higher than ± 1.0 $e/\dot{A}^2$). Despite the lower predicted relative solubility, the content of carnosic acid and rosmarinic acid were higher in the hydrated DES, Table S2. It means that water addition did not enhance the solubility but instead improved the mass transfer, overcoming its negative effect on the relative solubility of these molecules.

Based on the results from section 3.1, further supported by the COSMO-RS results, choline chloride:1,2-propanediol (CPH) was selected as the best solvent. In addition to the good performance showed in the TPC and chromatographic analyses, it should be highlighted that the presence of 1,2-propanediol in foods is authorized by the main regulatory agencies [11]. Finally, the ease of CPH preparation and the wide industrial use of 1,2-propanediol [49] contributed to this choice.

Besides the solvent selection and its relative solubility analysis, it is also necessary to evaluate the solid-liquid extraction conditions. In the following section, the water content of CPH, extraction temperature, and the L:S ratio was optimized. The dependent factors were TPC and the antioxidant activity of the extracts.

3.3 Optimization of solid-liquid extraction

After the preliminary screening of the various DES and their aqueous solutions at fixed conditions, CPH composed of 1,2-propanediol as HBD and choline chloride as HBA was selected for further optimization. A full factorial design (2^3) combined with response surface methodology (RSM) was applied to optimize the TPC and antioxidant activity of rosemary extracts. Table 4 shows the experimental data. The TPC and antioxidant activity values were determined as a function of extraction temperature (X_1, ºC), L:S ratio (X_2), and water content (X_3, wt%).

Table 4. Results of phenolic content and antioxidant activity of rosemary extracts obtained from different experimental conditions according to the factorial design

Samples	T (X_1) °C	L:S ratio (X_2)	Water content (X_3) wt %	TPC mg GAE/g	Antioxidant activity mg TE/g
1	25	10:1	10	$32.90^{f} \pm 1.35$	$39.56^{c} \pm 0.04$
3	25	40:1	10	$61.21^{c,d} \pm 0.63$	$20.19^{e} \pm 0.00$
5	25	10:1	50	$39.93^{e,f} \pm 0.66$	$35.87^{c,d} \pm 0.81$
7	25	40:1	50	$50.41^{c,e} \pm 1.03$	$19.54^{e} \pm 0.04$
2	65	10:1	10	$64.02^{d} \pm 1.03$	$79.31^{a} \pm 1.62$
4	65	40:1	10	$81.50^{a} \pm 1.74$	$32.90^{d} \pm 0.06$
6	65	10:1	50	$42.87^{e,f} \pm 2.37$	$79.66^{a} \pm 0.00$
8	65	40:1	50	$77.80^{a,b} \pm 4.47$	$80.71^{a} \pm 0.63$
9	45	25:1	30	$67.27^{b,d} \pm 5.19$	$52.00^{b} \pm 1.37$
10	45	25:1	30	$69.23^{b,d} \pm 3.27$	$51.03^{b} \pm 2.04$
11	45	25:1	30	$71.19^{b,d} \pm 4.41$	$52.96^{b} \pm 1.73$
		ANOVA (p- value)		< 0.01	< 0.001

Results expressed as mean ± standard deviation. Different letters in the same column represent significant difference according to Fisher's LSD test ($p < 0.05$). Independent variables: T- extraction temperature, L:S (liquid: solid ratio); Water added to the solvent.

The F test and *p*-value were applied to evaluate the significance of each coefficient, and the results are in Table S3. The high *F*-value associated with a small *p*-value indicates significant corresponding variables [50]. It is worth noticing that the higher the correlation coefficients (R^2), the lower the difference between experimental and predicted values. The obtained R^2 of the response variables were 0.86 and 0.99 for TPC and antioxidant activity, respectively, indicating good predictive power of the models.

For TPC, the significant model terms ($p < 0.05$) were X_1, X_2, X_3 and $X_1.X_2.X_3$. It means that extraction temperature, L-S ratio, and water content, and their interaction, influenced significantly the TPC of the rosemary extract. According to Table 4, the highest TPC (81.5 mg GAE/ g) was found at 65 ºC, 40:1 L:S ratio, and 10 wt% of water addition. For antioxidant activity, all variables were significant, except the interaction between temperature with L:S ratio ($X_1.X_2$). The maximum antioxidant activity (80.71 mg Trolox /g) was found at 65ºC, 40:1 (L:S ratio), and 50 wt% of water addition.

The plots (Fig. 4A-F) showed the positive effect of temperature on the variable responses. This means that the increase in temperature enhances TPC and antioxidant activity, which reinforces the biomolecules stabilization capacity of DES [15]. In addition, the positive effect of temperature seems to be a result of its influence on diffusion, solubility rates, and solvents viscosity reduction [51].

In this work, the L:S ratio effect on TPC was positive, mainly at higher temperatures (> 45 ºC), as shown in Fig.4-D. The higher amount of solvent promoted favorable interactions with the matrix and the DES due to enhanced contact between the parts. Furthermore, a raise in L:S ratio increases the contact between the two phases, which enhances the extraction until the equilibrium is reached [45].

For antioxidant activity (Fig 4-E), the interaction effect between variables X_2 (L:S ratio) and X_3 (water content) show a significant dependence ($p < 0.05$). Apparently, the negative impact caused by water addition on the solubility of rosemary compounds, highly hydrophobic, was lower than its benefit on viscosity reduction, which intensified mass transfer phenomena. This hypothesis was further confirmed by the higher content of carnosic and rosmarinic acid in extracts obtained using hydrated DES, compared to neat DES.

The optimal conditions to extract phenolics from rosemary, obtaining high antioxidant activity were: extraction temperature 65 ºC, L:S ratio 40:1, and water addition of 50 wt%. At these conditions, the antioxidant activity was 80.71 mg TE/g and TPC of 77.80 mg GAE/g.

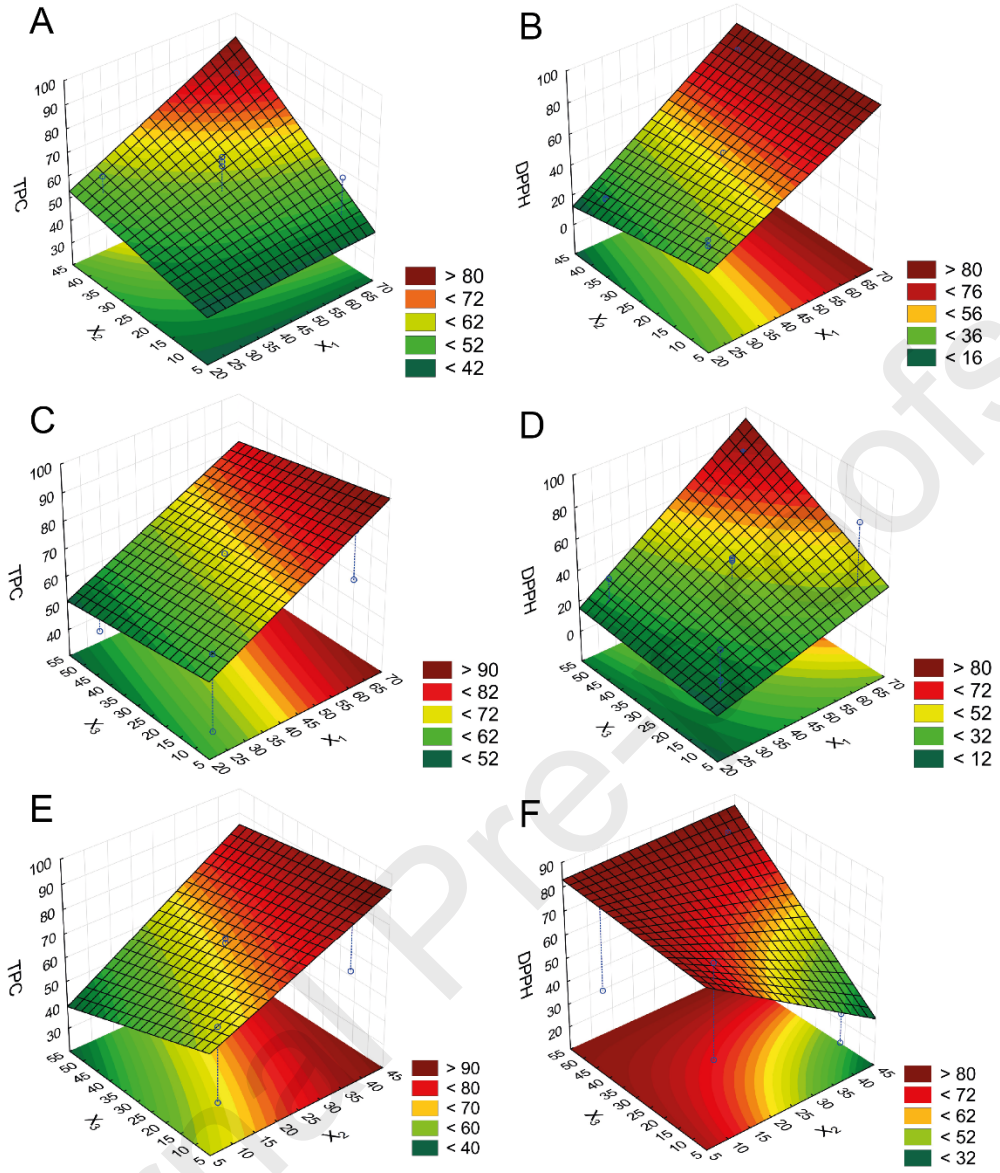


Fig 4 – 3D plots of the: **(A-C-E)** total phenolic content (TPC, mg GAE/g) and **(B-D-F)** antioxidant activity (DPPH, mg TE/g) of rosemary extracts with the combined effects of extraction temperature (X_1, ºC), L:S ratio (X_2) and water content (X_3, wt%).

3.4 Antibacterial assays

The study of the antibacterial properties of the rosemary extract was carried for the extract obtained at the optimal conditions (sample 8, obtained at a temperature of 65ºC, L:S ratio of 40:1, 50 %wt of water) and the results are presented in Table 5. Among

the studied bacteria, the lowest inhibition effect of the extract was observed for *E.coli.* Their resistance could be related to the gram-negative status and the lower permeability of their surface for phenolic compounds [52]. Besides, according to t-student test, no significant difference between %IE obtained by the extract or solvent was found for this bacteria ($p>0.05$).

Gram-positive bacteria (*Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens*) showed intermediate results for inhibition and values between 46-57 %IE for the extract and 40-51 %IE for pure DES. At 95% of confidence, according to t-student test results, rosemary extract increased significantly the % IE for *S.aureus* and *C.perfringes*.

The gram-negative bacteria *Salmonella sp*. demonstrated the highest susceptibility to the CPH-based extract. The compounds extracted from rosemary leaves can suppress *Salmonella* sp. strain with 64% of the antibiotic power. Moreover, significant differences ($p < 0.05$) between the %IE provided by the extract and the pure DES were observed for these bacteria. It indicates that the bioactive molecules extracted by CPH from the plant contributed positively to the inhibition of *Salmonella* sp.

The CPH rosemary extract showed a similar performance to that reported by Jordán and co-workers using methanolic rosemary extracts against *S.aureus (*about 51 % of inhibition) and superior results for *L.monocytogenes,* 57% against 47% of inhibition obtained by Jordán et al. (2012).

Solvents such as methanol, ethanol and water do not affect bacterial growth [4]. On the other hand, the current findings of CPH showed 39-51% of inhibition effect on all bacteria tested, either gram-positive or gram-negative. Part of this effect is due to the hydrogen bond donor, 1,2-propanediol. According to Kinnunen and Koskela, 1,2-

propanediol was effective against *S. aureus* and *E. coli*, and could be valuable in ointments for eczema treatment [54].

Table 5. Antibacterial results as inhibition zones (mm) and inhibition effect (%) of rosemary optimized extract (CPH, temperature of 65ºC, L:S ratio of 40:1, 50 %wt of water addition) tested against different bacteria.

Bacteria	S/I/R*	Inhibition zones (mm)	% IE[1]		p-value**
		Antibiotic	Extract	Solvent	
Listeria monocytogenes	I	28.37 ± 0.95	57.24[b]	51.22[a]	0.056
Staphylococcus aureus	I	29.86 ± 0.84	51.07[c]	47.92[a]	0.034
Clostridium perfringens	I	28.37 ± 1.66	46.70[c]	40.61[b]	0.006
Escherichia coli	R	28.88 ± 0.48	40.20[d]	39.61[b]	0.906
Salmonella sp.	S	28.94 ± 1.28	64.27[a]	43.54[b]	0.002

[1]Inhibition effect (%) regarding the antibiotic results.
*Status of the bacteria inhibition with extract sample.
** p-value < 0.05 (t-student test) indicates significant difference between %IE of extract and solvent, for the corresponding bacteria.
Different letters in the same column represent significant difference among bacteria according to Fisher's LSD test ($p < 0.05$).

These results show that rosemary extracts obtained using DES, especially those composed of propanediol, have considerable antimicrobial activities. Note that no plant extract is expected to present the full antibiotic power of bacterial inhibition. Thus, the findings were encouraging, with more than 40 %IE for all bacteria with the rosemary extracts.

4. Conclusions

The current work presents an efficient approach to extract phenolic compounds from rosemary leaves using choline chloride-based DES. The best solvent identified was the CPH, composed by [Ch]Cl: 1,2-propanediol, with a more hydrophobic character than

the other evaluated DES and the traditional organic solvent, ethanol. Moreover, water addition reduced DES viscosity, improving mass transfer and extraction yield of phenolics. It was demonstrated that COSMO-RS allows the evaluation of the relative solubilities of biocompounds from rosemary, which exhibited good correlation with TPC values from DES-based extracts, supporting the choice of the proper solvent. This method can be applied as a guide for the next experiments regarding solid-liquid extraction of biocompounds from natural sources.

The optimization carried out by experimental design, led to the following conditions: extraction temperature of 65 ºC, L:S ratio of 40:1, and 50 wt% water content, using CPH as solvent. DES-based extract showed 39-51% of inhibition effect compared to antibiotic results to all bacteria tested, either gram-positive or gram-negative. Regarding salmonella, the extract reached 64% inhibition (compared to antibiotic). This work revealed CPH as a suitable solvent to obtain extracts from rosemary leaves, rich in phenolic compounds, that could be used as a food additive with antioxidant and antimicrobial properties.

Appendix A. Supplementary data

Supplementary data to this article can be found online at "…".

The relative solubility data of rosemary antioxidants provided by COSMO-RS into different neat DES; the chromatographic results of carnosic and rosmarinic acids extracted through solid-liquid extraction using DES; the sigma-profiles of rosemary

biomolecules obtained by COSMO-RS; the ANOVA statistics table of phenolic content and antioxidant activity.

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Supplementary Material

Extraction of Phenolic Compounds from Rosemary using Choline Chloride – based Deep Eutectic Solvents

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Contents

Number of pages: 3

Number of figures: 1

Number of tables: 3

Figures/Tables

Table S1. Relative solubility of rosemary antioxidants into different neat DES.

Neat DES	log (x_{solute})		
	Rosmarinic acid	**Carnosic acid**	**Carnosol**
CAA	-1.570	-0.404	-0.219
CLA	-2.259	-0.540	-0.317
COA	-3.908	-2.389	-2.063
CPH	-1.568	-0.404	-0.287
CEH	-1.593	-0.768	-0.672
CGH	-2.577	-1.103	-0.916
CXH	-0.435	-0.327	-0.319
CSH	-0.788	-0.469	-0.157
CXY	0.000	0.000	0.000
CZC	-3.845	-2.262	-1.715

Table S2. Experimental data of carnosic acid and rosmarinic acid contents of different extracts obtained through solid-liquid extraction using neat and hydrated DES (30 wt.%)

Hydrated DES	carnosic acid	rosmarinic acid
CAA	$10.79^{b} \pm 0.22$	$13.65^{b} \pm 0.23$
CLA	$11.85^{a} \pm 0.28$	$12.07^{c} \pm 0.05$
COA	$4.50^{g} \pm 0.24$	$8.82^{e,f} \pm 0.25$
CPH	$9.01^{e} \pm 0.12$	$8.69^{f} \pm 0.16$
CEH	$5.33^{f} \pm 0.02$	$9.05^{e} \pm 0.11$
CGH	$9.45^{d} \pm 0.09$	$14.45^{a} \pm 0.37$
CXH	$8.98^{e} \pm 0.15$	$13.45^{b} \pm 0.11$
CSH	$9.78^{d} \pm 0.14$	$7.15^{g} \pm 0.03$
CXY	$10.15^{c} \pm 0.21$	$9.87^{d} \pm 0.12$
CZC	$4.12^{g} \pm 0.08$	$6.21^{h} \pm 0.38$

Neat DES	carnosic acid	rosmarinic acid
CAA	$8.45^{b} \pm 0.12$	$7.94^{b} \pm 0.08$
CLA	$9.32^{a} \pm 0.18$	$9.34^{a} \pm 0.11$
CEH	$5.48^{c} \pm 0.09$	$7.45^{c} \pm 0.05$
CPH	$5.32^{d} \pm 0.10$	$1.51^{d} \pm 0.03$

Results expressed as mean ± standard deviation. Different letters in the same column represent significant difference according to Fisher's LSD test ($p < 0.05$). Carnosic acid and rosmarinic acid expressed in mg/g.

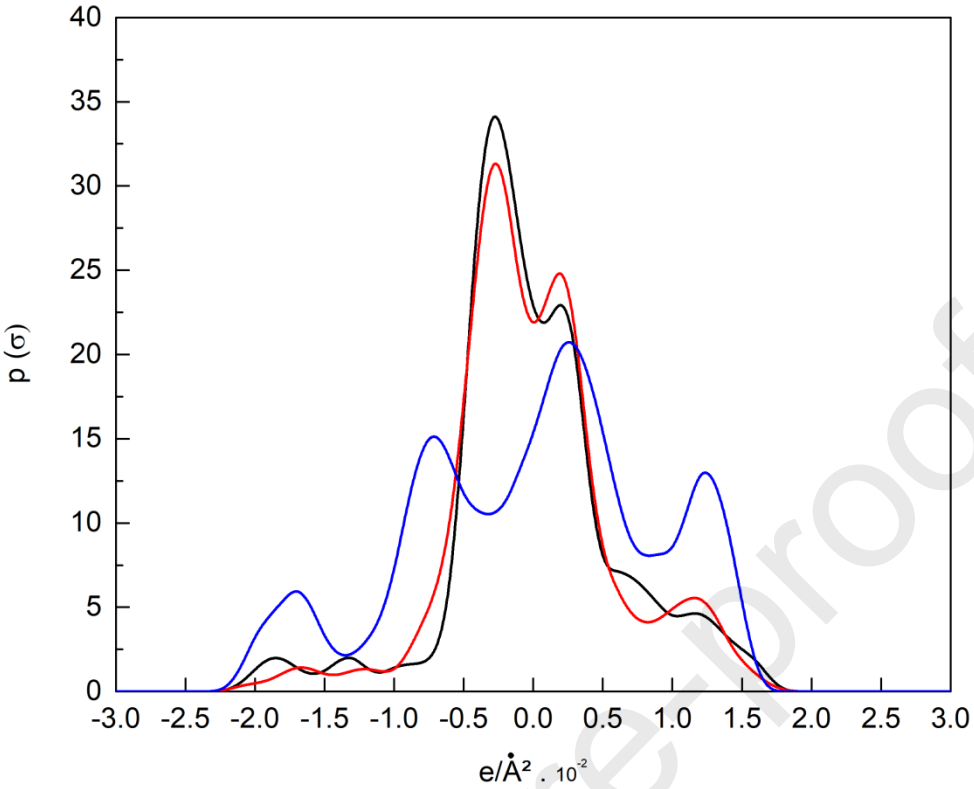
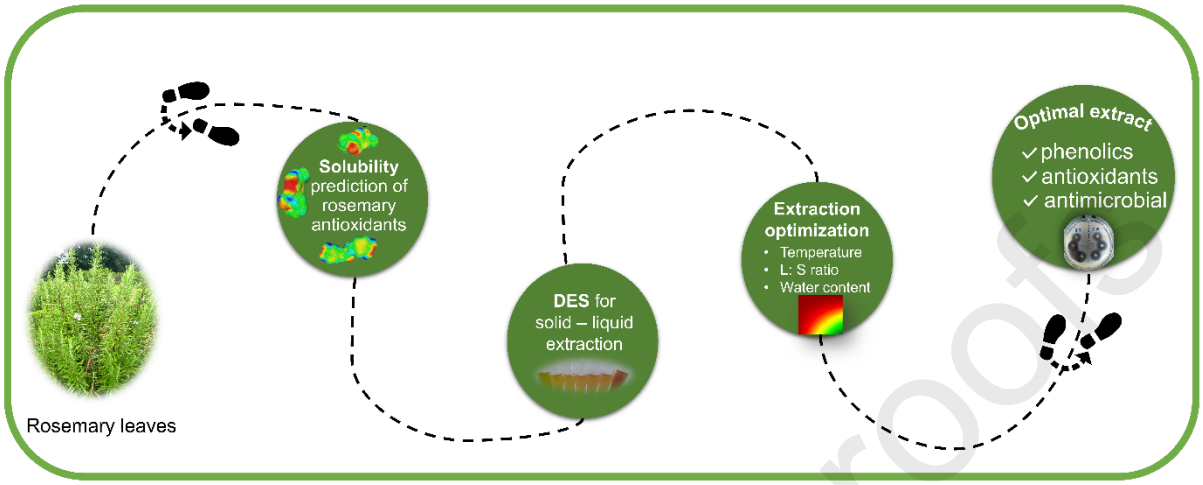


Fig. S1 – Sigma profiles obtained by COSMO-RS of rosemary biomolecules: carnosic acid (___), carnosol (___) and rosmarinic acid (___).

Table S3. ANOVA statistics of phenolic content and antioxidant activity models from rosemary extracts.

Variables	**TPC** mg GAE/ g			**Antioxidant activity** mg TE/ g		
	Means square	**F-value**	***p*-value**	**Means square**	**F-value**	***p*-value**
X_1	835.21	217.30	0.00457	3097.79	3326.56	0.00030
X_2	1039.71	270.50	0.00368	821.35	882.01	0.00113
X_3	102.40	26.64	0.03554	240.03	257.76	0.00386
$X_1.X_2$	23.26	6.05	0.13305	11.69	12.55	0.07126
$X_1.X_3$	55.55	14.45	0.06275	344.40	369.83	0.00269
$X_2.X_3$	0.02	0.00	0.95163	318.74	342.28	0.00291
$X_1.X_2.X_3$	155.61	40.48	0.02382	246.63	264.84	0.00375
R^2	0.856			0.994		



Highlights

1. Ten DES (neat and hydrated) were used for antioxidants extraction from rosemary leaves.
2. COSMO-RS was applied to estimate the solubility of rosemary biomolecules.
3. Choline chloride: 1,2-propanediol – based DES led to promising results.
4. The optimal extract suppressed Salmonella strain with 64% of the antibiotic power.

CRediT author statement

Title: Extraction of Phenolic Compounds from Rosemary using Choline Chloride – based Deep Eutectic Solvents

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

none