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Optimized Ultrasonic Extraction of Essential Oil from the Biomass of *Lippia graveolens* Kunth Using Deep Eutectic Solvents and Their Effect on *Colletotrichum asianum*

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Abstract: Essential oils are emerging as alternatives to conventional pest control chemicals. *Lippia graveolens* Kunth (Mexican oregano) is a source of essential oils and during conventional extraction, the biomass generated is discarded as waste; however, reports show that this biomass is still a rich source of essential oils. Conventional essential oil extraction causes contamination and utilizes toxic solvents. Deep eutectic solvents (DESs) offer low toxicity, biodegradability, high selectivity, and yields comparable to organic solvents. This study obtained essential oil from *Lippia graveolens* biomass via hydrodistillation with ultrasound-assisted DES pretreatment. This research aimed to optimize the extraction of essential oil from *Lippia graveolens* biomass using ultrasound-assisted DESs and assess its in vitro and in vivo inhibitory effect on *C. asianum*. The response variables were extraction yield and total reducing capacity. Optimal conditions were determined using a central composite rotatable design, considering solid-to-liquid ratio (0.38 g/mL), ultrasonic amplitude (45.05%), and time (7.47 min). The optimized oil, with thymol (48%) as the predominant component, exhibited more volatile compounds than conventional hydrodistillation. Fungicidal assays highlighted its potential in controlling anthracnose in papaya fruits caused by *C. asianum*, making ultrasound-assisted DES pretreatment a promising alternative for obtaining essential oil from botanical byproducts.

Keywords: essential oils; deep eutectic solvents; ultrasound-assisted extraction; Mexican oregano; *Lippia graveolens*; antifungal activity

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1. Introduction

The specie *Lippia graveolens* Kunth, also known as Mexican oregano, the most commercialized in Mexico, contains the compounds carvacrol and thymol, which are used industrially in multiple applications; oregano is sold as packaged leaves, in bottled oil or extract, in products for the perfume, and in the pharmaceutical, alcoholic beverage, natural preservative, cosmetic, and mechanical oil industries, among others [1]. In this context, the processing of plant raw materials generates solid organic residues, also known as biomass. These residues have been reported to be a source of bioactive compounds such as terpenes, alkaloids, flavonoids, polyphenols, and proteins, among others. These types of plant byproducts derived from industrial processes tend to be discarded, which

impacts the environment as they emit large quantities of greenhouse gases such as CO_2. Considering this, it is important to minimize the environmental impact of these residues through processes that utilize their content [2].

There are multiple methods for obtaining secondary metabolites from plants. Among these, hydrodistillation is the most commonly used operation in the industry for the extraction of essential oils, which utilizes high temperatures that can alter the chemical composition and affect their quality; however, it achieves low extraction yields [3–5]. Also, an important content of solids is generated after hydrodistillation (biomass), which is usually discarded; however, reports show that biomass is a rich source of bioactive compounds that could be extracted by other technologies. Another widely used operation for obtaining essential oils is extraction with organic solvents; nonetheless, it presents disadvantages such as environmental harm and the tendency to contaminate extracts with toxic solvent residues. On the other hand, extraction with deep eutectic solvents has been proposed as an alternative to the conventional extraction processes of bioactive compounds like essential oils [6]. These solvents, composed of a mixture of hydrogen bond acceptors and donors, are characterized by being biodegradable, non-toxic, having high selectivity based on their composition, and being economical and easy to synthesize [7,8]. Also, ultrasound-assisted extraction has been coupled with deep eutectic solvents to increase the extraction yield of bioactive compounds, due to the reduced extraction times by increasing the contact surface between the solvent and the raw material [8].

Oregano essential oils (OEOs) have been extensively studied for their antimicrobial properties. Various studies indicate that these oils could be used in important crop plants to control and protect against phytopathogens. In this sense, Zhao et al. [9] found that essential oil of *Origanum vulgare* L., along with its components thymol and carvacrol, effectively inhibited *Botrytis cinerea* growth and spore germination, suggesting their potential as natural fungicides. Their study highlights the effectiveness of these natural compounds in combating one of the most common postharvest pathogens, providing an alternative to traditional chemical treatments. Similarly, Medina-Romero et al. [10] demonstrated that essential oil of *Lippia graveolens* Kunth inhibited the growth of various Fusarium species in vitro and on cherry tomato fruits, indicating its use as a biopesticide. This research underscores the versatility of *Lippia graveolens* Kunth essential oil in managing different fungal pathogens, enhancing the sustainability and safety of crop-protection practices. Additionally, Yılmaz et al. [11] identified essential oils of *Origanum vulgare* L., *Salvia officinalis* L., *Rosmarinus officinalis* L., *Eucalyptus* sp., and *Foeniculum vulgare* Mill. as effective inhibitors of *Colletotrichum gloeosporioides*, with *O. vulgare* L. being the most effective. Their findings revealed significant inhibitory effects in both in vitro and in vivo assays, showcasing the potential of these oils to act as natural protective agents against fungal infections. This supports the use of essential oils in postharvest operations due to their low toxicity, biodegradability, and multifunctionality, making them suitable for sustainable agricultural practices.

Due to the substantial losses in food crops caused by such pathogenic agents, it is crucial to address this issue by promoting research and the development of products that protect and enhance plant health [12]. Furthermore, essential oils could be extracted from oregano biomass; therefore, the objective of this research is to optimize the extraction of essential oil from *Lippia graveolens* Kunth biomass using ultrasound-assisted DESs (deep eutectic solvents) and to evaluate its inhibitory effect on *Colletotrichum asianum* using an in vitro and in vivo approach with papaya fruit as a model.

2. Materials and Methods

2.1. Plant Material

Oregano (*Lippia graveolens* Kunth) was acquired from Santa Gertrudis, Durango, México (coordinates: N 23°32′43.8″ W 104°22′20.8″). Oregano biomass from the hydrodistillation process was donated by the company Oreganic. *L. graveolens* Kunth samples were identified at the Herbarium of the School of Agriculture from the Universidad Autónoma

de Sinaloa and given the catalogue number FA-UAS-017005. All aerial parts of the plants (leaves, flowers, and stems) were ground into a powder using an Ika Werke M20 grinder (Wilmington, NC, USA).

2.2. Chemical Reagents

Ethanol (Fermont CAS 64-17-5, Monterrey, NL, México), Folin–Ciocalteu (Sigma F9252), sodium carbonate (Na_2CO_3, Sigma 223530), gallic acid (Sigma G7384), choline chloride (Sigma C1879), and lactic acid (CTR 69775) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Preparation of Deep Eutectic Solvents

The preparation of deep eutectic solvents for the extraction of essential oils from *L. graveolens* biomass followed the methodology reported by Chen, Wu, Zhu, Wang, Su, and Yi [6] and Chen et al. [13]. Briefly, choline chloride was mixed as the hydrogen bond acceptor, and lactic acid was used as the hydrogen bond donor in a molar ratio of 1:2. A stirring and heating plate was used, maintaining the mixture at 80 °C until the solution was clarified. Thirty percent distilled water (*w*/*w*) was then added.

2.4. Ultrasonic Extraction

L. graveolens biomass powder was mixed with the choline chloride/lactic acid eutectic solvent in a 1:2 ratio and subjected to an ultrasonic extraction system (SONICS VCX 500, Newtown, CT, USA) following the methodology of Chen, Wu, Zhu, Wang, Su, and Yi [6]. The variables of time, ultrasonic amplitude, and solid/liquid ratio were studied according to the experimental design (Table 1). The sample container was placed in an ethylene glycol bath, maintaining a temperature range of 20 to 40 °C to prevent the degradation of the volatile compounds due to the ultrasonic power.

Table 1. Central composite rotatable design of response surface methodology for the optimization of ultrasonic extraction of Mexican oregano biomass.

	Process Variables			Response Variables	
Run	**X_1: Solid/Liquid Ratio (g/mL)**	**X_2: Ultrasonic Amplitude (%)**	**X_3: Extraction Time (min)**	**Essential Oil Yield (%)**	**Total Reducing Capacity (mg GAE/g Biomass)**
1	−1 (0.18)	−1 (36.22)	−1 (4.5)	0.9691	0.5949
2	−1 (0.18)	1 (83.78)	−1 (4.5)	0.6845	0.5783
3	−1 (0.18)	−1 (36.22)	1 (15.5)	0.6621	0.4489
4	−1 (0.18)	1 (83.78)	1 (15.5)	0.77	0.4656
5	1 (0.42)	−1 (36.22)	−1 (4.5)	1.2359	1.2170
6	1 (0.42)	1 (83.78)	−1 (4.5)	0.8689	1.002
7	1 (0.42)	−1 (36.22)	1 (15.5)	0.9030	1.0149
8	1 (0.42)	1 (83.78)	1 (15.5)	1.0488	1
9	0 (0.3)	−1.68 (20)	0 (10)	1.0310	1.1433
10	0 (0.3)	1.68 (100)	0 (10)	0.98	0.8152
11	0 (0.3)	0 (60)	−1.68 (0.75)	0.8575	1.085
12	0 (0.3)	0 (60)	1.68 (19.25)	0.8025	0.7625
13	−1.68 (0.1)	0 (60)	0 (10)	0.5106	0.5321
14	1.68 (0.5)	0 (60)	0 (10)	1.0181	1.1836
15	0 (0.3)	0 (60)	0 (10)	1.01	1.3382
16	0 (0.3)	0 (60)	0 (10)	1.03	1.2695
17	0 (0.3)	0 (60)	0 (10)	1.025	1.2650
18	0 (0.3)	0 (60)	0 (10)	1.02	1.4
19	0 (0.3)	0 (60)	0 (10)	1.02	1.4
20	0 (0.3)	0 (60)	0 (10)	0.9639	1.4913

GAE/g biomass: gallic acid equivalents per gram of biomass.

2.5. Optimization

The optimization of the ultrasonic extraction process was conducted using a rotatable central composite design of the response surface methodology with three factors (Table 1). The selected process variables were solid/liquid ratio (X_1, g/mL), ultrasonic amplitude (X_2, %) and extraction time (X_3, min), while the response variables were essential oil yield (%) and total reducing capacity (mg GAE/g biomass) [14]. The ranges for the process variable values were 0.1 to 0.5 g/mL for the solid/liquid ratio, 20 to 100% for ultrasonic amplitude, and 0.75 to 19.24 min for ultrasonic time. We used a design of 3 factors and high (1), low (−1), and axial (−1.68, 1.68) levels with a set of 20 runs. The optimal levels were obtained by contour plots of the graphs. The optimal treatment was carried out for quintupled. The optimization design and data analysis were performed using the statistical package Minitab 18 (Minitab Inc., State College, PA, USA).

2.6. Hydrodistillation

Essential oil extraction was carried out using a Clevenger-type apparatus. A measure of 80 mL of the extract from ultrasonic extraction was used, diluted with distilled water to a volume of 500 mL. The diluted extract was subjected to a temperature of 280 °C in a stirring plate (Cimarec, Thermo Scientific, Waltham, MA, USA) and stirred at 7000 rpm for 1.5 h. The oil yield was estimated based on dry weight (% *w*/*w*) (Equation (1)). Essential oil samples were stored in vials at 4 °C for chromatographic analysis [13,15].

$$\text{Essential oil yield}(\%) = \frac{\text{Essential oil weight (g)}}{\text{Total biomass weight (g)}} * 100 \quad (1)$$

2.7. Total Reducing Capacity

The total reducing capacity of the essential oils from *Lippia graveolens* Kunth was determined using the Folin–Ciocalteu method [16]. Ten microliters of oil were taken and placed in a 96-well microplate to which 230 µL of distilled water and 10 µL of 2 N Folin–Ciocalteu reagent were added. The samples were incubated for 3 min. Subsequently, 25 µL of 4 N Na_2CO_3 was added, and the samples were incubated for 2 h in the absence of light at room temperature. Absorbance was determined at a wavelength of 725 nm using a Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). A gallic acid curve was used, and the results were expressed in milligrams of gallic acid equivalents per gram of biomass (mg GAE/g biomass).

2.8. Gas Chromatography Analysis

The chromatographic analysis was performed using an Agilent 7890B gas chromatography system coupled with a 7000D GC/TD tandem mass spectrometry and an Agilent Technologies 7693 autosampler. High-purity helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The chromatographic column used was TG-WAXMS 30 m × 0.25 mm × 0.25 µm. The column inlet pressure was set at 9.37 psi. The column temperature was initially maintained at 40 °C for 1 min and then increased at a rate of 10 °C min^{-1} to 280 °C, where it was held for 6 min. A one-microliter volume of the optimized essential oil replicas was injected.

2.9. Mycelial Growth Inhibition

The mycelial growth assay was conducted using the methodology of Amini et al. [17] with modifications. The essential oil from the *Lippia graveolens* Kunth biomass from the optimal treatment was used. The assay was carried out on Petri dishes with potato dextrose agar (PDA) culture medium. Essential oils were dissolved in Tween 80 (0.5% *v*/*v*), and six concentrations (10, 25, 50, 75, 100, and 250 ppm) were evaluated. Culture media were prepared and sterilized, and the oils were added at the proposed concentrations before pouring into Petri dishes. After solidification of the PDA, a 5 mm mycelial disc of the *Colletotrichum asianum* fungus was added. The plates were incubated for 7 days at 27 °C or

until the control plates (medium without fungicide) showed mycelial growth across their diameter. After incubation, the diameters of the mycelium on each plate were measured with a digital caliper (Gwong 150 mm). The inhibition diameter was calculated using Equation (1), where *Dc* and *Dt* are the radial growth (mm) of the pathogen in control plates and treated plates, respectively. Finally, the effective concentration at 50% (EC50) in parts per million for each treatment was calculated using Equation (2). This was performed through linear regression in Minitab 19 software. The assay was performed in triplicate.

$$\text{Micelial grownt inhibition}(\%) = \frac{Dc - Dt}{Dc} * 100 \quad (2)$$

2.10. Determination of Minimum Inhibitory Concentration and Minimum Fungicidal Concentration

The minimum inhibitory concentration of the essential oils from the optimal treatment was determined using the microdilution method in broth reported by Alhajj et al. [18] with modifications. A spore suspension of 1×10^5 conidia/mL was prepared from 7-day-old *Colletrotrichum asianum* growth; the phytopathology laboratory of CIAD Unidad Culiacán provided the isolated fungus. The assay was conducted in a 96-well microplate, testing concentrations of 600, 500, 475, 450, 425, 400, 375, 350, 325, 300, 275, 250, 225, and 200 ppm for the essential oils of *Lippia graveolens* Kunth. A measure of 180 microliters of potato dextrose broth (BD Difco), 20 µL of essential oil, and 50 µL of spore solution were added to each well for a total volume of 250 µL. The spore suspension without fungicide was used as a positive control, and the medium without extract served as a negative control. The plates were incubated at 27 °C for 7 days. The lowest concentration of treatments that showed no visible growth was considered the minimum inhibitory concentration (MIC). Wells showing growth were used to determine the minimum fungicidal concentration (MFC) by taking 10 µL and adding them to Petri dishes with potato dextrose agar (PDA). The assay was performed in triplicate.

2.11. In Vivo Fungicidal Activity

The in vivo antifungal activity was determined on papaya fruits (*Carica papaya* var. Maradol). Commercially mature fruits of comparable sizes, not subjected to fungicide application, and without mechanical damage, insect damage, or pathogen infection were selected. The fruit surface was disinfected by immersion in 2% sodium hypochlorite for 2 min, followed by a rinse with distilled water. Two methods, preventive (1) and corrective (2), were traced on the same fruit with two well-separated lines, each with four points. In the corrective method, the inoculum (spore suspension) was first applied at each point of line 1 on the upper surface of the fruit and allowed to dry. Subsequently, the fruit was immersed in the fungicide for 5 min; again, it was allowed to dry before inoculating the points of line 2, corresponding to the preventive method. The assay was conducted on unwounded fruits and repeated on wounded fruits. The wounds were made with a sterile needle at the four points of each line (1 and 2) on the upper surface of the fruit, and 5 µL of a 5×10^5 spores/mL suspension was applied. The fungicide concentrations were as follows: 0 (control), 300, 1000, and 2000 ppm for the optimized oregano essential oil, Timorex gold (Syngenta), and Tecto 60 (Syngenta). Treated fruits were stored in polyethylene bags for 14 days at 27 °C and 95% relative humidity. After 14 days, the fruits were examined for anthracnose lesions. In case of lesions, the lesion diameter was measured, and the effectiveness percentage was reported using Equation (3), where *Lc* is the lesion in the control fruit and *Lt* is the lesion in the treatment. The assay was performed in triplicate. For statistical analysis, a three-factor ANOVA (fungicide, concentration, method) was employed, and the response variable was the effectiveness percentage calculated using Equation (3) [19,20].

$$\text{Effective concentration}(\%) = \frac{Lc - Lt}{Lc} * 100 \quad (3)$$

2.12. Statistical Analysis

The data on essential oil yield (% w/w), total reducing capacity (mg GAE/g biomass), mycelial growth inhibition, and in vivo fungicidal activity were analyzed using the statistical package Minitab 19 (Minitab Inc., State College, PA, USA), which was employed for all data analyses. The assays were conducted in triplicate, and the results were reported as mean ± standard deviation. In the in vivo fungicidal activity assay, a three-factor ANOVA (fungicide, concentration, and extraction method) was employed, and the response variable was the effectiveness percentage. A Tukey test was performed to analyze mean differences, with a significance level set at $p < 0.05$.

3. Results

3.1. Predictive Models

Table 1 shows the combinations of process variables for ultrasonic-assisted extraction (amplitude, time, solid/liquid ratio) used during the essential oil extraction process of Mexican oregano (*Lippia graveolens* Kunth) biomass. These combinations display the obtained values of the response variables (essential oil yield and total reducing capacity).

Based on the experimental data included in Table 1, predictive models were constructed:

$$\hat{Y}_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 \quad (4)$$

Equation (4) relates the selected process variables (X_1: solid/liquid ratio; X_2: ultrasonic amplitude; X_3: extraction time) to the response variables (essential oil yield and total reducing capacity).

3.1.1. Essential Oil Yield (EOY)

For the variable essential oil yield (EOY), a data range between 0.5106 and 1.2359% was found (Table 1). The analysis of variance (ANOVA) for the prediction model (Table 2) indicates that the overall, linear, and quadratic models are significant ($p < 0.001$), with a nonsignificant lack of fit ($p < 0.088$), a determination coefficient of R^2 = 96.26%, and an adjusted determination coefficient (adjusted R^2) = 94.54%. Due to the significance of the model, a high contribution to variability (R^2), similar determination and adjusted coefficients, and null significance to the lack of fit, the model is shown to be adequate and reproducible. For the linear model, it presents the highest contribution to total variability (52.22%; $p < 0.000$), where the process variable with the highest contribution to variability is the solid/liquid ratio (45.01%; $p < 0.000$); this is followed by ultrasonic amplitude (3.23%; $p < 0.005$) and time (3.01%; $p < 0.006$), with the latter two contributing very little to the model. For the quadratic model, the contribution to variability (24.72%; $p < 0.000$) was lower than that of the linear model; here, the quadratic variable that contributes the most to variability is the solid/liquid ratio (17.79%; $p < 0.000$), followed by time (6.93%; $p < 0.000$). The interaction of variables contributes moderately to variability (19.32%; $p < 0.000$), where the combination of amplitude*time variables provides this value (Equation (5)).

$$\text{EOY } (\%) = 1.01 + 0.13X_1 - 0.035X_2 - 0.034X_3 + 0.11X_2X_3 - 0.081{X_1}^2 - 0.057{X_3}^2 \quad (5)$$

The prediction model for the response variable essential oil yield using coded and uncoded variables was represented by Equations (5) and (6), respectively, where the solid/liquid ratio variable was represented by SL, ultrasonic amplitude was represented by A, and extraction time was represented by T:

$$\text{EOY}(\%) = 0.646 + 4.538SL - 0.010A - 0.020T + 0.000865AT - 5.692A^2 - 0.00189T^2 \quad (6)$$

After conducting the analysis of variance and the prediction model for the essential oil yield variable, the contour and response surface plots were obtained (Figures 1 and 2). These plots illustrate the behavior of the process variables (amplitude, time, and solid/liquid ratio) on the response variable, i.e., the essential oil yield. Analyzing the contour and response surface plots, the process variable that most positively affects the response variable to

maximize it is the solid/liquid ratio. The trend shows that as this variable increases, the essential oil yield is maximized. In contrast, for the variables time and ultrasonic amplitude, they have an opposite effect to the solid/liquid ratio, where a lower ultrasonic amplitude and time maximize the response variable.

Table 2. Analysis of variance for the essential oil yield variable.

Source	Degrees of Freedom	Contribution	SC Adjusted	MC Adjusted	F Value	*p* Value
Model	6	96.26%	0.510	0.085	55.83	0.000
Lineal	3	52.22%	0.276	0.092	60.57	0.000
S/L	1	45.01%	0.243	0.243	159.98	0.000
Amplitude	1	3.23%	0.017	0.017	11.25	0.005
Time	1	3.01%	0.015	0.015	10.48	0.006
Square	3	24.72%	0.131	0.065	43.01	0.000
$Time^2$	1	6.93%	0.047	0.047	31.38	0.000
S/L^2	1	17.79%	0.094	0.094	61.91	0.000
Interaction of two factors	1	19.32%	0.102	0.102	67.24	0.000
Amplitude × Time	1	19.32%	0.102	0.102	67.24	0.000
Error	13	3.74%	0.019	0.001		
Lack of fit	8	3.18%	0.016	0.002	3.59	0.088
Pure error	5	0.55%	0.002	0.0005		
Total	19	100%				

S = 0.0390341; R^2 = 96.26%; R^2 (adjusted) = 94.54%; R^2 (predicted) = 87.35%.

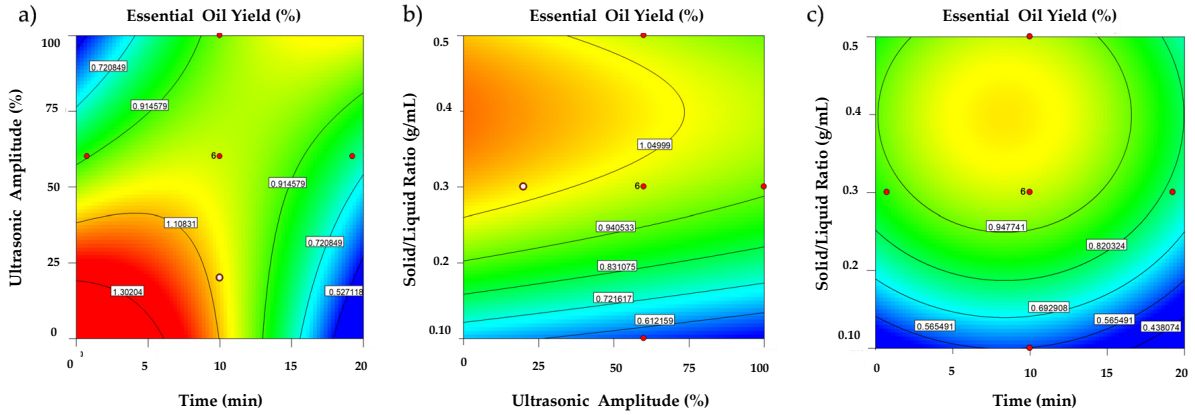

Figure 1. Contour plots for EOY with fixed S/L ratio (**a**) at 0.3 g/mL, fixed time (**b**) at 10 min, and fixed amplitude (**c**) at 60%.

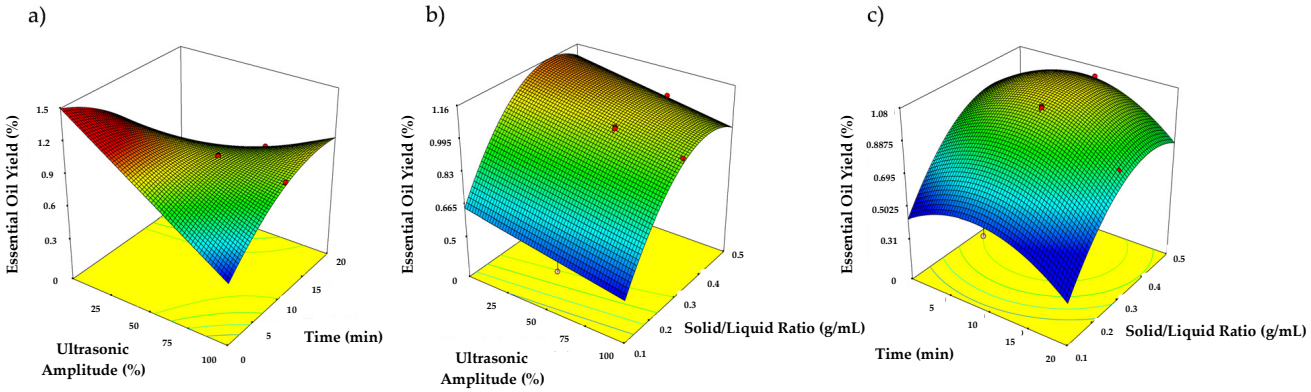

Figure 2. Response surface plots for EOY with fixed S/L ratio (**a**) at 0.3 g/mL, fixed time (**b**) at 10 min, and fixed amplitude (**c**) at 60%.

3.1.2. Total Reducing Capacity

The range of data obtained for the variable total reducing capacity by the Folin–Ciocalteu method is from 0.4489 to 1.4913 mg GAE/g biomass (Table 3). The prediction model, according to the analysis of variance, shows that the general, linear, and quadratic models are significant ($p < 0.000$), with an adjusted R^2 coefficient (91.34%) close to R^2 (94.08%) and a lack of fit p-value of 0.346. Since the model exhibits high significance, a high contribution to variability, and close values between the determination and adjusted coefficients, that the model is adequate and reproducible. The quadratic model is the largest contributor to variability with 53.11% and a p-value < 0.000. Regarding the process variables, the solid/liquid ratio proves to be the most significant contributor to variability (26.48%; $p < 0.000$), followed by time (16.44%; $p < 0.000$) and ultrasonic amplitude (10.19%; $p < 0.000$), which has the lowest contribution. The contribution to variability from the linear model (40.97%; $p < 0.000$) is less than that of the quadratic model; in this case, the variable with the greatest contribution to the linear model is the solid/liquid ratio (35.49%; $p < 0.000$), followed by time (3.41%; $p < 0.000$), and finally ultrasonic amplitude (2.06%; $p < 0.000$). Considering that the quadratic model contributes the most variability to the general model, it is possible to find the optimal conditions of the selected process variables that maximize the response variable total reducing capacity, highlighting the solid/liquid ratio and time variables as the most significant for the quadratic model. The prediction model for the response variable total reducing capacity using coded and uncoded variables was expressed by Equations (7) and (8), respectively:

$$TRC = 1.36 + 0.24X_1 - 0.057X_2 - 0.074X_3 - 0.20{X_1}^2 - 0.16{X_2}^2 - 0.18{X_3}^2 \quad (7)$$

$$TRC = -1.806 + 10.463SL + 0.030A + 0.103T - 14.111SL^2 - 0.000276A^2 - 0.005827T^2 \quad (8)$$

Table 3. Analysis of variance for the variable total reducing capacity (TRC).

Source	Degrees of Freedom	Contribution	Adjusted SS	Adjusted MS	F Value	*p* Value
Model	6	94.08%	2.039	0.339	34.41	0.000
Lineal	3	40.97%	0.888	0.296	29.97	0.000
S/L	1	35.49%	0.769	0.769	77.89	0.000
Amplitude	1	2.06%	0.044	0.044	4.53	0.053
Time	1	3.41%	0.073	0.073	7.49	0.017
Square	3	53.11%	1.151	0.383	38.84	0.000
Amplitude^2	1	10.19%	0.353	0.353	35.80	0.000
Time^2	1	16.44%	0.447	0.447	45.33	0.000
S/L^2	1	26.48%	0.574	0.574	58.10	0.000
Error	13	5.92%	0.128	0.009		
Lack of Fit	8	4.17%	0.090	0.011	1.48	0.346
Pure error	5	1.76%	0.038	0.007		
Total	19	100%				

S = 0.0994003; R^2 = 94.08%; Adjusted R^2 = 91.34%; Predicted R^2 = 80.17%.

To observe the behavior of the selected process variables (solid/liquid ratio, time, and ultrasonic amplitude) on the response variable total reducing capacity, contour and surface plots were generated (Figures 3 and 4). By observing these plots with the aim of maximizing the response variable and obtaining an optimum, it is possible to notice that the quadratic process variables exhibit similar behavior on the response in all arrangements. In this case, the quadratic model contributes more to the response variable than the linear model, resulting in curves instead of straight lines in the contour plots and domes or peaks in the surface plots. This indicates a nonlinear relationship between the independent variables and the response variable and indicates that the quadratic model is more suitable for describing the variability in the data. Considering that the quadratic variables with

the highest contribution are the solid/liquid ratio and time, we focus our attention on the plots that include these variables. It can be seen in these plots that the optimal process conditions that maximize the response variable total reducing capacity are achieved, with these conditions being close to the central points of these variables. Regarding the rest of the plots, a very similar behavior is observed, where the region corresponding to the optimal conditions that maximize the response variable is close to the central values of the points.

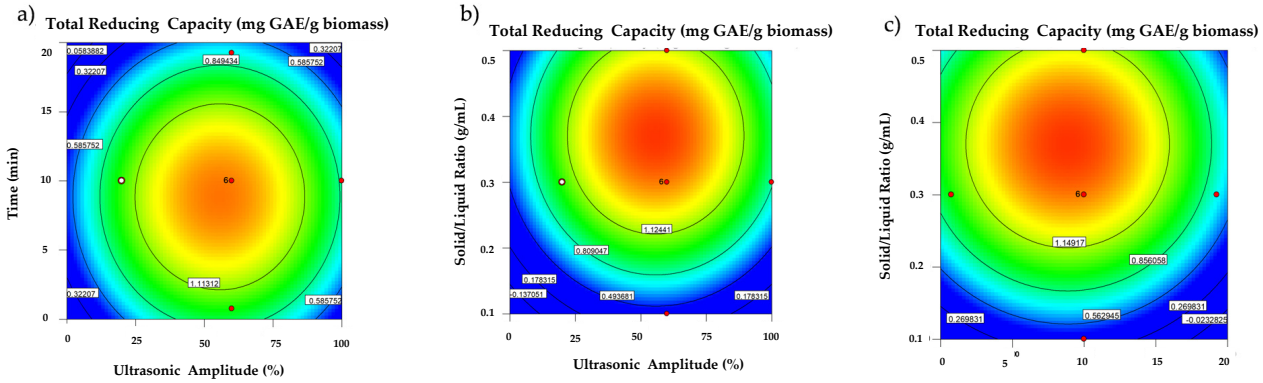

Figure 3. Contour plots for TRC with fixed S/L (**a**) at 0.3 g/mL, fixed time (**b**) at 10 min, and fixed ultrasonic amplitude (**c**) at 60%.

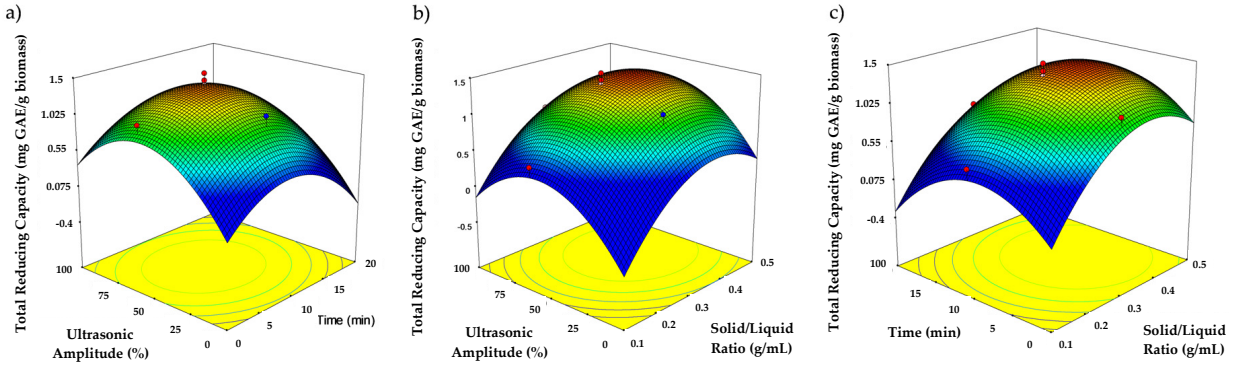

Figure 4. Response surface plots for TRC with fixed S/L ratio (**a**) at 0.3 g/mL, fixed time (**b**) at 10 min, and fixed amplitude (**c**) at 60%.

In a study by Michalaki et al. [21], the ultrasound-assisted extraction of phenolic compounds from *Origanum vulgare* ssp. hirtum was optimized. The authors reported that the total phenolic content determined by the Folin–Ciocalteu method increased with longer ultrasound extraction times, reaching a maximum of 362.1 ± 1.8 mg GAE/g DW after 40 min of extraction at 80 °C and 60% (*v*/*v*) ethanol. This finding is consistent with observations in this study, where total reducing capacity values also increased with longer extraction times, peaking at 1.4913 mg GAE/g DW around 10 min, 60% amplitude, and a S/L ratio of 0.3 g/mL, corresponding to the central values, before decreasing with further increases in time. The differences in results between these studies could be attributed to the solvents used, the nature of the raw materials, and the chemical profile of the extracts, as essential oils tend to have lower amounts of phenolic compounds.

3.1.3. Optimization of Ultrasonic-Assisted Extraction by DES of *Lippia graveolens* Biomass

To find the optimal process conditions to maximize the response variables (essential oil yield and total reducing capacity), the superposition of graphs from the contour plots was performed. The combination of process variables that maximizes the response variables was solid/liquid ratio = 0.38; ultrasonic amplitude = 45.05%; extraction time = 7.47 min (Figure 5). The predicted values for the response variables were essential oil yield = 1.127%

and total reducing capacity = 1.403 mg GAE/g biomass. Considering the optimal conditions and the predicted values obtained, five replicates (n = 5) were performed to confirm that the theoretical models are adequate and reproducible. The experimental values obtained under optimal conditions are included in Table 4: essential oil yield = 1.0932 ± 0.055%; total reducing capacity = 1.4788 ± 0.06 mg GAE/g biomass. Comparing the predicted values with the experimental values from the replicates, we can observe that they are close to each other, as these experimental values fall within the 95% confidence interval of the prediction model, indicating no significant difference. Therefore, the optimal ultrasound-assisted DES extraction conditions for essential oils to maximize essential oil yield and total reducing capacity are adequate and reproducible.

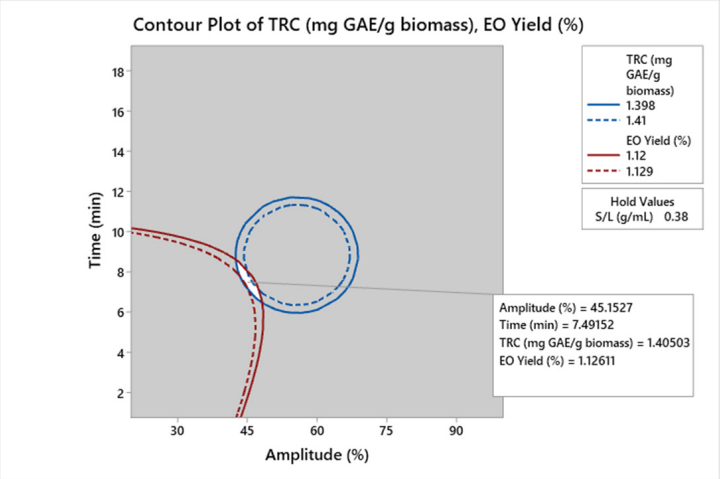

Figure 5. Graph showing the region with the best combination of process variables to maximize EOY and TRC.

Table 4. Confirmation report of optimized DES ultrasonic extraction.

Factor	Name	Optimal Level	Low Level	High Level	Code
A	Solid/Liquid Ratio (g/mL)	0.38	12	36	Actual
B	Amplitude (%)	45.15	12.5	37.5	Actual
C	Time (min)	7.49	32	42	Actual
Response	Predicted Value	Measured Value Mean	Predicted SE	CI 95%	PI 95%
Yield (%)	1.127	1.093	0.015	(1.09, 1.16)	(1.17, 1.63)
TRC (mg GAE/g biomass)	1.403	1.478	0.039	(1.318, 1.488)	(1.03, 1.21)

GAE/g biomass: gallic acid equivalents per gram of oregano biomass; SE: standard error; CI: confidence interval.

The optimal conditions obtained in this research (Table 4) to maximize the response variables (essential oil yield and total reducing capacity) differ from those reported by Biru et al. [22]. They optimized the ultrasound-assisted extraction process of essential oil from *Vernonia amygdalina*, obtaining optimal conditions of 17.263 min, sonication power of 150.677 W, and 6.811 mL/g, under which the essential oil yield was maximized at 4.185% g/g. The difference between the optimal conditions in this work and those of the mentioned study can be attributed to the different raw materials used, i.e., *Vernonia amygdalina* and *L. graveolens* biomass, respectively. It should be noted that the biomass in this study had already undergone a previous essential oil extraction process, so a lower extraction yield is expected. Additionally, the ultrasonic extraction of *Vernonia amygdalina* leaves did not include hydrodistillation as a separation step, which can degrade volatile compounds due to high temperatures and negatively impact the essential oil yield. Thus, thermolabile compounds were protected from degradation, favoring the oil yield.

In another study by Liu et al. [23], the extraction of essential oil from *Iberis amara* seeds was optimized using ultrasound-assisted hydrodistillation; they found that the optimal conditions for maximizing essential oil yield were 240 min, ultrasonic power of 45 W, and a solid/liquid ratio of 6.7 mL/g. The difference in values between this work and the study in question could be due to the nature of the raw materials used, as well as the different plant parts selected for extraction: seeds (*I. amara*) versus leaves, flowers, and fruit (*L. graveolens*).

Xu et al. [14] optimized the extraction of essential oil from turmeric (*Curcuma longa* L.) using microwave-assisted DES as a pretreatment for hydrodistillation. They found the following optimal conditions: NADES composed of choline chloride/oxalic acid in a 1:1 molar ratio, 60 g of plant material, pretreatment time of 5 min, pretreatment temperature of 84 °C, and hydrodistillation time of 76 min. Under these conditions, an essential oil yield of 0.85% was obtained. This differs from the results of this work; one of the causes could be the use of microwave technology, the different DES mixture used, and the nature of the plant material itself.

3.2. Gas Chromatography Analysis

All the essential oil samples from the *L. graveolens* biomass were analyzed using gas chromatography coupled to mass spectroscopy (GC-MS). Forty-six volatile compounds were identified in the optimized essential oil (EAU-DES-HD), while only twenty volatile compounds were detected in the oil extracted by conventional hydrodistillation (HD) (Table 5). Thymol was the most abundant essential oil in EAU-DES-HD samples, and its content was higher in the optimized than in the hydrodistillation samples (47% vs. 38%, respectively). Most of the remaining compounds were detected in amounts below 9%. The increased presence of compounds in the optimized oil through the use of DESs and ultrasound may be attributed to the capacity of eutectic solvents, particularly those with acidic properties, to dissolve the cellulose structures found in the plant cells of *L. graveolens*. This dissolution may lead to a greater release of secondary metabolites, specifically volatile compounds present in the intracellular medium.

Table 5. Profile of volatile compounds present in the biomass oil of *L. graveolens* optimized with ultrasonic–DES pretreatment and conventional hydrodistillation.

No	Compound	RT	Molecular Formula	Relative Content (%)		Classification
				UAE-DES-HD	HD	
1.	α-Fellandrene	3.46	$C_{10}H_{16}$	0.07 ± 0.04	ND	Monoterpene
2.	1,4-Cineole	3.50	$C_{10}H_{14}$	0.01 ± 0.004	ND	Monoterpene
3.	(+)-4-Carene	3.51	$C_{10}H_{16}$	0.22 ± 0.01	ND	Monoterpene
4.	p-Cymene	3.55	$C_{10}H_{14}$	1.66 ± 0.53	1.27 ± 0.45	Monoterpene
5.	Eucalyptol	3.59	$C_{10}H_{18}O$	0.68 ± 0.10	0.98 ± 0.36	Monoterpene
6.	γ-Terpinene	3.70	$C_{10}H_{16}$	0.16 ± 0.05	0.04 ± 0.02	Monoterpene
7.	α-Terpineyl propionate	3.78	$C_{13}H_{22}O_2$	0.16 ± 0.01	ND	Ester
8.	p-Propenyltoluene	3.85	$C_{10}H_{14}$	1.22 ± 0.17	ND	Aromatic hydrocarbon
9.	Linalool	3.87	$C_{10}H_{18}O$	ND	0.33 ± 0.11	Monoterpene
10.	Cis-3-Hexenyl butyrate	3.89	$C_{10}H_{18}O_2$	0.03 ± 0.007	ND	Ester
11.	Endo-borneol	4.25	$C_{10}H_{18}O$	0.54 ± 0.07	ND	Byciclic monoterpene
12.	Terpinen-4-ol	4.28	$C_{10}H_{18}O$	0.44 ± 0.05	ND	Monoterpene alcohol
13.	p-Tolyl acetate	4.36	$C_9H_{10}O_2$	0.24 ± 0.02	ND	Ester
14.	α-Terpineol	4.37	$C_{10}H_{18}O$	0.36 ± 0.23	ND	Monoterpene alcohol

Table 5. *Cont.*

No	Compound	RT	Molecular Formula	Relative Content (%)		Classification
				UAE-DES-HD	HD	
15.	Thymol methyl ether	4.50	$C_{11}H_{16}O$	0.55 ± 0.05	0.74 ± 0.23	Monoterpenoid
16.	Acetaldehyde O-methyloxime	4.82	$C_3H_5NO_2$	0.01 ± 0.01	ND	Oxime
17.	Thymol	4.82	$C_{10}H_{14}O$	47.04 ± 1.94	38.2 ± 0.01	Monoterpene
18.	Carvacrol	4.86	$C_{10}H_{14}O$	3.63 ± 0.97	4.86 ± 2.65	Monoterpene
19.	3-Methyl-4-isopropylphenol	4.86	$C_{10}H_{14}O$	3.64 ± 1.10	4.15 ± 1.84	Phenolic
20.	p-(Pentyloxy)acetophenone	4.90	$C_{13}H_{18}O_2$	0.03 ± 0.009	0.05 ± 0.02	Ketone
21.	Thymol acetate	5.09	$C_{12}H_{16}O_2$	0.40 ± 0.09	0.49 ± 0.18	Ester
22.	1,3-Indandione, 2-acetyl-	5.16	$C_{11}H_8O_3$	0.01 ± 0.0006	ND	Ketone
23.	1,1,5-Trimethyl-1,2-dihydronaphthalene	5.18	$C_{13}H_{16}$	0.06 ± 0.01	ND	Sesquiterpene
24.	(−)-Aristolene	5.22	$C_{15}H_{24}$	0.15 ± 0.06	ND	Sesquiterpenoid
25.	Isoledene	5.28	$C_{15}H_{24}$	0.41 ± 0.03	0.47 ± 0.18	Sesquiterpene
26.	β-selinene	5.61	$C_{15}H_{24}$	0.26 ± 0.05	ND	Sesquiterpene
27.	Aromadendrene	5.68	$C_{15}H_{24}$	3.08 ± 0.24	ND	Sesquiterpenoid
28.	1,4,7,-cicloundecatrieno, 1,5,9,9-tetrametil-, Z,Z,Z-	5.77	$C_{15}H_{24}$	6.15 ± 0.45	8.71 ± 3.08	Sesquiterpene
29.	Alloaromadendrene	5.82	$C_{15}H_{24}$	0.19 ± 0.01	ND	Sesquiterpenoid
30.	β-Panasinsene	5.85	$C_{15}H_{24}$	0.46 ± 0.03	0.54 ± 0.23	Sesquiterpene
31.	Cis-Calamenene	6.15	$C_{15}H_{22}$	ND	0.78 ± 0.01	Sesquiterpenoid
32.	Dehydro-aromadendrene	6.17	$C_6H_{12}O_2$	1.00 ± 0.05	ND	Sesquiterpenoid
33.	4′,6′-dihydroxy-2′,3′-dimethylacetophenone	6.23	$C_9H_{12}O_3$	0.76 ± 0.99	0.14 ± 0.03	Aromatic ketone
34.	Nerolidol 2	6.30	$C_{15}H_{26}O$	ND	0.88 ± 0.07	Sesquiterpene
35.	Ethanone, 1,1′,1″-(1,3,5-bencenethril)tris-	6.38	$C_{12}H_{12}O_3$	0.02 ± 0.003	ND	Aromatic ketone
36.	Cyclohexane, 1,2-dimethyl-3,5-bis(1-methylethenyl)-	6.40	$C_{14}H_{24}$	1.10 ± 0.23	ND	Cycloalkane
37.	1,3-Dimethyl-5-n-hexyladamantane	6.47	$C_{18}H_{32}$	0.03 ± 0.003	ND	Alkane
38.	Caryophyllenyl Alcohol	6.54	$C_{15}H_{26}O$	0.59 ± 0.11	ND	Alcohol
39.	(−)-spatulenol	6.57	$C_{15}H_{24}O$	0.92 ± 0.07	ND	Sesquiterpenoid
40.	(−)-globulol	6.62	$C_{15}H_{26}O$	1.75 ± 0.10	ND	Sesquiterpenoid
41.	Humuleno-1,2-epoxide	6.80	$C_{15}H_{24}O$	0.28 ± 0.02	1.14 ± 0.33	Epoxide
42.	(+)-rosifoliol	6.87	$C_{15}H_{26}O$	0.74 ± 0.44	0.92 ± 0.36	Sesquiterpene alcohol
43.	10-epi-γ -eudesmol	6.92	$C_{15}H_{26}O$	0.15 ± 0.01	ND	Sesquiterpene
44.	Humulenol-II	6.95	$C_{15}H_{24}O$	1.07 ± 0.03	2.38 ± 0.10	Sesquiterpene
45.	α-eudesmol	7.10	$C_{15}H_{26}O$	1.00 ± 0.02	ND	Sesquiterpenoid
46.	14-Hydroxycaryophyllene	7.19	$C_{15}H_{24}O$	0.65 ± 0.04	1.59 ± 0.67	Sesquiterpene
47.	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	7.23	$C_{15}H_{18}$	0.33 ± 0.65	ND	Aromatic hydrocarbon
48.	p-anisole	9.62	$C_{10}H_{14}O$	0.07 ± 0.01	ND	Aromatic ether

RT: retention time; UAE-DES-HD: ultrasound-assisted extraction with deep eutectic solvents and hydrodistillation; HD: conventional hydrodistillation. Monoterpene: 3.11 ± 1.51; Ester: 0.86 ± 0.12; Aromatic hydrocarbon: 1.55 ± 0.65; Bicyclic monoterpene: 0.54 ± 0.07; Monoterpene alcohol: 0.80 ± 0.28; Monoterpenoid: 0.55 ± 0.05; Oxime: 0.01 ± 0.01; Phenolic: 3.64 ± 1.10; Ketone: 0.04 ± 0.02; Sesquiterpene: 11.68 ± 5.36; Sesquiterpenoid: 9.34 ± 4.01; Aromatic ketone: 0.78 ± 0.33; Cycloalkane: 1.10 ± 0.23; Alkane: 0.03 ± 0.003; Alcohol: 0.59 ± 0.11; Epoxide: 0.28 ± 0.02; Sesquiterpene alcohol: 0.74 ± 0.44; Aromatic ether: 0.07 ± 0.01.

Fan and Li [24] reported this pattern in a study on the extraction of essential oil from *Angelica sinensis* radix using microwave-assisted hydrodistillation with eutectic solvents. The oils extracted with DES and microwaves were analyzed and compared to those obtained by conventional hydrodistillation. The results indicated that the oils from the DES treatment (choline chloride and citric acid 1:3) contained a higher concentration of ligustilide, the major compound reported for *A. sinensis*.

Similarly to the findings in this study, Chen, Xu, Pang, Jin, Lv, Li, and Lee [13] extracted essential oil from cloves (*Syzygium aromaticum*) using microwave-assisted hydrodistillation with eutectic solvents. They found that the DES composed of choline chloride and lactic acid in a 1:2 ratio, the same used in our study, generated oils with a more diverse chemical profile than those generated through conventional hydrodistillation.

The ability of acidic DES to dissolve lignin was reported by Yu et al. [25]. They investigated the effect of DES composed of carboxylic acids as hydrogen bond donors (HBD) as a pretreatment to dissolve lignin from pine and poplar, with lactic acid being particularly effective. The authors suggest that the carboxyl groups (-COOH) in HBDs provide active protons, enhancing the strength of hydrogen bonds and facilitating the breaking of various chemical bonds in lignin. They observed that the β-O-4 bond content in lignin decreased after contact with acidic DES, highlighting these solvents as promising agents for treating plant biomass.

3.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The minimum inhibitory concentration (MIC) of the optimized essential oil from *L. graveolens* biomass was 275 ppm, at which no visible growth was observed in the microplate. The minimum fungicidal concentration (MFC) was 300 ppm, confirmed by taking a sample from the well of the microplate at this concentration and applying it to a new PDA agar medium, where no growth was observed after 4 days. Similarly, Andrianjafinandrasana et al. [26] evaluated the effect of clove essential oil (*Syzygium aromaticum*) on the spore germination of *Colletotrichum asianum*. The authors reported a minimum inhibitory concentration (MIC) of 250 ppm. In this case, the values from that investigation and our work are close to each other. This closeness in MIC values could be attributed to the similarity in the lipophilic polarity of the major bioactive compounds present in the oils, which can penetrate the fungal cell and alter its function. For *S. aromaticum* essential oil, eugenol has been reported as the major compound, while for *L. graveolens* it is thymol, as confirmed in this work through chromatographic analysis. Both eugenol and carvacrol contain a phenol group in their structure, consisting of a benzene ring bonded to a hydroxyl group (-OH). This hydroxyl group can donate protons, giving the compound acidic properties. Phenolic groups can interact with the cell membranes of fungi through hydrogen bonds, potentially leading to membrane destabilization and loss of integrity. This interaction with cell membranes can alter essential functions of the fungi, such as nutrient and metabolite transport, ultimately leading to their death.

In another study by Muniz et al. [27], it was found that thyme essential oil (*Thymus vulgaris*) at 200 ppm completely inhibited the mycelial growth of *C. gloeosporioides*, considering this concentration as the MIC. It has been reported that essential oils have the capacity to damage the cytoplasmic membrane of microorganisms, affecting its structure and making it more permeable [28]. This means that the membrane becomes more permeable to substances that normally would not easily cross it, such as ions and large molecules. This can interfere with essential cellular processes and ultimately lead to the death or inhibition of the growth of the microorganism.

3.4. Mycelial Growth Inhibition

According to the results of the linear regression corresponding to the percentage of mycelial growth inhibition by the optimized essential oil from *L. graveolens* biomass, the effective concentration at 50% inhibition (EC_{50}) was 78.05 ppm. The highest concentration

tested, 250 ppm, showed 100% inhibition. Therefore, the maximum concentration range was limited to 100 ppm (Figure 6).

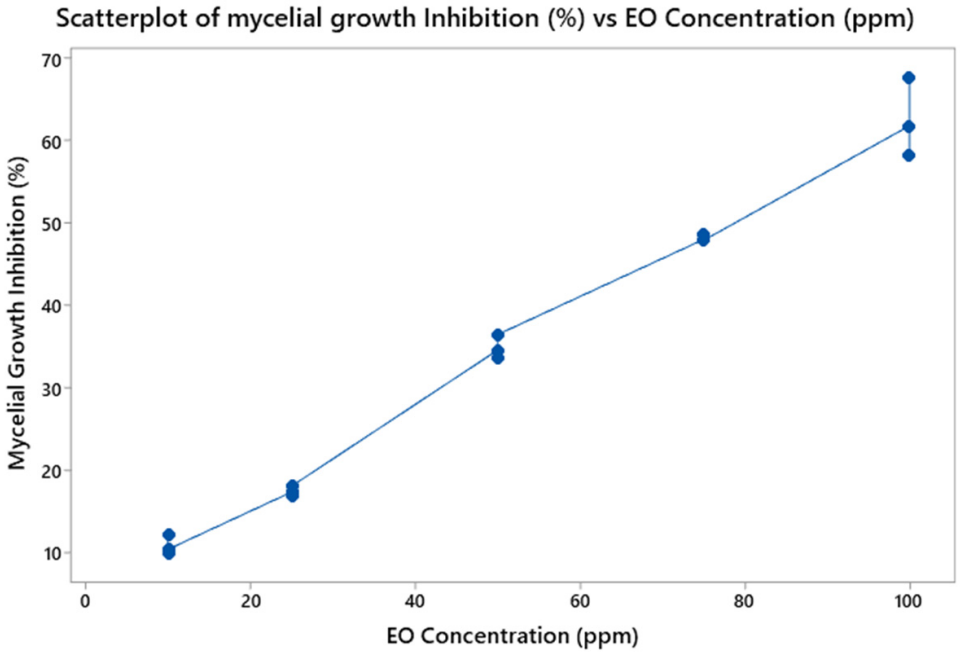

Figure 6. Graph showing the effective concentration at 50% (EC_{50}) of the optimized essential oil from *Lippia graveolens* biomass that inhibits the mycelial growth of *Colletotrichum asianum*. (y = 0.5838x + 4.4342; R^2 = 98.79%; Adjusted R^2 = 98.70%; EC50: 78.05 ppm).

The linear regression analysis presents a coefficient of determination R^2 = 98.79% and adjusted R^2 = 98.70%, which are very close. In linear regression, this suggests that the model is capturing the variability in the data well and is not overfitting. This indicates that the model is robust and reliable in explaining and predicting the dependent variable.

Huang et al. [29] evaluated the effect of *Artemisia scoparia* essential oil on *Colletotrichum gloeosporioides* using an agar diffusion assay, where they obtained an EC_{50} of 9320 ppm. Additionally, the chemical profile of the oil was determined using gas chromatography–mass spectrometry. The major compounds reported were ethenyl-naphthalene (23.5%), 2,4-pentadienyl-benzene (11.8%), 1,2-dimethoxy-4-(2-propenyl)-benzene (10.0%), β-pinene (8.0%), and 1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene (6.3%). The large difference between the EC_{50} values of the study in question and this work could be attributed to the chemical profile of the essential oils, as the major compound in *A. scoparia* was ethenyl-naphthalene, while in *L. graveolens* it was thymol. Previous reports in the literature indicate that thymol is a compound with high antimicrobial activity due to its ability to penetrate the fungal cell wall and disrupt its function, which could be the mechanism against this phytopathogenic species.

In addition, it is important to mention that, although they are pathogens of the same genus and share certain characteristics, they also present significant differences in terms of morphology, pathogenicity, genetics, and metabolism. These differences can affect their ability to infect different host plants and their responses to various treatments, including antifungal agents and essential oils.

On the other hand, Lima Oliveira et al. [30] applied *Cymbopogon citratus* (D.C. ex Nees) Stapf essential oil alone and in combination with chitosan on different species of *Colletotrichum*. When evaluating the essential oil alone at its highest concentration of 1250 ppm, they found a mycelial growth inhibition percentage of 37% on *C. asianum*. This greatly differs from the results obtained in this work, as the EC_{37} of *L. graveolens* was 55.78 ppm. Comparing both values, the oil of *L. graveolens* is 22.4 times more effective in inhibiting *C. asianum*. A possible reason for the difference between these values is

the compounds present in the essential oils. According to the authors of the mentioned study, the major compounds in *C. citratus* oil were geranial (51.39%) and neral (29.29%). Although these compounds can affect the fungal cell wall, thymol (the major compound in *L. graveolens*) is generally considered to be more effective in terms of cell wall disruption and antifungal activity.

3.5. In Vivo Fungicidal Activity

The results of the fungicidal activity of the optimized essential oils and commercial fungicides are presented in Tables 6–9. The ANOVA for wounded fruits (Table 6) shows that the fungicide factor is the most contributive to total variability (48.68%, $p < 0.000$), followed by concentration (33.22%, $p < 0.000$), with the method factor contributing the least to variability (1.58%, $p < 0.000$). For the interaction's fungicide/concentration and fungicide/method, both have similar contributions to variability (6.23%, $p < 0.000$) and (6.38%, $p < 0.000$), respectively. The remaining interactions, concentration/method, and the three-way interactions have very low contributions to variability (0.46%, $p < 0.027$) and (1.25%, $p < 0.002$). The ANOVA shows a coefficient of determination $R^2 = 97.92\%$ and an adjusted coefficient of determination $R^2 = 96.93\%$. The close values between these coefficients indicate that the model has a good capacity to explain the data.

Table 6. Analysis of variance for fungicide effectiveness on wounded fruits.

Source	Degrees of Freedom	Contribution	Adjusted SS	Adjusted MS	F Value	*p* Value
Fungicide	2	48.68%	12,808.6	6404.31	420.89	0.000
Concentration	2	33.22%	8767.6	4383.79	288.10	0.000
Method	1	1.58%	414.6	414.59	27.25	0.000
Fungicide × Concentration	4	6.23%	1639.9	409.98	26.94	0.000
Fungicide × Method	2	6.38%	1678.8	839.39	55.16	0.000
Concentration × Method	2	0.46%	122.2	61.09	4.01	0.027
Fungicide × Concentration × Method	4	1.25%	330.2	82.54	5.42	0.002
Error	36	2.08%	547.8	15.22		
Total	53	100%				

According to the results in Table 7, the most effective combination for preventing anthracnose in papaya fruits caused by *Colletotrichum asianum* in the presence of wounds consists of the corrective application of oregano essential oil at 2000 ppm using the corrective method. Our proved to be more effective than the chemical product Tecto 60 (thiabendazole). In this case, the highest mean, corresponding to the corrective method, does not share a letter with any other, indicating a significant difference in terms of fungicide effectiveness. This means that the group treated with the corrective method has a significantly higher effectiveness compared to the other groups. The least effective treatments correspond to Timorex (*Melaleuca alternifolia* essential oil) at concentrations of 1000 and 300 ppm, both applied with the corrective method (Figure 7). Consequently, it can be stated that these treatments are not suitable for correcting anthracnose.

Additionally, Sarkhosh, Schaffer, Vargas, Palmateer, Lopez, Soleymani, and Farzaneh [20] investigated the fungicidal effect of five essential oils from different plants on anthracnose caused by *C. gloeosporioides* in papaya fruits. The results of the assay on wounded and pathogen-inoculated papaya fruits showed that essential oils of *Satureja khuzistanica* and *Thymus* spp., at a concentration of 2000 ppm, caused a 59.26% and 58.40% reduction in lesion diameter and a 64.07% and 54.82% reduction in fruit decay, respectively. The application of these oils resulted in better preservation of fruit firmness. The results of our research showed greater effectiveness at the same concentration of 2000 ppm for the treatment with optimized oregano essential oil. The difference between the results of both studies could be due to the different chemical profiles of the essential oils used, as the nature of the compounds in the oils directly impacts their biological activity. Additionally, the species of

phytopathogenic fungus must be considered, as one species may be more susceptible to the chemical compounds of the oil than the other.

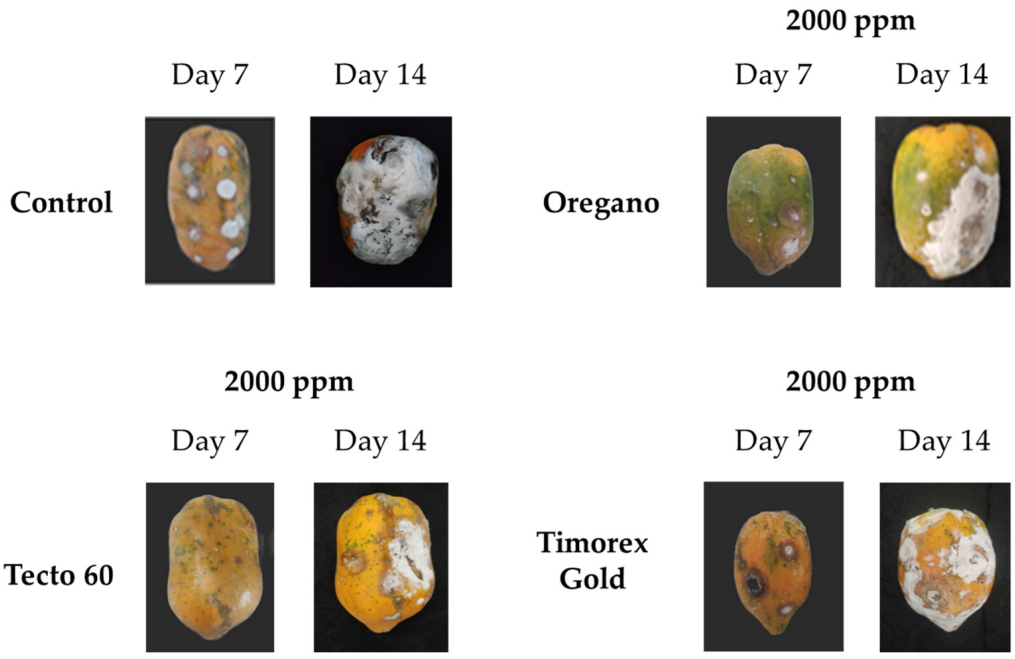

Figure 7. Wounded papaya fruits 7 days and 14 days after inoculation.

Table 7. Tukey's method comparisons of mean fungicide effectiveness on wounded fruits.

Fungicide × Concentration × Method	Mean (% Effectiveness)	Groups
Oregano 2000 corrective	85.60	A
Tecto 2000 corrective	76.25	A B
Tecto 2000 preventive	74.30	A B
Tecto 1000 corrective	69.90	B
Tecto 1000 corrective	64.45	B C
Oregano 1000 corrective	56.41	C D
Oregano 2000 preventive	52.43	D E
Oregano 300 corrective	47.61	D E F
Oregano 1000 preventive	45.33	D E F G
Timorex 2000 preventive	40.93	E F G H
Tecto 300 corrective	36.22	F G H
Timorex 2000 corrective	35.20	G H
Oregano 300 preventive	32.69	H
Tecto 300 preventive	30.34	H I
Timorex 1000 preventive	20.61	I J
Timorex 300 preventive	20.56	I J
Timorex 1000 corrective	14.30	J
Timorex 300 corrective	10.03	J

Different letters indicate statistical significance ($p < 0.05$) according to Tukey's test. The results show the means of three replicates ($n = 3$).

The ANOVA for unwounded fruits (Table 8) shows that the concentration factor is the most contributive to total variability (38.82%, $p < 0.000$), followed by the method (11.73%, $p < 0.000$), with the fungicide factor contributing the least (2.53%, $p < 0.000$). For the interactions, the triple interaction was the most contributive (21.16%, $p < 0.000$), followed by the fungicide/method interaction (11.45%, $p < 0.000$) and the concentration/method interaction (8.45%, $p < 0.000$). The interaction with the least contribution to total variability was fungicide/concentration (3.98%, $p < 0.000$). The closeness between the coefficient of determination $R^2 = 98.12\%$ and the adjusted coefficient of determination $R^2 = 97.23\%$ indi-

cates that the model can explain the variability of the data adequately and that unnecessary or redundant factors are not being included in the model. This gives us confidence in the model's predictive ability.

Table 8. Analysis of variance for fungicide effectiveness on unwounded fruits.

Source	Degrees of Freedom	Contribution	Adjusted SS	Adjusted MS	F Value	*p* Value
Fungicide	2	2.53%	698.3	349.17	24.23	0.000
Concentration	2	38.82%	10,718.8	5359.38	371.89	0.000
Method	1	11.73%	3238.9	3238.91	224.75	0.000
Fungicide × Concentration	4	3.98%	1099.7	274.93	19.08	0.000
Fungicide × Method	2	11.45%	3162.7	1581.34	109.73	0.000
Concentration × Method	2	8.45%	2332.4	1166.20	80.92	0.000
Fungicide × Concentration × Method	4	21.16%	5841.4	1460.36	101.34	0.000
Error	36	1.88%	518.8	14.41		
Total	53	100%				

S = 3.79, R^2 = 98.12%, R^2 (adjusted) = 97.23%, R^2 (predicted) = 95.77%.

In the case of the fungicidal activity assay on unwounded papaya fruits, according to Table 9, eight groups had the same mean effectiveness corresponding to 100%, including oregano essential oil with the corrective method at 2000, 1000, and 300 ppm. Within the same group A, Timorex Gold (*Melaleuca alternifolia* essential oil) at 2000 ppm with both methods and at 1000 ppm with the preventive method were also included, indicating that, at the highest concentration, this product is equally effective as a corrective and preventive treatment on papaya fruits; meanwhile, at a lower concentration of 1000 ppm, it is equally effective as a preventive treatment. The remaining means in group A correspond to the chemical control Tecto 60 (thiabendazole) at 2000 and 1000 ppm with the corrective method, confirming its high effectiveness as a corrective treatment. The least effective treatment in the assay corresponds to Timorex at 300 ppm with the preventive method, indicating that this treatment is not effective as a preventive fungicide (Figure 8).

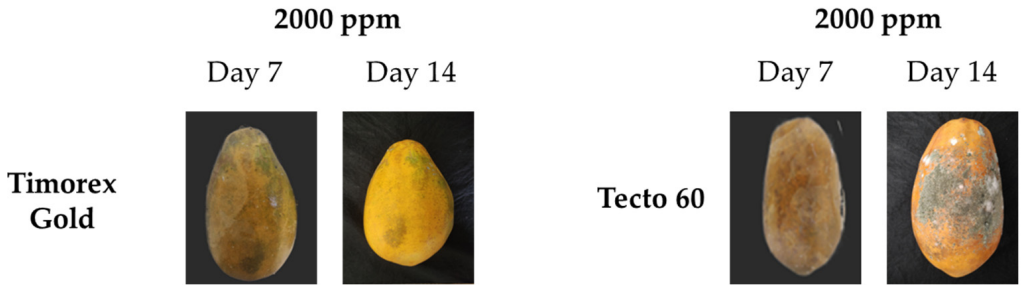

Figure 8. Papaya fruits without wounds 7 days and 14 days after inoculation.

Uclaray et al. [31] studied the effect of encapsulated phenolic extracts of Cuban oregano (*Plectranthus amboinicus*) with plasmolyzed yeast on papaya fruits infected by *C. gloeosporioides*. When applied as a postharvest spray treatment for papaya, the microcapsules exhibited significant inhibitory action on the mycelial growth against *C. gloeosporioides* and greatly reduced anthracnose symptoms on the papaya fruits. At concentrations of 0.1, 1, and 10 mg/L of the non-encapsulated Cuban oregano phenolic extract, the severity percentage was around 50% for all three inoculated samples. Comparing these results with those of our research, corresponding to the lowest concentration of Mexican oregano by the preventive method, at 300 ppm, an effectiveness percentage of 48.28% was obtained. This indicates that the Cuban oregano phenolic extract is more effective in protecting the fruit from fungal attack, requiring a much lower concentration to achieve this effect. These differences could be attributed to the different fungal species tested (*C. gloeosporioides* and *C. asianum*), as the virulence of the species could be influencing the assay results. Additionally, differences in the chemical compositions of the phenolic extract and the essential oil play a crucial

role in their respective fungicidal capacities. The phenolic extract of Cuban oregano may contain various phenolic compounds, including flavonoids and polyphenols, which could contribute to its fungicidal properties. In contrast, the essential oil of Mexican oregano is a complex mixture of volatile compounds, such as terpenes and phenylpropanoids, which may also exhibit fungicidal activities.

Table 9. Tukey's method comparisons of fungicide effectiveness means on unwounded fruits.

Fungicide × Concentration × Method	Mean (% Effectiveness)	Groups
Oregano 300 corrective	100	A
Oregano 1000 corrective	100	A
Tecto 1000 corrective	100	A
Timorex 1000 preventive	100	A
Tecto 2000 corrective	100	A
Timorex 2000 preventive	100	A
Oregano 2000 corrective	100	A
Timorex 2000 corrective	100	A
Tecto 2000 preventive	93.01	A B
Oregano 2000 preventive	88.12	B
Timorex 300 corrective	75.28	C
Oregano 1000 preventive	71.45	C D
Tecto 300 corrective	62.87	D E
Tecto 1000 preventive	57.81	E F
Tecto 300 preventive	48.42	F G
Oregano 300 preventive	48.28	F G
Timorex 1000 corrective	47.57	F G
Timorex 300 preventive	39.20	G

Different letters indicate statistical significance ($p < 0.05$) according to Tukey's test. The results show the means of three replicates ($n = 3$).

In another study by Barrera-Necha et al. [32], a fungicidal assay was conducted on papaya fruits inoculated with *C. gloeosporioides*, testing the oils of *C. zeylanicum* and *S. aromaticum* on papaya fruits during storage at room temperature and 14 °C. The lowest infection percentage of 3.3% was observed in papaya fruits treated with *S. aromaticum* at 47 ppm and 12.4% for *C. zeylanicum* at a concentration of 236 ppm at both evaluated temperatures. *S. aromaticum* oils could be an alternative for controlling *C. gloeosporioides* in papaya fruits. Comparing both studies, it can be noted that the results vary significantly. In this work, oregano essential oil and Timorex Gold showed high effectiveness in treating papaya fruits. However, in the study by Barrera-Necha, Bautista-Baños, Flores-Moctezuma, and Estudillo [32], *C. zeylanicum* and *S. aromaticum* oils were more effective. These differences could be attributed to variations in the concentrations used, storage conditions, and specific properties of the essential oils, including the presence of eugenol in clove oil, which could influence its antifungal activity. Both compounds, eugenol and thymol, could interact with fungi, causing anthracnose (*C. gloeosporioides* and *C. asianum*) similarly by affecting their cell membrane and vital metabolic processes. However, due to their structural differences and distinct mechanisms of action, each compound may exhibit varying affinities for specific pathways and cellular components of the fungus. This could explain the variations in effectiveness observed in previous studies and how each can have a distinct impact on the inhibition of growth and development of these Colletotrichum species. Given that they are different species, their responses to antimicrobial compounds, such as eugenol and thymol, may vary due to their distinct biochemical profiles and sensitivities to essential oils. Although both compounds may have general antimicrobial properties, the way they interact with the species may depend on the unique molecular and metabolic characteristics of each one. For example, eugenol and thymol may have different affinities for the specific metabolic pathways present in *C. gloeosporioides* and *C. asianum*, which could affect their viability and cellular function differently in each species. Additionally, the species may

have differences in the composition of their cell membranes and other essential components, which could influence how the compounds interact and exert their effect.

4. Conclusions

The optimal process conditions for the ultrasound-assisted DES extraction of essential oil from Mexican oregano (*Lippia graveolens* Kunth) biomass as a pretreatment for hydrodistillation were 0.38 g/mL for solid/liquid ratio, 45.15% ultrasonic amplitude, and 7.49 min of ultrasonic time. The estimated values according to the response surface design were 1.127% for essential oil yield and 1.403 for TRC (mg GAE/g biomass). The experimental values obtained under optimal conditions were 1.0932 ± 0.055 and 1.4788 ± 0.06 for essential oil yield and TRC, respectively. The fact that these values are very close to the predicted ones indicates that the prediction models used are accurate and valid for predicting the results. A total of 47 compounds were found in the optimized essential oil and 20 compounds obtained by conventional hydrodistillation. In both cases, the major compound was thymol. The in vitro fungicidal activity of the optimized essential oils against *C. asianum* revealed that the essential oil of *L. graveolens* Kunth was effective in inhibiting the mycelial growth and spore germination of the fungus. The optimized essential oil of *L. graveolens* was effective on unwounded fruits at all concentrations using the corrective method. For wounded fruits, the highest concentration of oregano essential oil was the best treatment against the fungus *C. asianum*, deriving even better results than the positive controls corresponding to commercial products.

Therefore ultrasound-assisted DES extraction is a promising process to extract essential oils with antifungal potential against *C. asianum* from Mexican oregano biomass.

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