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Original Article

# Synergistic Effect of Cloves (*Syzygium aromaticum*), Thyme (*Thymus vulgaris*) and Lemon (*Citrus limon*) Blended Essential Oils Optimized by Mixture Design for Improving the Antioxidant Activity

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## Abstract

**Background:** Combining various essential oils (EOs) for developing pharmaceutical formulations has been the focus of attention in recent years.

**Objectives:** This study aimed to determine the antioxidant effect of the combination of three Eos obtained from clove (*Syzygium aromaticum* L.), lemon peel (*Citrus limon* L.), and thyme *(Thymus vulgaris* L.*)* by using mixture design.

**Methods:** The EOs of lemon peel (EOL), clove (EOC), and thyme (EOT) as well as their combination were analyzed using a gas chromatograph with flame ionization detector (GC/FID). The antioxidant activities of the EOs from EOL, EOC, and EOT as well as their combination were measured adopting DPPH assay. The construction and statistical analysis of the experiment were designed using the NemrodW (LPRAI, version 2000) software.

**Results:** EOL, EOC, and EOT were found capable of neutralizing DPPH radical. EOC was distinguished by its strongest antiradical activity with $IC_{50}=15.02\pm0.02$ µg/mL. EOT had an $IC_{50}=29.20\pm0.12$ µg/mL while EOL had $188.69\pm0.95$ µg/mL. The positive standard BHT was detected to be $IC_{50}=24\pm0.02$ µg/mL. The optimal, combinative mixture of essential oils may have been determined based on these iso-response curves which allowed fixing the ideal combinations of ingredient in terms of quantity to obtain an EO mixture possessing appreciable and optimal antioxidant characteristics. The predicted antioxidant properties determined by the mixing plan model were retained and the experiments were carried out respecting the contents of proposed ingredients of 25.7% EOT, 32.3% EOL, and 41.9% EOC equivalent to 15.42 mg, 19.38 mg and 25.14 mg, respectively. This resulted in arriving at an essential oil mixture with an experimental $IC_{50}=11.023\pm0.145$ µg/mL which was similar to those of the predicted antioxidant properties with an order of $10.907\pm0.212$ µg/mL and a non-significant difference of $P<0.05$, based on which the validity of the proposed mixing plan model was determined. The combined EO was also found to be rich in eugenol ($32.35\pm1.13$%), thymol ($25.49\pm0.03$%), and limonene ($21.30\pm0.02$%).

**Conclusion:** Statistical planning and the development of utility profiles for mixtures of essential oils may have been used to predict the optimal composition as well as to determine their antioxidant profile.

**Keywords:** Clove (*Syzygium aromaticum* L.), Lemon peel (*Citrus limon* L.), Thyme (*Thymus vulgaris* L.), Essential oil, Antioxidant activity, Mixture design

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## Background

Oxidative stress occurs naturally as an outcome of aerobic cellular metabolism (1). The free radicals exert harmful effects on human body due to their unstable complexes responsible for DNA mutations, lipid oxidation, and peroxidation of proteins. All these processes appear to enormously contribute to several disorders including cardiovascular and neurodegenerative diseases (2). Since developing a new effective strategy for protecting human health from free radical damage has been the focus of attention in recent years, natural products obtained from plant species have attracted considerable research attention (3). Among these various kinds of natural substances, plant-derived essential oils (EOs) have been historically

confirmed to be valuable sources of bioactive molecules which could be exploited as bio-products for industrial purposes (e.g., pharmaceutical and food industries) (4).

EOs have been used not only in monotherapy but in combinations for many years. The interaction between EO compounds can produce four possible effects, namely indifferent, additive, antagonistic, or synergistic effects (5). Generally, the antagonistic effect is attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons (6). The additive and synergism effects are associated with phenolic and alcohol compounds (7). Therefore, the compounds with similar structures exhibit additive rather than synergistic effect. The possible synergistic effect produced by the combination of plant EOs has been determined to be an efficient strategy to inhibit or reduce the natural oxidation process of foods. In light of these considerations, three local aromatic and medicinal plant species, namely clove (*Syzygium aromaticum*), lemon peel (*Citrus limon*), and thyme (*Thymus vulgaris*) were selected for synergistic combination screening of their EOs after considering their popular use in traditional medicine. Since there was no study investigating the combination of their EOs in the literature, this study aimed to determine the antioxidant effect of the combination of three EOs obtained from clove, lemon peel, and thyme using mixture design.

## Materials and Methods

### *Plant Materiel*

In this study, three aromatic and medicinal plants including cloves (*Syzygium aromaticum*), thyme (*Thymus vulgaris* L.), and citrus (*Citrus limon* L.) were examined. Tunisian cloves were purchased from the local market in the form of dried flower buds and then were stored in a dry, tightly closed bottle. Thyme was collected in March from the mountain of Bou Garnine and was transferred to a laboratory where it was dried at room temperature before being subjected to the extraction of EO. Lemon peel was obtained from the lemon tree (four seasons due to its great floridity) variety 'Eureka'. Voucher specimens were deposited in the herbarium of our laboratory (*Syzygium aromaticum* Sa-LPAM-2021; *Citrus limon* Cl-LPAM-2021; *Thymus vulgaris* Tv-LPAM-2021).

### *Essential Oil Extraction Techniques*

#### *Essential Oils Extraction From Thyme Leaf and Lemon Peel by Clevenger*

The apparatus used for performing hydro distillation was of the Clevenger type. It consisted of a flask heater, a 2 L Pyrex glass flask where we placed 100 g of dried material, a vapor condensation column (refrigerant), and a collector in Pyrex glass which received the extracts from the distillation for 3 hours. The condensed vapor led to the EO which was separated from the hydrolate (aromatic waters) by performing decantation after adding magnesium sulfate (MgSO4) to remove traces of water. The EOs were collected directly by using a Pasteur pipette over the distillate without adding any solvent. The quantity of the obtained EO was weighed in order to calculate the yield and, then, was stored in opaque bottles at 4°C (8).

#### *Extraction of Clove Essential Oil by Distillation*

As for extraction, 500 g of cloves were soaked in 4 L of distilled water in a stainless steel still and, then, the still was heated under pressure to bring its contents to the boil for 3 hours. After the condensation, the EO was separated from the distillate by decantation after adding MgSO4 to remove traces of water. The quantity of EO obtained was weighed in order to calculate the yield, and then it was stored in opaque bottles at 4°C.

### *Characterization by Chromatographic Analysis of the Obtained Essential Oils*

#### *Identifying the Volatile Compounds by GC/MS*

CPG/MS coupling makes it possible to identify volatile compounds. The principle behind this identification is based on the fragmentation of compounds to follow their bombardment by a flow of electrons as well as their exposure to electric fields. In our study, the released ions were classified according to their mass/charge ratio (m/z). The analysis was carried out by a chromatograph coupled to a quadrupole type mass spectrometer (HEWLET-PACKARD 5972 A) and equipped with a HP-5ms column and an oven temperature program (50°C to 240°C at a rate of 5°C/min).

#### *GC-FID Quantification Method*

The analysis was carried out by Hewlett-Packard 6890 chromatograph equipped with an electronic pressure control injector, a flame ionization detector, and a HP-INNOWax (polyethylene glycol capillary) column (30 m x 0.25 mm; 0.25 µm). The flow of the carrier gas (N2) was 1.6 mL/min, and the split ratio was 60:1. When conducting the analysis, the following temperature program was followed: oven temps isotherm at 35°C for 10 minutes, from 35 to 205°C at the rate of 2°C/min, and isotherm at 205°C for 10 minutes. Injector and detector temperatures were maintained at 250 and 300°C, respectively. The injection volume was 1 µL.

### *Identifying Volatile Compounds by GC/MS*

The GC/MS coupling facilitated the identification of volatile compounds. The principle behind the identification was based on the fragmentation of compounds through their bombardment by a flow of electrons as well as their exposure to electric fields (9). The released ions were classified according to their mass/charge ratio (m/z). The analysis was carried out by employing a chromatograph coupled to an Agilent mass spectrometer (5975C inert XL MSD) and performing electron impact ionization (70 eV). An HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) coated with 5 % phenyl methyl silicone and 95% dimethylpolysiloxane was used. The oven temperature was programmed at 40°C for 1 minute, and

then it was raised from 40 to 100°C at a rate of 8°C/min and kept constant at 100°C for 5 minutes. The temperature was increased to 200°C with a rate of 10°C/min and kept constant at 200°C for 3 minutes and, then, the final temperature was set up at 300°C with a rate of 2°C/min. Injector temperature was set at 250°C. The carrier gas was helium with a flow rate of 1 mL/min, and the split ratio was 100:1. Scan time and mass ranges were 1 second and 50-550 m/z, respectively. Individual peaks corresponding to the volatile components were identified by comparing their retention indices (RI) relative to (C8-C40) n-alkanes with those of literature or those of authentic compounds available in the authors' laboratory. Further identification was made by matching their recorded mass spectra with those stored in the Wiley 09 NIST 2011 mass spectral library of the GC/MS data system.

### *Antioxidant Activity Assay*

Following the method developed by Hatano et al (9), the anti-free radical activity was evaluated by the percentage inhibition of the degradation of the DPPH radical measured by spectrophotometry at 517 nm. A 250 µL aliquot of the solution DPPH was added to 1 mL of EO (sample) at different concentrations (5, 10, 15, 100 mg/mL). The variation in absorbance was measured after keeping the mixtures for 30 minutes at obscurity by referring to a reference without EO. The anti-free radical activity was estimated as a percentage inhibition using the following formula:

$IP = (OD\ control - OD\ sample/\ OD\ control) \times 100$

Where IP: percentage inhibition, OD control: reading of the control absorbance, OD sample: reading of the absorbance of the solution of the sample. The $IC_{50}$ value was expressed in µg/mL. The lower value corresponded to the effectiveness of the higher antioxidant activity of the sample.

### *Optimization of the Antioxidant Activity of the Blended Essential Oil by Using Mixture Design Methodology*

The studied optimal response, which was the antioxidant activity of the blended EO expressed in median inhibitory concentration $IC_{50}$, was obtained by using NemrodW software (LPRAI, version 2000) in order to define the optimal blending condition made with three main ingredients. These were the EOs from three medicinal plants including clove (*Syzygium aromaticum*) from the flower ($HE_C$), Lemon tree (*Citrus limon* L.) from the zest ($HE_{CL}$), and Common thyme (*Thymus vulgaris* L.) from the leaves ($HE_T$). The results obtained by software are presented in two graphs (i.e., mixing contour graph and 3-dimensional graph) of the optimum desirable response. The mixing plan design employed in this study was developed after referring to an earlier study by Crespo et al (10).

### *Studied Factors and Experimental Fields*

Table 1 summarizes three ingredients of the matrix of mixing plan (i.e., EOT ($X_1$), EOL ($X_2$) and EOC ($X_3$)), which was created using the NemrodW software (LPRAI 2000, France). Likewise, the studied factors as well as their experimental fields are shown in Table 1.

### *Experimental Matrix for the Design of Mixtures*

The experimental matrix was prepared by making 10 blends of the following ingredients in different amounts (µg/mL): EOT ($X_1$), EOL ($X_2$) and EOC ($X_3$). The various blends obtained were subjected to a test to assess the DPPH antioxidant activity (Table 2).

### *Statistical Analysis*

The results reported in this study are mean values of at least three repetitions (n = 3) unless otherwise stated. Pearson correlation and principal component analysis (PCA) was calculated by XLStat. Pro® Version 2014.5.03 statistical software (XLStat, Paris, France) was used to analyze the data, and significance level was set at $P < 0.05\%$. The construction and the statistical analysis of the experimental were design using the NemrodW (LPRAI, version 2000) software.

## Results

### *Optimization of Antioxidant Activity by Mixture Design*

The antioxidant activities of the EOs from lemon peel (EOL), clove (EOC), and thyme (EOT) were measured using DPPH assay. The results showed that EOL, EOC, and EOT had the potential to neutralize DPPH radical. EOC was distinguished by its strongest antiradical activity with $IC_{50} = 15.02 \pm 0.02$ µg/mL. EOT had an $IC_{50} = 29.20 \pm 0.12$ µg/mL while EOL had 188.69 ± 0.95µg/mL. The positive standard BHT showed an $IC_{50} = 24 \pm 0.02$ µg/mL. The desirable optimal response was elucidated by two curves,

**Table 1.** Studied Factors and Experimental Fields

| | Corposant | Constraint Inferior | Constraint Superior |
|---|---|---|---|
| X1 | EOT | 0.0000 | 1.0000 |
| X2 | EOL | 0.0000 | 1.0000 |
| X3 | EOC | 0.0000 | 1.0000 |
| | Total | 1.0000 | |

**Table 2.** Experimental Matrix of Mixture Design

| N° Exp | EOT ($X_1$) | EOL ($X_2$) | EOC ($X_3$) | DPPH $IC_{50}$ ($Y_1$) µg/mL |
|---|---|---|---|---|
| 1 | 1.0000 | 0.0000 | 0.0000 | 29.200 |
| 2 | 0.0000 | 1.0000 | 0.0000 | 188.690 |
| 3 | 0.0000 | 0.0000 | 1.0000 | 15.020 |
| 4 | 0.0000 | 0.5000 | 0.5000 | 120.250 |
| 5 | 0.5000 | 0.0000 | 0.5000 | 12.355 |
| 6 | 0.5000 | 0.5000 | 0.0000 | 80.560 |
| 7 | 0.6667 | 0.1667 | 0.1667 | 60.658 |
| 8 | 0.1667 | 0.6667 | 0.1667 | 94.655 |
| 9 | 0.1667 | 0.1667 | 0.6667 | 18.960 |
| 10 | 0.3333 | 0.3333 | 0.3333 | 40.236 |

one by the contours of the mixture (2D) and another one by 3D obtained based on the interaction between three ingredients including EOT ($X_1$), EOL ($X_2$), and EOC ($X_3$), respectively (Figure 1). As for each response, the predicted and experimental results were investigated to confirm the optimal results proposed by the mixture design. Figure 1A and 1B represent the iso-response curves of the optimal antioxidant responses in terms of the median inhibitory concentration ($IC_{50}$) of free radical's DPPH. The optimal mixture of the EO combination may have been determined through these iso-response curves which allowed fixing the ideal combinations of ingredient (EOT ($X_1$), EOL ($X_2$) and EOC ($X_3$)) in terms of quantity to obtain an EO mixture with appreciable and optimal antioxidant characteristics.

### *Meaning the Factors for the Response Y ($IC_{50}$: DPPH)*

The significance coefficients of the factors involved for the response Y: ($IC_{50}$: DPPH), are shown in Table 3. The results showed that certain ingredients such as the EO of citrus Lemon ($HE_C$ ($X_2$)) (b2) may have significantly influenced the antioxidant activity of the obtained EO mixture with significance of *P* value < 1% (level of significance 99%). In fact, the addition of lemon EO was capable of influencing the antioxidant activity negatively by increasing the value of $IC_{50}$. For the same response (Y: ($IC_{50}$: DPPH)), the other ingredients (EOT ($X_1$) and EOC ($X_3$)) were found to be insignificant with percentage order of 13.8% and 60.0%, which showed a considerable decrease in $IC_{50}$ and, consequently, a considerable increase in the antioxidant activity of EO mixture (Table 3). These results indicated that the interactions among the ingredients may have contributed to the antioxidant properties of EO mixture.

### *Predicted and Experimental Values of Responses Under Optimal Conditions*

Analysing the results in Table 4 regarding the residues of the Y response ($IC_{50}$: DPPH), it was found that the predicted antioxidant properties proposed by the mixing plan model were retained and the experiments were carried out respecting the contents of 'proposed ingredients of 25.7% EOT ($X_1$), 32.3% EOL ($X_2$), and 41.9% EOC ($X_3$) equivalent to 15.42 mg, 19.38 mg and 25.14 mg, respectively, per 50 g of microcapsule powder.

This facilitated arriving at an EO mixture with experimental $IC_{50}$ values of the order of 11.023 ± 0.145 µg/mL which were similar to those of the predicted antioxidant properties which were of the order of 10.907 ± 0.212 µg/mL, with a non-significant difference of $P < 0.05$, based on which it was found that the proposed mixing plan model was valid (Table 5). The optimization procedure by using the mixing plan resulted in a considerable improvement in the antioxidant activity of the EO samples ($IC_{50}$ = 11.023 ± 0.145 µg/mL) formulated based on a combination of clove, thyme, and lemon peel EOs.

Moreover, Table 6 shows that the "ratio-F" regression which was the ratio between the mean square of the regression and the residue for the response Y were greater than the tabulated value: Freg Y (5.4, 0.05) = 10.2927 > 6.26) with a probability less than 5%, which confirmed that the coefficients of the factors of the postulated model were significant. What consolidated the previous result was the fact that the postulated model was valid (Table 6).

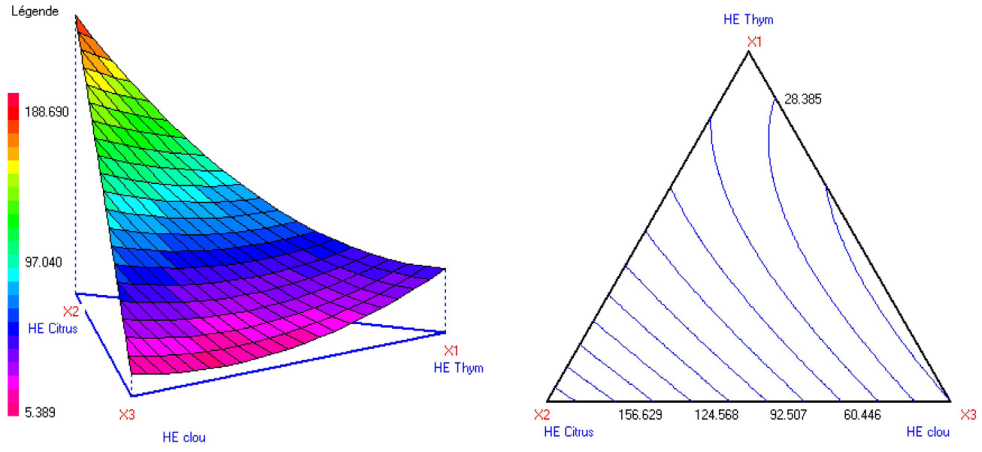
Therefore, the antioxidant property model (DPPH) of the optimized blended EO proposed by the mixture design was written as follows:

$$\mathbf{Y}_{(\mathbf{DPPH,\ IC50})} = 40.43\ X_1 + 184.89\ X_2 + 12.56\ X_3 - 151.21\ (X_1{*}X_2) - 74.00\ (X_1{*}X_3) + 8.50\ (X_2{*}X_3)$$

**Table 3.** Factor Significance of Y ($IC_{50}$ : DPPH)

| Nom | Coefficient | F. Inflation | Ecart-Type | t.exp. | Signif. % |
|---|---|---|---|---|---|
| | | Y: ($IC_{50}$: DPPH) | | | |
| b1 | 40.43 | 1.96 | 21.96 | 1.84 | 13.8% |
| b2 | 184.89 | 1.96 | 21.96 | 8.42 | ** |
| b3 | 12.56 | 1.96 | 21.96 | 0.57 | 60.0% |
| b12 | -151.21 | 1.98 | 101.22 | -1.49 | 20.9% |
| b13 | -74.00 | 1.98 | 101.22 | -0.73 | 50.9% |
| b23 | 8.50 | 1.98 | 101.22 | 0.08 | 93.5% |

**represents the significance at *P* value < 1%. Y ($IC_{50}$; DPPH): resistance at transaction (Y1). $b_1$: coefficient of X1 factor (EOT); $b_2$: coefficient of $X_2$ factor (EOL); $b_3$: coefficient of X3 factor (EOL)




(A) 3D graphical study (A) 2D graphical study

**Figure 1.** Iso-response Variation of YDPPH $IC_{50}$ in the Mixture Design (X1: EOT, X2: EOL, X3: EOC).

**Table 4.** Response Residues of Y ($IC_{50}$: DPPH)

| Number | Yexp. | Ycalc. | Difference | Nome | SE | Student-R | R-Student | D-Cook |
|---|---|---|---|---|---|---|---|---|
| 1 | 29.2000 | 40.4395 | -11.2395 | -0.494 | 0.930 | -1.8675 | -4.5192 | 7.7420 |
| 2 | 188.6900 | 184.8949 | 3.7951 | 0.167 | 0.930 | 0.6306 | 0.5755 | 0.8827 |
| 3 | 15.0200 | 12.5690 | 2.4510 | 0.108 | 0.930 | 0.4073 | 0.3602 | 0.3682 |
| 4 | 120.2500 | 100.8593 | 19.3907 | 0.851 | 0.736 | 1.6561 | 2.5580 | 1.2720 |
| 5 | 12.3550 | 8.0033 | 4.3517 | 0.191 | 0.736 | 0.3717 | 0.3276 | 0.0641 |
| 6 | 80.5600 | 74.8639 | 5.6961 | 0.250 | 0.736 | 0.4865 | 0.4343 | 0.1098 |
| 7 | 60.6580 | 35.0843 | 25.5737 | 1.123 | 0.251 | 1.2975 | 1.4765 | 0.0940 |
| 8 | 94.6550 | 114.1895 | -19.5345 | -0.858 | 0.251 | -0.9911 | -0.9881 | 0.0548 |
| 9 | 18.9600 | 34.4620 | -15.5020 | -0.681 | 0.251 | -0.7865 | -0.7408 | 0.0345 |
| 10 | 40.2365 | 55.2194 | -14.9829 | -0.658 | 0.250 | -0.7596 | -0.7111 | 0.0320 |

Yexp.: Y experimental; Ycalc.: Y calculated; SE, standard error

**Table 5.** Optimization of Antioxidant Activity of the Essential Oil Mixture

| | Ingredient Proportion (g) Proposed by the Mixture Design | Predicted Antioxidant Activity (µg/mL) | Experimental Antioxidant Activity (µg/mL) |
|---|---|---|---|
| EOT ($X_1$) | 25.7%eq (15.42 mg) | | |
| EOL ($X_2$) | 32.3%eq (19.38 mg) | 10.907 ± 0.212 | 11.023 ± 0.145 |
| EOC ($X_3$) | 41.9%eq (25.14 mg) | | |

**Table 6.** Variance Analysis of the Optimal $Y_{(DPPH, IC50)}$

| Variation | Total | Liberty Grade | Mean | (Ratio F) | *P* |
|---|---|---|---|---|---|
| Regression | 2.66905E+0004 | 5 | 5.33810E+0003 | 10.2927 | * |
| Residues | 2.07453E+0003 | 4 | 5.18632E+0002 | | |
| Total | 2.87650E+0004 | 9 | | | |

*statistically significant at *P* value < 0.05

Finally, EO mixture was obtained and considered to be an excellent additive with an optimized antioxidant property. Taking into account the statistical examination, moreover, it was detected that the content of the EO from EOL ($X_2$)) (b2) may have influenced the antioxidant activity (Y: ($IC_{50}$: DPPH)).

### *Evaluating Antioxidant Activity of the Essential Oil Blend Optimized by Mixture Design Methodology*

Taking into account the results of the antioxidant activity through evaluating the median inhibition concentration of free radical's DPPH, the EO mixture obtained from clove, lemon peel, and thyme showed a considerable antioxidant activity ($IC_{50}$ = 11.023 ± 0.145 µg/mL) compared to those of BHT (positive standard), EOC, EOT, and EOL characterized by $IC_{50}$ of 24 ± 0.11 µg/mL, 15.02 ± 0.02 µg/mL, 29.2 ± 0.12 µg/mL, and 188.69 ± 0.95 µg/mL, respectively (Figure 2).

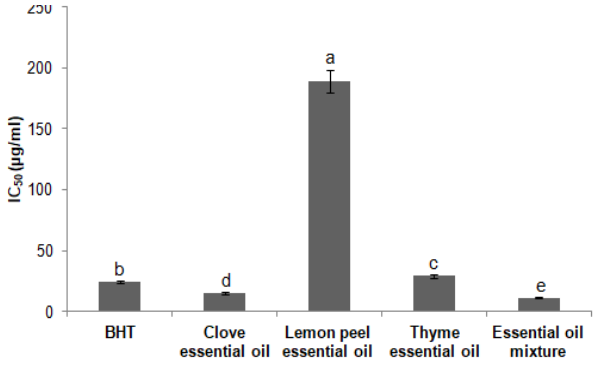



**Figure 2 .** Antioxidant Activity of Clove, Lemon Peel and Thyme Essential Oils and Their Combination. $IC_{50}$ values with different letters (a,b,c,d,e) are significantly different at *P* < 0.05.

### *Study of the Chemical Composition of Essential Oils and the Synergistic Effect Between Volatile Compounds on the Variation of Antioxidant Activity*

The yields of lemon peel, clove, and thyme EOs were determined to be 1.30 ± 0.78, 5.11 ± 0.99 and 1.25 ± 0.57%, respectively, based on dry weight of plant material. GC-MS analysis of lemon peel, clove, and thyme EOs as well as their combination are given in Figure 3. Twenty-one volatile compounds were identified in lemon peel composing 99.07 ± 0.83% of EO, eight compounds in clove encompassing 99.92 ± 0.78% of EO, and sixteen compounds in thyme covering 99.99 ± 0.71% of EO. For the combination, 26 compounds were identified having 99.27 ± 0.11% of EO. Lemon peel EO was characterized by the predominance of limonene (71.81 ± 0.78%). Eugenol (87.3 ± 3.70%) was the main component in clove EO, and thymol (78.54 ± 4.50%) was the major one in thyme EO. In other words, the combined EO was mainly rich in eugenol (32.35 ± 1.13%), thymol (25.49 ± 0.03%), and limonene (21.30 ± 0.02%).

One of the major objectives of this study was the evaluation of the synergistic effect of the volatile

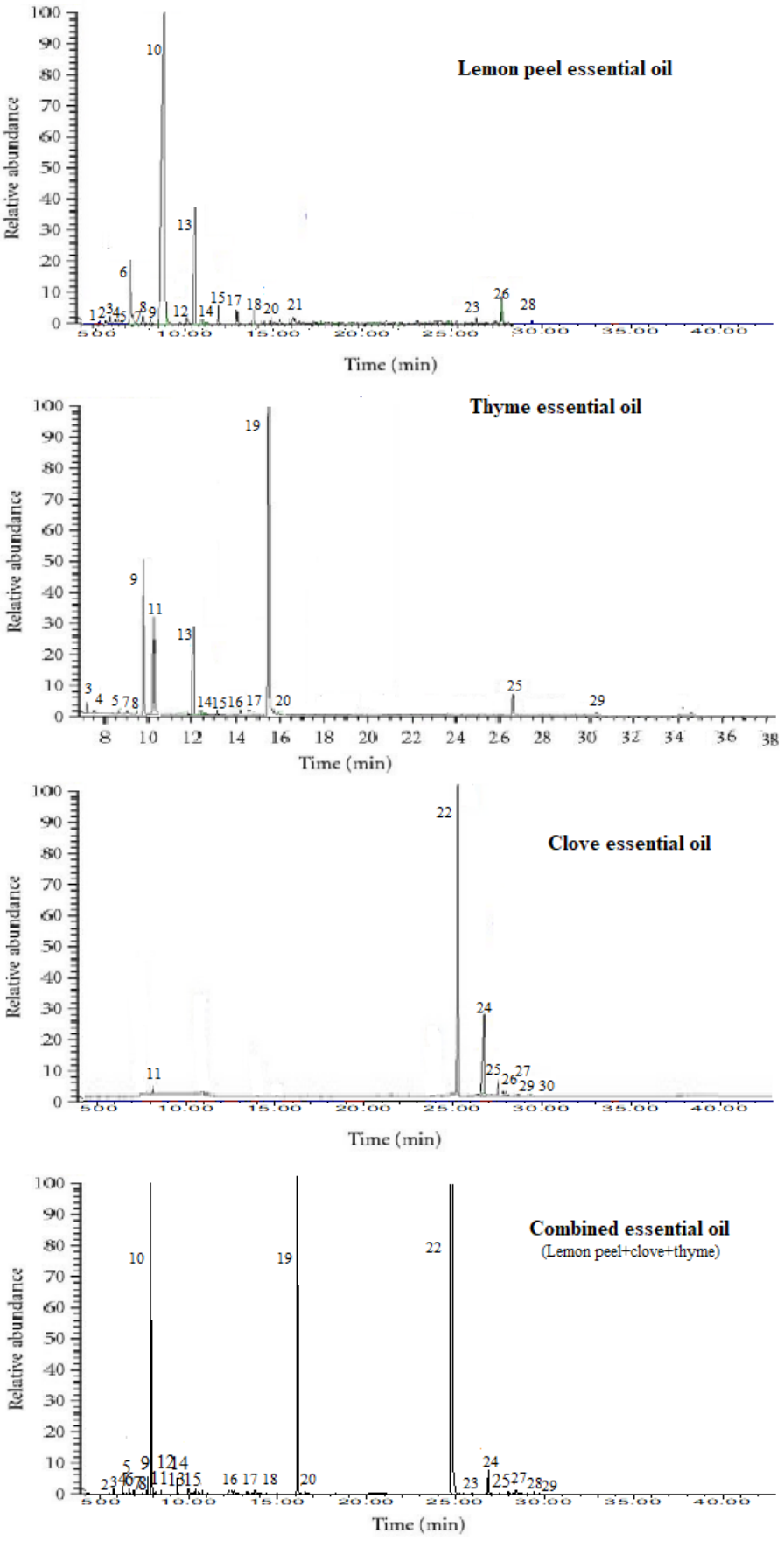



**Figure 3 .** Gas Chromatography Chromatograms of Lemon Peel, Thyme and Clove Essential Oils and Their Combination. 1: Tricyclene; 2: α-Thujene; 3: α-Pinene; 4: β-Pinene; 5: Camphene; 6: Sabinene; 7: β-Myrcene; 8: α-Terpinene; 9: *p*-Cymene; 10: limonene; 11: 1,8-Cineole; 12: *E*-β-Ocimene; 13: γ-terpinene; 14: linalool; 15: Borneol; 16: Terpinene-4-ol; 17: α-Terpineol; 18: Camphor; 19: Thymol; 20: Carvacrol; 21: Bornyl acetate; 22: Eugenol; 23: geranyl acetate; 24: eugenol acetate; 25: *E*-Caryophyllene; 26: Germacrene-D; 27: α-Humulene; 28: Valencene; 29: Caryophyllene oxide; 40: Chavicol. Compounds are eluted using a HP-5 column.

compounds on the variation of the antioxidant activity of the optimized blended EO obtained by mixture design. To this end, a phytochemical characterization of the EO mixture was established at the end of the qualitative analysis of the main volatile compounds present in the mixture in order for detecting the impact of synergistic effect on the antioxidant activity of the product (Table 7).

Likewise, for accurately assessing the synergistic effect of volatile compounds on the variation of antioxidant activity, a statistical analysis was performed using multivariate analysis through PCA. This analysis was interesting regarding the laws of probability with several variables for revealing the relations between the individuals to be tested (Figure 4).

The statistical analysis obtained by carrying out the principal component analysis and the Person correlation analysis (which assess the correlation between the free radical scavenging activity and the chemical composition of these EOs) and their optimized mixture by a mixing plan showed that the free radical scavenging activity is positively correlated with the EO of lemon peel that proved a weak antioxidant activity. Secondly, a positive correlation was identified between free radical scavenging activity and the EO of clove, thyme, and lemon peel of the optimized mixture, which was suggestive of a considerable antioxidant activity.

These results were consolidated by the results from Person's correlation analysis which proved that the antioxidant activity of each EO was largely attributable to the synergy between certain volatile compounds present in the composition of each EO. In effect, the considerable activity in the EO of clove resulted from the presence of the compounds 1,8-cineole, eugenol, α.-humulene, eugenol acetate, and *E*-caryophyllene with the negative

**Table 7.** Chemical Composition of Lemon Peel, Clove and Thym Essential Oils and Their Combination

| Volatiles Compounds* | RI[a] | RI[b] | Lemon Peel EO | Clove EO | Thyme EO | Combined EO |
|---|---|---|---|---|---|---|
| Tricyclene | 919 | 929 | 0.02 ± 0.01a | - | - | - |
| α-Thujene | 923 | 836 | 0.34 ± 0.11a | - | - | 0.22 ± 0.01b |
| α-Pinene | 934 | 982 | 1.14 ± 0.83a | - | 1.07 ± 0.22a | 0.52 ± 0.01b |
| β-Pinene | 937 | 1113 | 0,63 ± 0,1b | - | 0.16 ± 0.03c | 2.12 ± 0.02a |
| Camphene | 952 | 1077 | 0.03 ± 0.01c | - | 0.31 ± 0.06a | 0.10 ± 0.01b |
| Sabinene | 983 | 1111 | 5.82 ± 0.12a | - | - | 1.28 ± 0.02b |
| β-Myrcene | 991 | 1168 | 0.99 ± 0.06b | - | 0.58 ± 0.12a | 0.41 ± 0.02a |
| α-Terpinene | 1018 | 1255 | 1.05 ± 0.04a | - | 0.91 ± 0.19b | 0.33 ± 0.02c |
| p-Cymene | 1026 | 1277 | 0.23 ± 0.33c | - | 7.13 ± 1.49a | 3.59 ± 0.02b |
| Limonene | 1030 | 1031 | 71.81 ± 7.71a | - | - | 21.30 ± 1.21b |
| 1,8-Cineole | 1033 | 1214 | - | 0.03 ± 0.01b | 3.50 ± 0.73a | 0.09 ± 0.04b |
| *E*-β-ocimene | 1052 | 1022 | 0.5 ± 0.01a | - | - | 0.13 ± 0.01a |
| γ-Terpinene | 1059 | 1262 | 9.96 ± 0.05a | - | 3.44 ± 0.73b | 2.89 ± 0.02c |
| Linalol | 1098 | 1551 | 0.65 ± 0.03b | - | 0.39 ± 0.08a | 0.28 ± 0.02c |
| Borneol | 1165 | 1642 | 2.13 ± 0.12a | - | 0.85 ± 0.19b | 0.61 ± 0.01c |
| Terpinen-4-ol | 1178 | 1593 | - | - | 0.64 ± 0.13a | 0.37 ± 0.01b |
| α-Terpineol | 1185 | 1711 | 1.22 ± 0.11a | - | 0.14 ± 0.03c | 0.42 ± 0.03b |
| Camphor | 1192 | 1498 | 0.58 ± 0.02a | - | - | 0.06 ± 0.01b |
| Thymol | 1266 | 1263 | - | - | 78.54 ± 4.50a | 25.49 ± 0.03b |
| Carvacrol | 1278 | 1283 | 0.02 ± 0.01b | - | 0.18 ± 0.04a | 0.14 ± 0.03a |
| Bornyl acetate | 1295 | 1601 | 0.01 ± 0.01 | - | - | - |
| Eugenol | 1330 | 1329 | - | 87.3 ± 3.70a | - | 32.35 ± 1.13b |
| Geranyl acetate | 1383 | 1599 | 0.56 ± 0.04a | - | - | 0.24 ± 0.01b |
| Eugenol acetate | 1387 | 1360 | - | 10.4 ± 1.02a | - | 5.12 ± 0.01b |
| E-caryophyllene | 1446 | 1608 | - | 1.35 ± 0.30a | 1.58 ± 0.30a | 0.73 ± 0.03b |
| Germacrene D | 1480 | 1685 | 1.35 ± 0.04a | 0.14 ± 0.01b | - | - |
| α-Humulene | 1485 | 1691 | - | 0.19 ± 0.09a | - | 0.14 ± 0.01a |
| Valencene | 1495 | 1520 | 0.03 ± 0.01b | - | - | 0.12 ± 0.01a |
| Caryophyllene oxide | 1578 | 1699 | - | 0.20 ± 0.01c | 0.57 ± 0.09a | 0.22 ± 0.03b |
| Chavicol | 1652 | 1701 | - | 0.31 ± 0.02a | - | - |
| Total | | | 99.07 ± 0.83a | 99.92 ± 0.78a | 99.99 ± 0.71a | 99.27 ± 0.11a |

*Compounds in order of elution on HP-5 MS. Volatile compounds percentages in the same line with different letters (a–c) are significantly different at $P<0.05$.
RI[a] RI[b]: retention indices calculated using, respectively, an apolar column (HP-5) and polar column (HP-INNOWax).

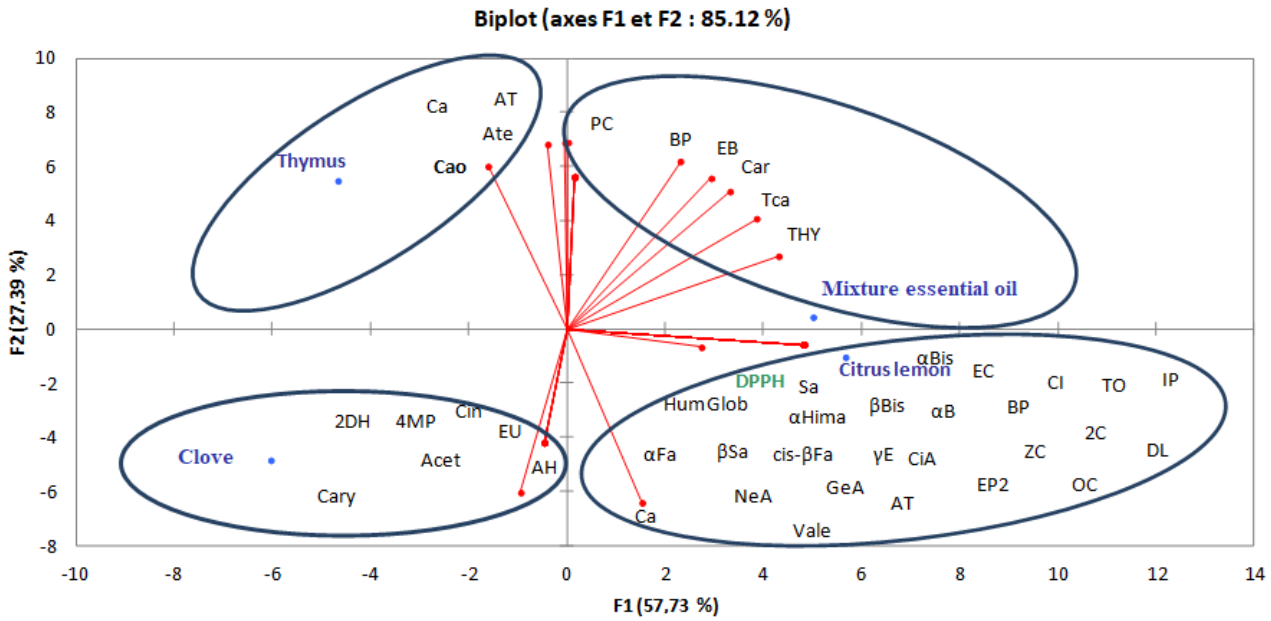



**Figure 4.** Principal Component Analysis to Assess the Correlation Between the Free Radical Scavenging Activity and the Chemical Composition of Clove, Thyme and Lemon Peel Essential Oils and Their Optimized Mixture by Mixing Plan. 1,8-Cineole (Cin), Eugenol (EU), alpha.-Humulene (AH), Acetyleugenol (Acet), Caryophyllene (Cary), Camphor (Ca); alpha-Terpineol (AT); alpha-Terpinene (Ate), Caryophyllene oxide (Cao), Thymol (THY); trans-Caryophyllene (Tca); p-Cymene (PC); beta-Phellandrene (BP); endo-Borneol (EB) and Carvacrol (Car).

correlation coefficients of -0.648, -0.648, -0.648, -0.648, and -0.414, respectively. In addition, the considerable antioxidant activity in the EO from the common thyme leaves was attributable to the presence of the compounds camphor, α-terpineol, α-terpinene, and caryophyllene oxide with the negative correlation coefficients of -0.333, -0.261, -0.272, and -0.600, respectively. However, the considerable antioxidant activity of the optimized blend EO occurred when it was formulated using the proposed ingredient contents of 25.7% EOT ($X_1$), 32.3% EOL ($X_2$), and 41.9 % EOC ($X_3$) resulted from the synergy between the following compounds: thymol; trans-caryophyllene, *p*-cymene, beta-phellandrene, borneol, and carvacrol with the negative correlation coefficients of -0.552, -0.552, -0.552, -0.552, -0.552, and -0.552, respectively.

## Discussion

In this study, the optimization of the antioxidant activity was investigated by adopting the mixing plan of a mixture of clove, lemon peel, and thyme EOs.

According to the plot graphs, it was shown that the optimization using the mixing plan resulted in the potent antioxidant activity of the EO mixture ($IC_{50} = 11.023 \pm 0.145$ µg/mL). This result was proportional to the proposed ingredient levels 25.7% EOT (X1), 32.3% EOL (X2) and 41.9 % EOC (X3) equivalent to 15.42 mg, 19.38 mg and 25.14 mg of the total EO mixture. In addition, taking into account the statistical examinations, it was found that the EO content of lemon peel (EOC (X2)) (b2) may have influenced the antioxidant activity (Y: ($IC_{50}$: DPPH)).

Similar results were reported by Baj et al (11) who highlighted the usefulness of statistical modeling of antioxidant activity, which was used to design a mixture of marjoram, basil, and rosemary EOs. Indeed, it was detected that the highest antioxidant activity was obtained for a mixed percentage of 75, 8, 17, respectively. However, the designed mixture showed a higher inhibition percentage (90%) compared to that of marjoram oil (88%) which was the most active oil.

Similarly, a qualitative analysis of the main volatile compounds of the EOs' mixture based on the antioxidant activity facilitated the evaluation of the effect of the synergy between the volatile compounds and the variation of antioxidant activity. In our study, lemon peel EO was characterized by the predominance of limonene (71.81 ± 0.78%). Eugenol (87.3 ± 3.70%) was the main component in clove EO, and thymol (78.54 ± 4.50%) was the major one in thyme EO. In other words, the combined EO was mainly rich in eugenol (32.35 ± 1.13%), thymol (25.49 ± 0.03%) and limonene (21.30 ± 0.02%). Our results regarding the chemical composition of lemon peel were in line with the findings from other studies confirming that limonene was the main component ranging from 29.52 to 98.40% (12-22). The major component of clove EO is usually considered eugenol (34.10-88.58%) (23-26). Numerous other studies have also introduced the EO composition of thyme with thymol as the main constituent ranging from 22 to 71% (27-30).

According to the results from PCA and Person's correlation analysis, the considerable activity in the EO of clove results from the presence of the compounds 1,8-cineole, eugenol, α.-humulene, eugenol acetate, and *E*-caryophyllene which have the following negative correlation coefficients of -0.648, -0.648, -0.648, -0.648; and -0.414, respectively. In addition, the considerable antioxidant activity in the EO from common thyme leaves was attributable to the presence of the compounds camphor, α-terpineol, α-terpinene, and caryophyllene

oxide with the negative correlation coefficients of -0.333, -0.261, -0.272, and -0.600, respectively. However, the considerable antioxidant activity of the optimized blend EO occurred when it was formulated using the proposed ingredient contents of 25.7% EOT (X1), 32.3% EOL (X2), and 41.9 % EOC (X3). This considerable antioxidant activity results from the presence of the compounds thymol; trans-caryophyllene, p-cymene, beta-phellandrene, borneol and carvacrol which have the following negative correlation coefficients of -0.552, -0.552, -0.552, -0.552, -0.552, and -0.552, respectively. These results were in agreement with findings of a study by Sonam and Guleria (31), which proved that the antioxidant potential may have been increased by the synergistic interactions among the different antioxidant compounds present in the mixture of synthetic antioxidants and natural products or the mixture of different EOs from aromatic and medicinal plants.

## Conclusion

In this work, statistical modelling was utilized to design a mixture of three EOs, namely thyme, lemon peel, and clove. The highest antioxidant activities were obtained for 25.7% EOT, 32.3% EOL, and 41.9% EOC. The EO mixture was mainly rich in eugenol (32.35 ± 1.13%), thymol (25.49 ± 0.03%), and limonene (21.30 ± 0.02%); and it had a stronger antioxidant activity than those of each individual EOs. Therefore, statistical planning and the development of utility profiles for mixtures of EOs may have been used to predict the optimal composition as well as to determine their antioxidant profile.

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### Authors' Contribution

Conceptualization, WY; Writing, WY, IM, WAW, TGA; Methodology, WY, MH, SK; Investigation, WY, Formal analysis, WY, IM, MH; Data curation, WAW; Supervision, MST; All authors have read and agreed to the published version of the manuscript.

### Conflict of Interests

None.

### Ethical Issues

None.

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