Ultrasound-assisted lactic acid based deep eutectic solvent extraction of phenolic antioxidants from *Thymus broussonetii*: A Box–Behnken design approach for optimization

S Kaoui¹*, B Chebli¹, G Ait Baddi², K Basaid¹, M Zaafrani¹ and Y Mir³

¹Laboratory of Mechanic Process Energy and Environment, National School of Applied Sciences, Ibn Zohr University, Agadir, Morocco
²Laboratoire des Sciences de l’Ingénieur et Management de l’Energie, Higher School of Technology, Ibn Zohr University, Agadir
³MIBCM, Faculty of Medicine and Pharmacy, Ibn Zohr University, Agadir, Morocco.

*Corresponding author: soukainakaoui@gmail.com

Abstract. Developing of an eco-friendly, sustainable and efficient solvents with low toxicity and cost has always been a tremendously important goal for industries. In this context, green solvent, such as natural deep eutectic solvent, have been developed as a promising solvent capable of replacing organic ones. The combination of lactic acid: glucose (5:1) was investigated as an extraction medium for bioactive phenolic compounds from *Thymus broussonetii*. The ultrasonication method was established and optimized by a systematic investigation of the influencing factors: water content in solvent (0/35/70 %), extraction time (30/60/90 min), and temperature (30/50/80°C). A Box–Behnken design was adopted including 17 experiments with three center points. The results obtained presented an excellent efficacy of polyphenols extraction ranging from 25.53±0.02 to 153.23±0.03 mg GAE/g dm and a high scavenging activity attending 80.64 %, the optimized conditions selected for both responses were 66.47 min, at 80°C and a 40.72 % of water, with an extraction yields of 144.39±0.02 mg GAE/g dm and 75.9 % for phenolic compounds and antioxidant activity, respectively. Regression analysis showed a good fit of the experimental data which indicates the suitability of the model employed and the successful application of Box–Behnken design in optimizing the extraction conditions. Furthermore, the developed procedure represents an excellent alternative for the extraction of natural products from sample matrices.

1. Introduction
Recently, it has been an increasing public concern about the risks and dangers caused by the usage of conventional petroleum-based solvents, including hexane, methanol ethanol and others, in different areas of applied chemistry, so the design of green solvents with safer ecotoxicological profiles is currently a hot research topic and one of the key subjects in any extraction process. To date, the number available of green solvents for extracting natural products has been rather limited, some of these new “Green Solvents” including water, subcritical water extraction, and other system supercritical fluids have received growing interest over the last two decades. Water and supercritical fluids like scCO₂ are beneficial because they are non-toxic, relatively inert, and easily removable when applied to extracting natural products [1]. However, they are hampered by the low yield of the active principle, the high initial investment, and difficulties to perform continuous extractions [2] during widespread application in...
research and development. In 2011, the pioneering study of Choi and coauthors [3] presented a new class of ecofriendly solvents named natural deep eutectic solvents (Nades) prepared by simply mixing two or more natural components which are generally primary plant-based metabolites mainly organic acids, sugars, alcohols, amines and amino acids [4] that form intramolecular hydrogen bonds together to obtain a eutectic mixture. The discovery of natural deep eutectic solvents (Nades) generally are considered today as proposed new greener generation of ionic liquids (ILs) with higher biodegradability, lower environmental and economic impact, adjustable viscosity, and lower toxicity[5]. Over the past few years, the numbers of publications on these types of solvents have grown exponentially. However, in such phytochemical analysis, ultrasound-assisted extraction (UAE) have been widely applied with DES, which considering today to be ‘greener’, more efficient, and faster extraction method than conventional ones [6,7].

Thymus broussonetii (Lamiaceae) was selected to develop an effective and completely environmentally friendly extraction process for phenolic antioxidant compounds using lactic acid-based deep eutectic solvent. In this study ultrasound-assisted extraction combined with eutectic solvent has been reported as an effective tool for the extraction. Response surface methodology RSM conducted by box benhk design (BBD) was also been used to find the optimal conditions of the extraction process mainly time, the water content in solvents, and temperature.

2. Materials and methods
2.1. Plant material
The dry sample of T.broussonetii aerial parts was pulverized in a laboratory mill to a fine powder. The pulverized sample was stored in plastic tubes at ambient temperature. Detailed information on this substance employed in the analytical method are shown in Table 1.

Table 1. Plant used in this study with common name, family, and origin of collection.

| Botanical name | Common name | Family | Collection site | GPS Coordinates |
|----------------|-------------|--------|----------------|-----------------|
| Thymus broussonetii | Zaatr | Lamiaceae | Sebt gzoula | DMS: 31° 47' 30" - 7° 5' -33" |

2.2. Nades preparation
Natural deep eutectic solvent was prepared according to the previously published procedure [8], lactic acid (hydrogen bond donor) was combined with glucose (hydrogen bond acceptor ) at (5:1) molar ratio. The mixture was then heated up with continual agitation until obtaining a homogeneous transparent liquid.

2.3. Deep eutectic solvent-based ultrasound-assisted extraction (UAE-DES) procedure
The extraction was performed in a 15 ml tube, 0.1 g of sample was added to 10 ml of eutectic solvent. The mixture was vigorously shaken to form a suspension, then extracted using ultrasound-Assisted extraction (UAE) method (Elmasonic, S 60 H, Elma). After the extraction process, the samples were filtered and centrifuged for 10 min at 15,000 rpm (MPW-350R, Med.Instruments) and stored at room temperature until ready to use [9].

2.4. Determination of total polyphenol yield
Phenolics contents of Nades T.broussonetii extracts were determined using Folin–Ciocalteu reagent according to previous study[10]. For this determination, samples were diluted 1:20 with water. In brief, 0.02 mL of sample was mixed with 0.05 mL of Folin-Ciocalteu reagent and 0.78 mL of distilled water, after 1 min, 0.15 mL of aqueous sodium carbonate (Na2CO3) 20% was added and the mixture was incubated for 1 hour in the dark, the phenol content was calculated at 750. In the concentration range of
25–500 mg L⁻¹ (R² = 0.9971). Polyphenols content was expressed as mg gallic acid equivalents (GAE) per g of dry weight (dw).

2.5. (DPPH) radical scavenging activity
Antioxidant activities were determined using radical scavenging (DPPH) assay described by [11] with slight modifications. Dilutions of the extracts were used at 19 percent with water. 3.5 mL of DPPH* methanolic solution (0.045 mg m⁻¹) was rapidly combined with 250 μL of each extract dilution. The mixture was incubated for half hour and at 515 nm, the absorbance was determined (AE). The plant extracts' radical scavenging activity was demonstrated by the decrease in DPPH* concentration. 1.36 was the initial absorbance of the DPPH* solution (ADPPH). As a reference (AREF), gallic acid solution (1000 μg mL⁻¹) was used. Radical scavenging activity of the extracts were determined as inhibition percentage (I %) (Eq. 1):

\[
\% I_{DPPH} = \left( \frac{ADPPH - AE}{ADPPH - AREF} \right) \times 100 \tag{1}
\]

The experiment was carried out in triplicate.

2.6. Extraction optimization using response surface methodology
In regrade to obtain the highest total antioxidant phenolics level, a systematic investigation of the influencing parameters was used to optimize the extraction process. Viscosity, temperature and extraction time were selected as the most vital parameters affecting the extraction capacity, A Box–Behnken design was adopted conducted by response surface methodology (RSM), as a statistical design technique for studying the interactions between factors in an appropriate variable range [12]. The extraction process was conducted using 3 factors (Table 2) and 3 levels (including low, medium, and high) with 17 total experiments.

| Table 2. Independent parameters, their code, range and levels selected in extraction process. |
|---------------------------------|---------------|--------|--------|--------|
|                                | Lactic: Glucose (5 :1) |
| Parameters factor              | Unit   | Low   | Medium | High   |
| Water content                  | %      | 0     | 35     | 70     |
| Time                           | Minutes| 30    | 60     | 90     |
| Temperature                    | °C     | 30    | 55     | 80     |

The general equation for predicting the extraction process optimal conditions is given in Equation 2 as follows:

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ij} X_{ij} + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \tag{2}
\]

where k is the number of variables X (Xi,j) were the coded variables for response Y; \( \beta \) (\( \beta_0, \beta_{ii}, \beta_{ij} \)) were the regression coefficients for intercept, linearity, square and interaction, respectively.

2.7. Data analysis
All the statistical analysis were processed in the Design Expert 7.1.0. statistical program (Stat-Ease Inc, Minneapolis, MN, USA), carried out in triplicate and were evaluated with the lack of fit, the regression coefficient (R²), and the F-value obtained from the analysis of variance (ANOVA).
3. Results And Discussions
3.1. Extraction Efficiency: Antioxidant phenolic compounds extraction
Based on the results in Table 3 the solvent tested show an impressive performance, *T.broussonnetii* extracts revealed an interesting phenolics contents ranging from 25.53±0.02 to 153.23±0.03 mg GAE/g dm. The highest TPC (153.23 mg AGE/g of dm) was recorded at 55°C, with 35% of water and after 60 min (Run 12) and did not increase further as the extraction proceeded, this indicated that 60 min was sufficient to obtain a satisfactory extraction efficiency, the lowest concentration (25.53mg AGE/g of dm) was discovered in the condition of 0% water, 30°C, and 60 minutes (Run 2). Confirming the high yield of total polyphenols obtained, the eutectic *T.broussonnetii* showed a high scavenging activity attending 80.64 %, at the concentration of 1,9 mg/ml (Table 4 and 5, Fig. 1), The highest value was obtained at 55 °C, 70% of water and for 30 min (Run 5) and the lowest value 13.63% was found at 55°C, 0% water and for 30 min (Run 14).

Table 3. Experimental responses obtained.

| Run | Water content (%) | Temperature (°C) | Time (min) | polyphenol yield (mg of GAE /g of dm) | DPPH Radical Scavenging Activity (%) |
|-----|-------------------|------------------|------------|--------------------------------------|-------------------------------------|
| 1   | 70.00             | 55.00            | 90.00      | 106,051282                           | 79,2424242                          |
| 2   | 0.00              | 30.00            | 60.00      | 25,5384615                           | 18,5606061                          |
| 3   | 35.00             | 55.00            | 60.00      | 112,717949                           | 68,9015152                          |
| 4   | 70.00             | 80.00            | 60.00      | 107,589744                           | 70,8712121                          |
| 5   | 70.00             | 55.00            | 30.00      | 132,717949                           | 80,6439394                          |
| 6   | 0.00              | 55.00            | 90.00      | 88,6153846                           | 38,2575758                          |
| 7   | 0.00              | 80.00            | 60.00      | 151,692308                           | 47,8030303                          |
| 8   | 70.00             | 30.00            | 60.00      | 98,8717949                           | 64,0530303                          |
| 9   | 35.00             | 80.00            | 30.00      | 127,076923                           | 75,4166667                          |
| 10  | 35.00             | 55.00            | 60.00      | 109,128205                           | 71,1742424                          |
| 11  | 35.00             | 80.00            | 90.00      | 137,846154                           | 70,6439394                          |
| 12  | 35.00             | 55.00            | 60.00      | 153,230769                           | 64,8863636                          |
| 13  | 35.00             | 55.00            | 60.00      | 106,051282                           | 56,5530303                          |
| 14  | 0.00              | 55.00            | 30.00      | 37,8461538                           | 13,6363636                          |
| 15  | 35.00             | 30.00            | 90.00      | 96,3076923                           | 48,4848485                          |
| 16  | 35.00             | 55.00            | 60.00      | 128,615385                           | 63,6742424                          |
| 17  | 35.00             | 30.00            | 30.00      | 97,3333333                           | 53,5984848                          |

According to the literature, this is the first study that used lactic acid based Nades as a green extraction solvent for *T.broussonnetii*, however, the efficacy of lactic acid-based deep eutectic solvents had been proved also recently in variety of Thymus plants.

In 2017, A study evaluated by Georgantzí et al [13] for polyphenol extraction from *Thymus vulgaris; Thymus capitatus*, as a result, lactic acid: nicotinamide and lactic acid: L-alanine, at of 7:1 was selected as the effective DESs components, exhibiting a significantly higher level of polyphenol yield compared with ethanol 60% and water.

3.2. Model fitting
As mentioned above, optimization of the extraction condition was performed by BBD with a total of 17 experiments were performed. The analysis of variance (ANOVA) was statistically significant, the model quality was evaluated in terms of the determination coefficient ($R^2=0.9240/ 0.8438$) and the low
probability values (P < 0.05) which indicate that the models were significant and adequate for reasonable prediction of the phenolic antioxidant’s extraction yields.

### Table 4: ANOVA Statistical Results.

| Model  | polyphenols P-value | Antioxidant Activity P-value |
|--------|---------------------|------------------------------|
| Model  | 0.0358              | 0.0036                       |
| A-water content | 0.0441              | 0.0001                       |
| B-T°  | 0.0091              | 0.0104                       |
| C-time | 0.5763              | 0.5814                       |
| AB     | 0.0238              | 0.2116                       |
| AC     | 0.9998              | 0.1547                       |
| BC     | 0.7811              | 0.9839                       |
| A²     | 0.9422              | 0.0201                       |
| B²     | 0.8962              | 0.5021                       |
| C²     | 0.5680              | 0.9633                       |
| R²     | 0.8438              | 0.9240                       |

#### 3.3. Effect of extraction parameters on the yield of polyphenols and scavenging activity

In the current study, three-dimensional (3D) response surface plots with one factor fixed and the other two factors varied within set limits were investigated for graphical interpretation of the significant interactions between the variables. Figure 1 shows the response surface plots of the phenolic antioxidant extraction yields as a function of water content, extraction time, and temperature.

**3.3.1 Effect of water content**

Viscosity is an important property extensively studied by researchers; a high viscosity is a big drawback for any deep eutectic solvents to be used for any application. To date, the majority of eutectic solvents that have been reported are extremely viscous which leading to some practical issues, suggesting a low fluidity, which is thought to be caused mostly by the numerous hydrogen bonds that occur between the components of deep eutectic solvents. According to Dai, Zhang and coauthors [14,15] sugars-formed systems and metal salt-based deep eutectic solvents presented higher viscosities to those formulated with acids or polyalcohols, since the smaller molecules such as polyalcohols leading to weaker intermolecular forces. Therefore, to overcome this issue adding water to a deep eutectic solvent is often applied and have been investigated by several authors [16, 17] as a good way to decrease viscosity, leading to a diminution in density and viscosity of the solvent, thus to a better penetration of the solvents in the sample. In our study, the response-surface plots showed the positive effect of water content on the variable’s responses, the total polyphenol content and the antioxidant activity increased with increasing water content from 0 to 52 % while further increases led to decreases slightly in content of these responses.

**3.3.2 Effect of extraction temperature**

Temperature is an important factor which affects extraction, generally increasing temperature enhance secondary metabolites desorption and solubility in extraction solvents. Furthermore, it is another tool used by researchers to reduce the viscosity of eutectic solvent which accelerate by consequence releasing of secondary metabolites from the plant to the solvent. As shown in Fig. 1, extraction efficiencies are markedly changed with an increase in temperature, especially when temperature was raised from 30 to 80°C. This might be due to high temperature which reduce also, same as water, viscosity, and interactions between the target components and matrices improving the extraction performance and make the penetration of the Nades easier.
3.3.3 Effect of extraction time

The extraction time was an important parameter contributing to improve the extraction efficiency, as shown in Fig. 1, the processing time was ranging from 30 to 90 minutes. As a result, the extract exhibit

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Figure 1. 3D response surface plots of the interactions between three extraction factors (extraction time, extraction temperature and the water content) for polyphenols yields and antioxidant activity.
better performance at medium extraction time, between 30 and 65 minutes, so it was considered as the appropriate time for the extraction. Nevertheless, as long as the time of the extraction extend over 65 the yield of two responses increases but slightly.

3.4. Optimization of extraction conditions and verification of the models
Although all three optimized parameters have a substantial impact on the extraction process, the effect of water content in solvents was relatively bigger. Using the developed models above for parameter optimization, the desirable conditions selected presented in Table 5. To confirm the fitness of the predicted responses values, we performed verification experiments under the selected conditions in two repetitions. A slight difference was noticed between the predicted extraction content of both responses and the experimental results, which proved the fitness of design used for determining the optimized processing conditions with the desired results.

Table 5: The optimized parameters used in the extraction process and comparative yield of the predicted and experimental results.

| Optimal Parameters | Predicted results | Experimental results |
|--------------------|-------------------|----------------------|
| Water content (%)  | Time (min)        | Temperature (°C)     |
| Phenolics yield (mg GAE /g dw) | Radical scavenging activity (%) |
| Phenolics yield (mg GAE /g dw) | Radical scavenging activity (%) |
| Lactic Acid: glucose (5:1) | 40.72 | 66.47 | 80 | 144.56 | 74.66 | 144.394 ±0.02 | 75.9±0.05 |

Conclusion
The current work presents an efficient green approach to extract bioactive compounds from Thymus broussonnetii using a lactic acid-based deep eutectic solvent. A fast and green Nades-UAE procedure was established for the extraction of phenolics antioxidant compounds and the optimization was carried out by an experimental design, revealing that Nades tested composed of Lactic Acid: glucose (5:1) improve an excellent extraction capacity achieving 144.39 mg GAE/g dw as a phenolic content and an excellent antioxidant activity higher than 70% under specified extraction conditions: 40.72% of water content, extraction temperature and time of 80°C, and 66.47 min respectively. The outstanding properties of Nades such as great biodegradability, low volatility, toxicity and environmental economic impact[5] highlight their potential as ecofriendly solvents for other active components extraction. Nevertheless, the effectiveness of this eutectic extracts such as antioxidant, antibacterial, anti-inflammatory properties, and other biological activities still extremely challenging and under investigation[18].

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