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Analyzing the Effect of Extraction Parameters on Phenolic Composition and Selected Compounds in Clove Buds Using Choline Chloride and Lactic Acid as Extraction Agents

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Abstract: Utilizing a deep eutectic solvent-like mixture such as choline chloride and lactic acid in a 1:2 molar ratio, clove buds underwent extraction. Furthermore, the determination of the primary components in the clove extracts, namely eugenol, eugenol acetate, and β-caryophyllene, was conducted using the HPLC-DAD method. The total phenolic content (TPC) was also established. Extraction conditions using choline chloride and lactic acid encompassed variables such as extraction temperature (within the range of 40 to 80 °C), water addition (ranging from 5.6 to 40%), and extraction time (ranging from 30 to 90 min). Optimal operational conditions for TPC were pinpointed at 77 °C, 30 min, and a water addition of 40%. The findings showed that clove extracts obtained at 60 °C, 22.8%, and 30 min had the highest amount of eugenol (307.26 ± 8.44 mg/g dry raw material).

Keywords: clove buds; choline chloride; lactic acid; extraction; design of experiment; eugenol

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1. Introduction

Syzygium aromaticum, commonly referred to as clove, is an esteemed medicinal plant currently undergoing investigation related to its antiviral and antioxidant properties. This botanical specimen is a valuable source of terpenes, mainly bioactive substances such as eugenol, eugenol acetate and β-caryophyllene [1]. Historically, clove has found applications in traditional medicine for addressing respiratory issues, serving as a warming and invigorating agent to alleviate digestion problems while enhancing circulation and metabolism [2–6].

The essential oil content in clove buds ranges from 15 to 20% by weight, with eugenol dominating (70–95%), accompanied by acetyleugenol (up to 20%) and β-caryophyllene (12–17%) in smaller proportions [7]. As proven by science, cloves and their essential oil are biologically beneficial for human health due to their exceptional qualities [4–6,8–13]. Previous research [13–18] has explored the potential of clove extractives in preventing and treating SARS-CoV-2-associated ailments, emphasizing their antiviral, antioxidant, antimicrobial, antifungal, antinociceptive, cytotoxic, anti-inflammatory, and antithrombotic activities [19–28]. Computational studies [15,16] propose that phytocompounds extracted from cloves, including kaempferol, target the main protease of SARS-CoV-2, which is essential for viral replication, and as such they could be potent drugs against COVID-19 [14]. Clove phytochemicals were tested as possible antiviral agents against the SARS-CoV-2 main protease in another study [26]. A vital technology is the extraction of substances from nature, which can replace materials derived from petrochemicals. Conventional extraction methods

possess drawbacks, including the use of toxic solvents, potential thermal degradation, and hydrolysis of constituents. Modern, environmentally friendly extraction techniques provide avenues for process development to enhance efficiency [29–31]. A crucial step in obtaining desired product yields is identifying the suitable method and conditions for isolation, and the representation of individual components varies in quantity and quality depending on these conditions. The selection of extraction conditions of particular interest, focusing on replacing organic solvents with green alternatives [32–34].

Green extraction techniques with non-toxic solvents have been used by several researchers to study the chemical composition and biological activity of cloves. Supercritical CO_2 extraction of clove buds resulted in remarkable yields (13–23.9 wt%) and eugenol content (56.97–87.41%) in studies [35–42]. Eugenol yields (11.5–21.2 wt%) and concentrations (50.3–87.26%) were lower when obtained via conventional extraction methods.

The conventional hydrodistillation method was used by Gonzales-Rivera et al. [43] to analyze clove essential oil. They determined the levels of eugenol (66.9%), β-caryophyllene (24.8%), α-humulene (3.1%), and acetyleugenol (2.7%) in the oil. Oliviera et al. [44] conducted extraction with supercritical CO_2 under the experimental temperatures of 40 °C and 50 °C and extraction pressure of 10, 20, and 30 MPa. The results revealed the qualitative chemical composition of the oil, which consisted mainly of eugenol (62.88%), E-caryophyllene (17.13%), eugenol acetate (21.20%), and alfa-humulene (2.62%), with eugenol being the most abundant component in all the oil fractions examined. The results also indicated that eugenol, the major constituent, had a significant role in enhancing phytotoxic activity.

Deep eutectic solvents (DES) encompass a variety of categories based on the nature of the complexing agents involved. Type I comprises quaternary ammonium salts and metal halides, although their use in lignocellulosic biomass processing is limited due to the high melting temperatures of nonhydrated metal halides. Type II includes quaternary ammonium salts and hydrated metal halides, offering better viability for industrial processes. Type III involves a mixture of quaternary ammonium salts and various hydrogen bond donors (HBDs); this type is commonly utilized in biomass processing due to its cost-effectiveness and ease of preparation. Type IV consists of inorganic transition metals and HBDs, although the metal salt typically does not ionize in nonaqueous environments. A relatively novel class, Type V, exclusively comprises non-ionic molecular substances and exhibits hydrophobic characteristics under normal conditions [45–49]. Terminological inconsistencies within the field have led to the adoption of the term "DES-like mixtures" for systems lacking a proven eutectic point, with the aim of maintaining clarity amidst evolving terminology.

Various studies have delved into the structural changes, interactions, and hydrogen bonding within systems involving choline chloride and lactic acid [50–54]. Fourier-transform infrared analysis has confirmed hydrogen bond formation within these systems [50,52,53]. Research by Alcade et al. [51] explored the impact of water on hydrogen bonding and observed minimal disruption within the studied range. ^{1}H NMR analysis has revealed interactions between the OH groups of lactic acid and choline chloride, leading to chemical shifts and the formation of new signals indicative of intermolecular interactions [52–54]. Alcalde et al. [51] identified intermolecular interactions facilitated by hydrogen bonding between the chloride ion from choline chloride and lactic acid, with the chloride ion acting as a bridge between choline chloride and multiple lactic acid molecules at a molar ratio greater than 1. In the last 20 years, green solvents have been intensively utilized for the extraction of value-added compounds. Among these, a group of solvents known as deep eutectic solvents (DES), including natural deep eutectic solvents, has gained prominence. Numerous original papers, patents, and conference contributions focus on various aspects of extracting valuable compounds from natural sources each year. Researchers also strive to consolidate these findings in books and review papers [55–57].

Deep eutectic solvent-like mixtures (DES-like mixtures) have gained prominence as promising sustainable solvents [58]. These mixtures, derived from natural components,

offer unique physicochemical properties aligned with ecological requirements, making them appealing for green extraction processes. Another crucial step in extraction is the setting of extraction conditions. Many factors affect how well the extraction process works, such as the type and quantity of solvents used, the temperature of the solution, the concentration of ions, the extraction time, the properties of the analyte, and the agitation of the mixture. These conditions and their interactions have a significant impact on the quantity and quality of the output of individual compounds [59–62].

One key component used to prepare these solvents is quaternary ammonium salt (such as choline chloride), which acts as the hydrogen bond acceptor (HBA). Additionally, hydrogen bond donors play a crucial role, and substances like urea, imidazole derivatives, amides, alcohols, and organic carboxylic acids are commonly employed. These solvents achieve promising results in the extraction of various classes of active substances, especially phenols [63–68].

In recent years, several articles were published in which these solvents were used for the extraction of these substances, while the combination of choline chloride and lactic acid was also often investigated.

In the work of Soukina et al. [63], the combination of choline chloride and lactic acid achieved the highest yield of polyphenols compared to the other six types of solvents used to extract polyphenols from *Mentha pulegium*.

However, based on our knowledge, only a few works have been published that used the extraction of DESs and cloves. In the work of Chen et al. [69], DESs were used as pretreatment solvents; they were combined with microwave-assisted hydrodistillation for the extraction of essential oils from the clove buds. They used five DES systems (Choline chloride Fructose 1:2; Choline chloride Lactic acid 1:2; Choline chloride Glucose 1:2; Choline chloride Phenol 1:2; Choline chloride Ethylene glycol 1:2) and the most suitable was evaluated system chloride and lactic acid (1:2). The extraction efficiency of 10 DESs mainly composed of tetrabutylammonium bromide and long-chain alcohols, including choline chloride and lactic acid (2:3), was evaluated for the extraction of terpenes from six spices (cinnamon, cumin, fennel, clove, thyme, and nutmeg); DESs were used as extraction agents for headspace single-drop microextraction [70].

This is an original work because the extraction of selected active substances and the determination of optimal conditions using the DES (deep eutectic solvent) for clove extraction have not yet been explored in published works. Our primary goal was to use choline DES-like mixtures, that is, a selected system of choline chloride and lactic acid for the extraction of substances with antiviral, antioxidant and anti-COVID properties. And it mainly concerns the monitored substances that we analyzed. In this study, we did not deal with the purification of phenolic substances, considering that these extracts can be used as nutritional supplements without subsequent purification, which should have positive effects in the field of prevention in the fight against COVID-19. The study examined the effects of changing the extraction parameters (temperature, water addition, extraction time) on the TPC and identified the major constituents of clove bud extracts, namely eugenol, eugenol acetate, and β-caryophyllene, by applying the HPLC-DAD method.

2. Materials and Methods

2.1. Chemicals

All chemicals, solvents, and reagents utilized in this research were of analytical grade. Choline chloride (ChCl) (≥98.0%), Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical, gallic acid, anhydrous sodium carbonate (Na_2CO_3), and analytical standards, including eugenol (≥98.0%), eugenol acetate (≥98.0%), and β-caryophyllene (≥80.0%), were procured from Sigma-Aldrich (Bratislava, Slovakia). Lactic acid (LacA) (90.0%) solution was obtained from VWR International (Bratislava, Slovakia). Choline chloride was dried in a vacuum oven prior to use to eliminate moisture contamination.

2.2. Plant Materials

Clove buds were acquired from the store Svet plodu (Czech Republic) [71]. These organic cloves originated from Molucca (Indonesia), where they were grown and dried. Before each experimental assay, the raw material underwent grinding in a knife mill and separation through sieves into 0.02–0.04 mesh.

2.3. Preparation of Deep Eutectic Solvents-like Mixtures

The solvent consisted of ChCl and lactic acid in a molar ratio of 1:2. The preparation of the solvent was carried out with constant stirring in a water bath (60 °C; 30 min) and a homogeneous liquid formed as a result [72].

2.4. Design of Experiment

A comprehensive description of the influence of the studied physical parameters on total phenolic content utilized the planned experiment method. A full 2^3 factorial design was employed, encompassing temperature, water addition, and extraction time as selected physical parameters. After five repetitions of the center point and twenty combinations to measure the standard deviation from the overall mean, the experimental design was complete. The design of the experiment method (DOE) was utilized to represent the mathematical-statistical data of complex formation. The factors that had statistical significance were assessed by analysis of variance (ANOVA). The acquired measurements for each parameter underwent processing based on the principles of experimental design theory using the computer program STATIS, developed by Alexy et al. [73] within MS Excel Visual Basic. The STATIS program output complete regression and statistical analyses, with ANOVA being the employed statistical test, conducted at a 95% probability level. All statistical tests were carried out at a probability level of 95%. For each parameter, the following type of regression Equation (1) was obtained:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}\ x_3^2 \quad (1)$$

where y is an output parameter, x_1, x_2, and x_3 are levels of factors in coded coordinates, b_0–b_{33} are regression coefficients, b_0 is a constant, b_1, b_2 and b_3 are the linear coefficients, b_{12}, $b_{13,}$ and b_{23} are the interaction or crossed coefficients, and b_{11}, $b_{22,}$ and b_{33} are the quadratic coefficients.

2.5. Extraction Using Deep Eutectic Solvents-like Mixtures

In the work of Chen et al. [70], different DES pretreatments were applied. Choline chloride and lactic acid (1:2) combined with microwave-assisted hydrodistillation for the extraction of essential oils from the clove buds proved to be the most suitable pretreatment. In our research, therefore, the extraction system used choline chloride and lactic acid in a molar ratio of 1:2. The extraction conditions were chosen in a sufficiently wide range and corresponded to the conditions of other works, in which researchers investigated the extraction of polyphenols from other plant sources [73,74].

Under the conditions of the experimental design, the extraction was performed with constant stirring (blender speed 12; Tube Revolver Rotator Thermo Fisher Scientific (Bratislava, Slovakia)) in a closed flask. DOE was used to examine the effect of extraction temperature (t = 40–80 °C), water addition (above 5.6 up to 40%), and extraction time (30–90 min) on total phenolic content. The choline chloride: lactic acid (1:2) mixture had a water content of 5.6%. The water content in the DES-like mixtures was determined by coulometric Karl Fischer titration. The ratio of clove buds to extraction solvent was kept at 1:10 (w/v) throughout the experiments.

2.6. Determination of Total Phenolic Content

The content of total phenolic compounds was determined by the Folin–Ciocalteu (FC) method as described in the study of Jablonský et al. [75].

2.7. HPLC Determination of Eugenol, Eugenol Acetate, β-Caryophyllene

Eugenol, eugenol acetate, and β-caryophyllene were determined by the modified HPLC-DAD method. The analysis was performed using Agilent Technologies HPLC system (series 1100) consisting of a degasser, a binary solvent delivery pump, an autosampler, a column thermostat, and a diode array detector (DAD). Separation of analytes was carried out in reverse-phase mode using a Nucleodur 100-5 C18ec (250 × 4.6 mm i.d., 5 µm particles) column (Macherey-Nagel, Düren, Germany). The mobile phase consisted of acetonitrile (A) and water (B). The gradient program was a 0–10 min linear gradient for (A) component from 40 to 80% then to 100% of (A) over 0.5 min and maintained at 100% of (A) for 10 min. This was followed by a reverse gradient over 0.5 min and maintained at 40/60 (A)/(B) for 4 min. The flow rate was 1.0 mL/min, the injection volume was 20 µL, and the column temperature was maintained at 25 °C. DAD was operated in the wavelength range of 190–400 nm, and the detection wavelengths were set at 210 nm for β-caryophyllene. Eugenol and eugenol acetate were detected at 280 nm. The cloves extracts were diluted 1:10 (*v*/*v*) with water before being injected into the HPLC.

Quantification of target analytes was performed using the calibration curve method. The calibration curve was constructed as the dependence of average peak areas of analyte versus concentration of analyte in standard solution. Mixed-analytes calibration solutions at six concentration levels ranging from 100 to 1000 µg/mL for eugenol and eugenol acetate, or from 15.5 to 100 µg/mL for β-caryophyllene, were analyzed in triplicate for the construction of the calibration curve. In Table 1, the values of the retention time, resolution, peak symmetry factor, high equivalence of the theoretical plate, linearity, limit of detection, and limit of quantification are summarized.

Table 1. Determination of eugenol, eugenol acetate, and β-caryophyllene by HPLC-DAD method: system suitability and validation parameters.

	Eugenol	**Eugenol Acetate**	**β-Caryophyllene**
	HPLC system suitability parameters [a]		
Retention time (t_R, min)	7.36	9.09	17.54
Repeatability RSD (%) t_R	0.21	0.25	0.22
Repeatability RSD (%) A	1.65	1.05	1.59
Peak symmetry	1.16	1.14	1.15
HEPT (µm)	17.27	9.33	2.26
R_S	7.53	39.12	
	Validation parameters		
Calibration curve	A = 145.32 + 18,809.18 × c	A = 101.13 + 10,523.43 × c	A = 23.55 + 46,563.62 × c
R^2	0.9998	0.9989	0.9990
Linear range (µg/mL) [b]	100–1000	100–1000	15.5–100
LOD (µg/mL) [c]	7.7	16.6	5.1
LOQ (µg/mL) [c]	23.2	50.3	15.5

[a] the concentrations of eugenol, eugenol acetate, and beta-caryophyllene in standard solution were 0.1 mg/mL, [b] expressed as the concentration of the analyte in the reference solution, [c] the values of (LOD) limit of detection, and (LOQ) limit of quantification were calculated according to Equations (1) and (2), t_R—retention time, A—peak area, HEPT—height equivalent of a theoretical plate, c—con-centration of analyte (in mg/mL), RSD—relative standard deviation, (n = 6).

Equations (2) and (3), respectively, were followed to calculate the LODs and LOQs using the standard deviation of the response (Sy) and the slope (S) of the calibration curve at levels around the LOD and LOQ.

$$\mathrm{LOD} = \frac{3.3 \times \mathrm{Sy}}{\mathrm{S}} \quad (2)$$

$$\mathrm{LOQ} = \frac{10 \times \mathrm{Sy}}{\mathrm{S}} \quad (3)$$

3. Results and Discussion

Various factors can exert independent or combined influences on extraction efficiency. Altering extraction parameters can impact the transport and solubilization of biological macromolecules, influence the extraction kinetics, and modify the physicochemical properties of DES-like mixtures [76,77]. This study delved into the individual and combined effects of extraction temperature, water addition, and extraction time on the overall yield of phenolic compounds obtained through the extraction process utilizing ChCl:LacA (1:2). Adjustments to water addition were made to enhance the diffusion of polyphenols. The chosen ranges for these factors were based on existing scientific knowledge derived from a literature search [78]. The experimental design conditions, detailed in Table 2, aimed to comprehensively elucidate the impact of selected physical parameters on the extraction process. A complete 2^3 factorial design of experiments was implemented, encompassing twenty individual experiments. The measured values of the TPCs are outlined in Table 3.

Table 2. Conditions of experimental design.

Factor	−1.682	−1	0	1	1.682
x_1	40	48.11	60	71.89	80
x_2	5.63	12.6	22.8	33.0	40
x_3	30	42	60	77	90

Table 3. Measured parameter TPC; (mg GAE/g dry raw material) and extraction factors—temperature (factor x_1; °C), water addition (factor x_2; %), time of extraction (factor x_3; min.).

Trial No.	Sample No.	Factor x_1	Factor x_2	Factor x_3	TPC (mg GAE/g Dry Raw Material)
1	Clove_1	48.11	12.6	42	48.8
2	Clove_2	71.89	12.6	42	33.4
3	Clove_3	48.11	33.0	42	89.1
4	Clove_4	71.89	33.0	42	80.5
5	Clove_5	48.11	12.6	78	40.7
6	Clove_6	71.89	12.6	78	34.6
7	Clove_7	48.11	33.0	78	70.3
8	Clove_8	71.89	33.0	78	75.9
9	Clove_9	40.00	22.8	60	43.4
10	Clove_10	80.00	22.8	60	31.6
11	Clove_11	60.00	5.63	60	39.5
12	Clove_12	60.00	40.0	60	111.9
13	Clove_13	60.00	22.8	30	55.8
14	Clove_14	60.00	22.8	90	56.8
15	Clove_15	60.00	22.8	60	59.8
16	Clove_16	60.00	22.8	60	55.9
17	Clove_17	60.00	22.8	60	59.4
18	Clove_18	60.00	22.8	60	74.3
19	Clove_19	60.00	22.8	60	67.4
20	Clove_20	60.00	22.8	60	76.2

The outcomes revealed a significant impact of adding water when utilizing ChCl:LacA (1:2) on the total amount of extracted phenolic compounds from clove buds, as indicated by the regression coefficient b_2 (Table 4). The solubility of compounds correlates with the polarity of DES-like mixtures and can be further enhanced by the addition of water. As shown in Figure 1, TPC exhibits variability based on water addition, ranging from approximately 36 mg GAE/g dry raw material to around 112 mg GAE/g dry raw material. As pointed out by several papers [61,62,79–82], water addition affects the physicochemical properties of solvents and how they interact with biological structures. The presence of a highly polar solvent like water could potentially impact extraction capabilities, such as viscosity. High viscosity is a hindrance for the analytical use of deep eutectic solvents-like mixtures, as it reduces the mass transfer between the sample and the extraction phase. The viscosity of the reaction medium can be lowered by the addition of water, which leads to a better mass transfer, higher extraction efficiency, and enhanced polyphenol diffusion [62,83].

Table 4. Results of ANOVA.

Coefficient	TPC
* b_0	65.164
b_1	0.869
* b_2	24.646
* b_3	−6.214
* b_{11}	−7.745
* b_{12}	9.337
b_{13}	−4.086
* b_{22}	5.780
* b_{23}	−9.064
b_{33}	−1.080

* statistically significant coefficients.

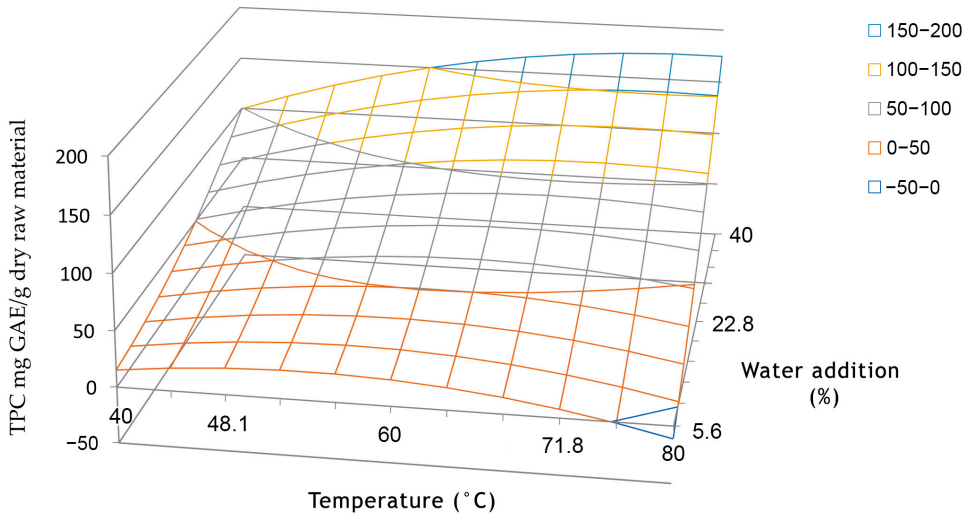

Figure 1. Response surface of total phenolic content in dependency on factor x_1 (temperature, °C) and factor x_2 (water addition %) in coded coordinates at constant factor x_3, (extraction time 30 min).

The outcomes presented in Table 4 indicate that, within the specified range of values, temperature did not significantly impact the yield of phenolics. Both an elevation in extraction temperature and addition of water led to an increase in TPC, as illustrated by the response surface in Figure 1. The *x*-axis (extraction temperature) demonstrates a peak in

total phenolic content, supported by the negative value of coefficient b_{11} (refer to Table 4). This mutual interaction between water addition and temperature is further affirmed by the significant interaction coefficient b_{12} (Table 4). Higher temperatures are known to soften plant tissues, facilitating the extraction of phenolic compounds into the solvent [84]. Additionally, there is a confirmed mutual interaction between water addition and extraction time, as indicated by the interaction coefficient b_{23} (Table 4). In the realm of deep eutectic solvents (DES), water holds substantial significance [85]. As shown in Figure 2, the viscosity of DES-like mixture decreases when water is added [86,87], improving the mass transfer and extraction efficiency and facilitating polyphenol diffusion [62,83]. The polarity of DESs is also influenced by water content, as observed by Craveiro et al. [76], who found that an increase in water quantity heightened solvent polarity. Besides the viscosity, the density of DESs was also affected by water, as reported by Francisco et al. [87]. They discovered that the density of the DESs they investigated declined with the increase in water content, but this effect was minor.

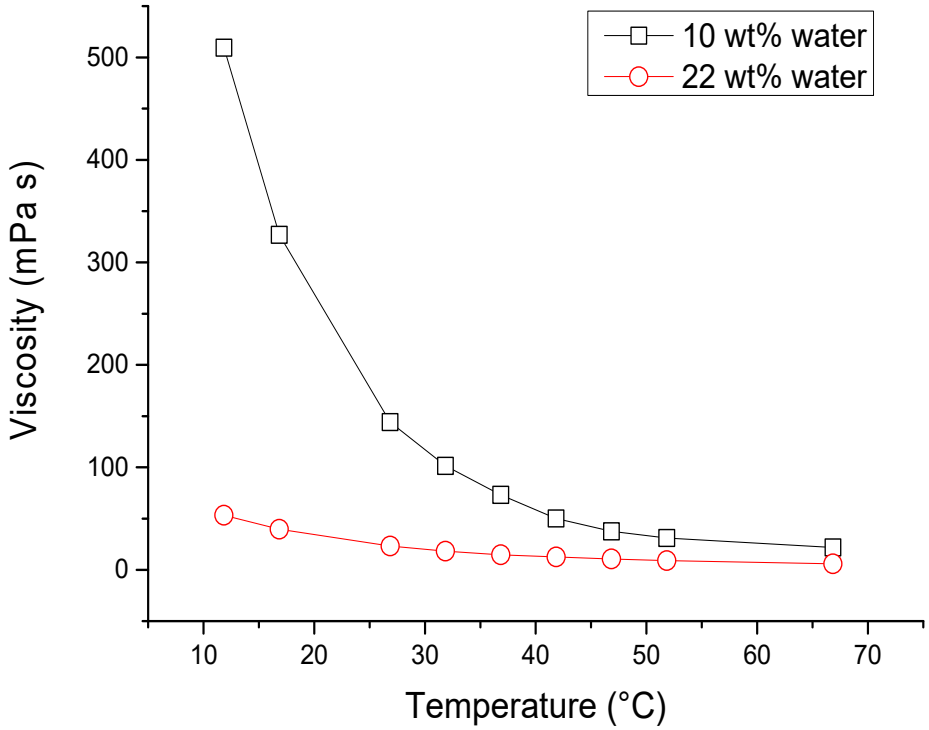

Figure 2. Dependence of viscosity on temperature and water content in DESs composed of choline chloride and lactic acid (molar ratio 1:2).

The response surface of phenols was influenced by extraction time in the range of 30–90 min, as shown in Figure 3. The maximum value of the response surface was observed at 30 min, demonstrating the significance of extraction time, which was also verified by our regression analysis results (Table 4). The extraction time had a negative correlation with the phenolics yield, which decreased from 30 to 90 min. Phenolic oxidation can occur at longer extraction times unless the solvent system contains reducing agents that can partially prevent these reactions [88].

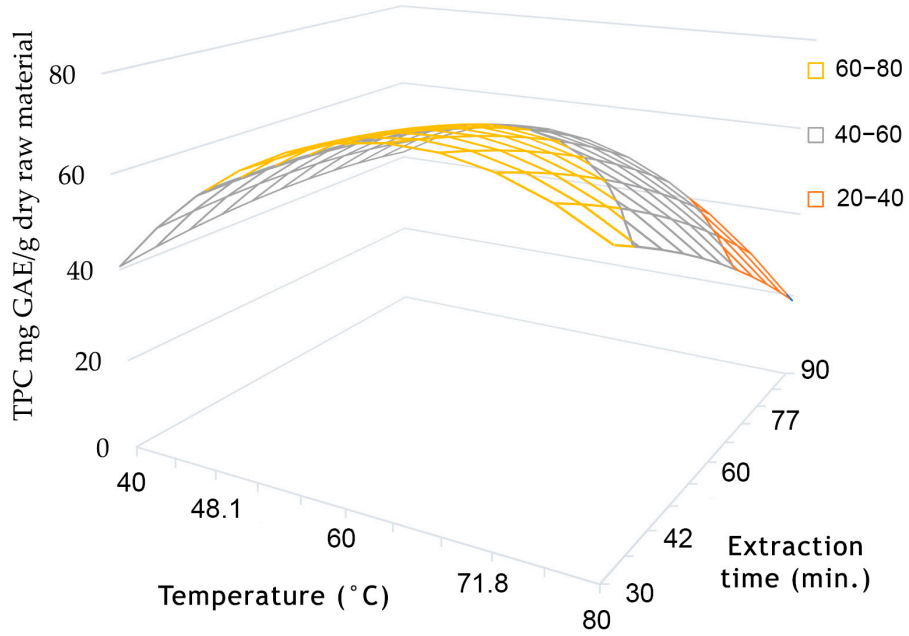

Figure 3. At constant factor x_2 (water addition 22.8%), the total phenolic content in coded coordinates depends on factor x_1 (temperature, °C) and factor x_3 (extraction time, min.).

The SOLVER subprogram from Microsoft Excel was used to optimize the computations and achieve the highest phenolic content. The optimal conditions for maximum phenolic content were an extraction temperature of 77 °C, time of 30 min, and water addition of 40%. The work also determined the phytogenic bioactive components in the clove extract, which is a mixture of various substances. The main compounds are eugenol, eugenol acetate, and β-caryophyllene. Eugenol and eugenol acetate have excellent antimicrobial activity against fungi and various Gram-negative and Gram-positive bacteria [89,90]. Eugenol has multiple benefits against many life-threatening diseases such as inflammation, oxidative stress, hyperglycemia, nerve disorders, elevated cholesterol, and cancer [71–93]. Eugenol has several benefits against various life-threatening diseases such as inflammation, oxidative stress, hyperglycemia, nerve disorders, elevated cholesterol, and cancer [91–93]. Eugenol also has antiviral activity against the influenza A virus, as demonstrated by Dai et al. [94]. Eugenol can limit viral infection and replication specifically against herpes simplex viruses, with IC50 values between 16.2 mg/mL and 25.6 mg/mL, as evaluated by the plaque reduction assay. Eugenol has been confirmed to be effective against clinical isolates of herpes simplex virus-1 [95,96]. Musthafa et al. [97] examined eugenol acetate for its antiviral potential against both Gram-negative and Gram-positive pathogens. The results of their work show the potential of eugenol acetate as an alternative candidate to control the pathogenicity of both Gram-negative and Gram-positive organisms. Eugenyl acetate at a concentration of 150 µg/mL significantly inhibited the production of virulence factors, such as pyocyanin and pyoverdin, by *Pseudomonas aeruginosa*. As shown by several studies, β-caryophyllene, a natural sesquiterpene, affects various organs, such as the liver [98], kidney [99] and brain, in a pharmacological way. β-caryophyllene has different therapeutic benefits, such as functioning as an antioxidant, decreasing inflammation [100], and fighting cancer [101,102]. The HPLC-DAD method was applied to measure eugenol, eugenol acetate, and β-caryophyllene in 20 samples that were extracted using DES-like mixtures. The results of the determination of target analytes by HPLC-DAD method are summarized in Table 5.

Table 5. HPLC determination of eugenol, eugenol acetate, and β-caryophyllene.

No.	Eugenol	Eugenol Acetate	β-Caryophyllene
		(mg/g) *	
1	168.61	11.63	0.87
2	200.70	18.93	0.89
3	216.14	15.23	0.96
4	218.51	13.96	0.46
5	196.01	19.64	1.05
6	205.67	18.08	0.64
7	222.11	14.22	0.76
8	217.64	13.08	0.22
9	222.99	20.45	0.46
10	211.89	18.51	0.37
11	188.14	19.63	1.09
12	218.41	32.70	0.57
13	307.26	28.64	0.56
14	221.18	18.53	0.27
15	211.18	17.87	0.51
16	215.14	18.23	0.37
17	210.66	17.60	0.45
18	218.22	19.29	0.31
19	209.85	17.55	0.22
20	208.67	17.37	0.39

* Expressed for dried sample.

Eugenol was present in the extracts in the highest concentration among the target analytes, according to the HPLC results obtained. The major component of clove extract, eugenol, increased from 168.61 mg/g dry raw material to 307.26 mg/g dry raw material, which is several times lower compared to that of eugenol acetate (11.63–32.70 mg/g dry raw material) and β-caryophyllene (0.57–0.87 mg/g dry raw material). Eugenol was more abundant in the extract obtained at higher temperatures (70 °C) and with less added water (15%).

The chemical composition and content of clove essential oils vary widely according to different studies. One study by Alma et al. [103] analyzed the clove bud essential oil obtained via steam distillation and identified 18 components, with eugenol being the predominant one (87%), followed by eugenol acetate (8.01%) and β-caryophyllene (3.56%). Another study by Razafimamonjison et al. [104] compared the clove bud essential oil from Indonesia, Madagascar, and Zanzibar, which was also extracted via steam distillation for 12 h. They found that eugenol was a major component in all samples, with its content ranging from 77.50% to 79.87%, and β-caryophyllene was the second most abundant, ranging from 4.06% to 6.91%. Chatterjee et al. [105] used a different extraction method, supercritical carbon dioxide extraction, to isolate eugenol from dried clove buds. They optimized the extraction conditions to achieve the highest yield of eugenol (129.86 mg/g dry clove buds), which were 60 °C, 25 MPa, 90 min, and 2 L/min of the CO_2 flow rate. Yazdani et al. [38] also used supercritical carbon dioxide extraction and obtained a similar yield of eugenol (86.7%) at 80 °C and 19 MPa. They also compared the eugenol yields from other extraction methods, such as hydrodistillation and microwave-assisted extraction, and found that supercritical extraction resulted in the highest percentage of eugenol (86.70%)

compared to hydrodistillation (81.47%) and microwave-assisted extraction (79.08%). The variation in the components and composition of clove essential oils can be attributed to several factors, such as the type and origin of the clove, the environmental conditions, the pretreatment of the plant material, the extraction method, and the extraction parameters.

This study applied the AGREE [106] (analytical environmental performance metric for sample preparation) to assess the environmental performance of the extraction method based on the principles of green chemistry, which aims to reduce the use of hazardous chemicals and energy to improve sustainability and lower the environmental impacts of processes, including extraction methods [107]. The AGREE metric considers several factors that affect the environmental impact of the procedure, such as the sample size, the type of reagents and instruments, the health and safety risks of any reagent, and the waste generation, among others. Figure 4 shows the results for a specific sample type and extraction procedure performed in optimal conditions. The AGREE pictogram shows a mostly yellow-green color rating of the individual aspects. The overall score was 0.63, which indicates a high level of environmental compatibility and low impacts of the proposed extraction procedure. The extraction technique does not use any organic solvents, only DES. The main source of environmental impact is the HPLC method used to analyze the extracts. Reducing the amount of sample used could improve the greenness evaluation of the sample processing.

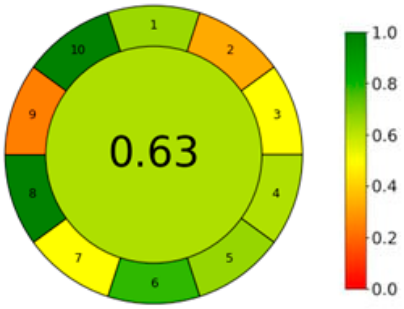

Figure 4. Evaluation of the greenness of the proposed extraction method using the AGREE (B) assessment metric. Legend: 1. Sample preparation placement, 2. hazardous materials, 3. sustainability, renewability, and reusability of materials, 4. waste, 5. size economy of the sample, 6. sample throughput, 7. integration and automation, 8. energy consumption, 9. post-sample preparation configuration for analysis, 10. operator's safety.

4. Conclusions

Upon analyzing the trial, our observations infer that extraction conditions significantly influence the total phenolic content, with particular emphasis on water addition and extraction time. Notably, water addition emerged as the most influential factor affecting the total phenolic content, closely followed by the extraction time. The interplay of extraction temperature with the addition of water further underscored the significance of temperature as a contributing factor to total phenolic content (TPC). The culmination of these factors pointed towards an optimal extraction scenario, where the highest TPC was achieved at an extraction temperature of 77 °C, extraction time of 30 min, and a water addition of 40%. The HPLC analysis further confirmed a notable abundance of eugenol in the extracts. Eugenol content exhibited a substantial increase from 168.61 mg/g dry raw material to 307.26 mg/g dry raw material. However, it is noteworthy that this concentration is significantly lower compared to that of eugenol acetate (ranging from 11.63 to 32.70 mg/g dry raw material) and β-caryophyllene (ranging from 0.57 to 0.87 mg/g dry raw material). In summation, this study demonstrates that choline chloride–lactic acid (molar ratio 1:2) is an effective solvent for extracting compounds from clove buds. This research provides useful information for improving the extraction parameters to increase the total phenolic content and the concentrations of specific substances in clove extracts.

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