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Evaluation of the synergistic effects of antioxidant activity on mixtures of the essential oil from *Apium graveolens* L., *Thymus vulgaris* L. and *Coriandrum sativum* L. using simplex-lattice design

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ABSTRACT

Essential oils (EOs) are known for their antioxidant properties, and are widely employed in the food industry as preservatives. They can be used as condiments or as preservatives to achieve certain organoleptic effects for consumers. The aim of this research was to evaluate antioxidant activity in mixtures of three EOs: *Apium graveolens* L., *Thymus vulgaris* L. and *Coriandrum sativum* L., using the Simplex Lattice Mixture Design. Ultimately, a linear model was used, as it best adjusted to the experimental behavior, and it allowed the prediction of EOs mixtures antioxidant activity, determined by FRAP and ABTS techniques. The mixture of the three EOs that showed the best antioxidant activity and also had the highest synergistic effect, was composed of 66.7% of *T. vulgaris*, 16.7% of *C. sativum* and 16.7% of *A. graveolens*. The greatest contribution to the potentiation of antioxidant activity was shown by *T. vulgaris* followed by *A. graveolens* and then *C. sativum*.

1. Introduction

Essential oils (EOs) are volatile aromatic compounds generated from secondary metabolism in plants. Each EO is composed of between a dozen and several hundred components, which mainly consist of terpenes and terpenoids including oxygenated derivatives, such as aldehydes, ketones, alcohols, ethers, esters and epoxides (Bajpai and Baek, 2016). Some EOs are also known to contain allyl- and propenylphenols (Bajpai et al., 2012, 2013), as well as nitrogen, sulfur or chlorine atoms in their structure (Bakkali et al., 2008; Nagella et al., 2012). Moreover, EOs contain conjugated double bonds and phenolic functional groups. Their significant free radical scavenging properties have been extensively studied in recent years (Aruoma, 1998; Baj et al., 2018; Kamatou and Viljoen, 2010). These chemical characteristics give them their antioxidant properties (Miguel, 2010). Thymol, carvacrol, and eugenol are the most powerful antioxidants contained in the EOs examined, and their effects on food are among their benefits to human health (Amerah and Ouwehand, 2016; Elgndi et al., 2017; Firenzuoli et al., 2014). Antioxidant properties play a pivotal role in some EOs' biological activity, related to oxidative stress pathologies (Valgimigli et al., 2000).

The antioxidant activity of the EOs extracted from *A. graveolens, T. vulgaris* and *C. sativum* could be attributed to their main components. These three species of plants are herbs which are widely used for culinary purposes, with their fresh or dried leaves often used as spices. EOs extracted from the leaves and flowers of these species may also be used as flavoring additives in food. The antioxidant activity and non-toxic properties of these EOs have been well established (Burdock and Carabin, 2009; Lee et al., 2005; Nagella et al., 2012). The major components of the *A. graveolens* volatile oil are 4-chloro-4.4-dimethyl-3-(1-imidazolyl)-valerophenone, 1-dodecanol, 9-octadecen-12-ynoic acid (Nagella et al., 2012), and limonene (Baananou et al., 2013; Rożek et al., 2016; Seo et al., 2015). *T. vulgaris* oil is composed of the chemical compounds α-pinene, thymol, caryophyllene (Al-Asmari et al., 2017) and carvacrol, depending on chemotype (Benzie and Strain, 1996; Mancini et al., 2015). The dominant compound of *C. sativum* is generally linalool (more than 70%) (Chahal et al., 2018). Studies about the antioxidant properties of EO mixtures are rare, and in case of *A. graveolens, T. vulgaris* and *C. sativum*, there are no previous studies using similar mixture design, nor demonstrating any type of synergy. "Synergy'' is a popular concept in the field of herbal medicine, which suggests that plant extracts contain

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compounds which potentiate one another (Mancini et al., 2015).

Research regarding the synergistic effects of these plants in other mixtures is still limited. Thus, the aim of the present study was to evaluate the synergistic effect of mixed EOs by Simplex Lattice Mixture Design (SL-MD). In this study, the antioxidant properties depend only on the percentage of each component in the mixtures. SL-MD was preferred, as it is a tool widely used for evaluating the constituent factors and their response effects (Cornell, 2011). Recently, there have been research papers that showed use of the SL-MD for optimizing the antioxidant activity of EO mixtures (Baj et al., 2018).

2. Materials and methods

2.1. Plant material

Four kilograms of fresh aerial parts for each herbs *A. graveolens*, *T. vulgaris* and *C. sativum*, harvested before flowering and belonging to only one batch, were purchased from a local market in Puyo, Pastaza in the Amazon region of Ecuador in June, 2018.

All the herbs were taken to the laboratory and air-dried at a temperature range between 20 - 25 °C, for 48 hours (additional airflow was not used). The equilibrium moisture content, as percent moisture, for each of the 3 herbs after drying under the conditions referenced here were: 7.72 ± 0.04% for *A. graveolens*, 4.25 ± 0.02% for *T. vulgaris* and 6.59 ± 0.03% for *C. sativum*.

2.2. Essential oil extraction by steam distillation

A FIGMAY brand (manufacturing in Argentina, www.figmay.com.ar) essential oil extraction apparatus was used to extract volatile oil from the three plant species.

The apparatus comprises: heating system made of transparent quartz and polytetrafluoroethylene (PTFE) provided with an internal electrical resistance inside a borosilicate glass boiler, as well as borosilicate glass condenser, loading and unloading lid, and stainless steel basket (capacity 2 L).

A sample of 500 grams of air-dried coarsely powdered, using a Thomas Scientific Model 4 Wiley mill, aerial parts with a particle size <0.5 mm, obtained by RETSCH test sieves with round hole, were placed in the distiller. Distillation with a continuous flow of water vapor (near to 100 °C due to the altitude) was stopped after 40 minutes when it was observed that successive readings of the volume of oil remained constant.

Multiple runs were carried out to obtain the EOs and only one fraction was collected for each individual herb. The receiver vessel was not cooled, it remained at room temperature (<20 °C). The oil was then separated and passed over anhydrous sodium sulfate. The oil was filtered using filter paper and kept at 4 °C in sealed vials in the dark, up to 24 hours with the aim of ensuring that oxidative degradation has not occurred, for later use. Test were done immediately after the extraction in order to avoid degradation or changes in the samples' composition. EOs were obtained in a yield of 0.13%, 0.33% and 0.23% w/w for *A. graveolens., T. vulgaris and C. sativum,* respectively.

2.3. Sample preparation

Each individual essential oil was put in plastic tube in the required proportion (Table 1), and afterwards, a dilution of 1:100 v/v with methanol was applied using a micropipette Eppendorf (SIGMA-ALDRICH), and finally, a vortex stir (Scientific Industries Vortex Genie2 Vortex Mixer G560E) was carried out for one minute. The final volume was 1 mL.

2.4. Total antioxidant activity

Both, the FRAP and ABTS radical scavenging methods were used because only one of them would not offer enough information for all of the antioxidant functional groups in the extracted compounds (Lai et al., 2018). The methods were simple and robust, and the assays were performed quickly, providing precise results (Prior et al., 2005).

2.4.1. FRAP assay

The reagent was freshly prepared for use by adding 2.5 mL of 10 mM 2,4,6-tripyridyl -*s*-triazine in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3 6H_2O$ to 25 mL of 0.3 M of an acetate buffer (pH = 3.6) solution. At the beginning, 80 μL of each EO mixture was placed in a 10 mL volumetric flask and 5 mL of a FRAP reagent was added. After being filled with distilled water, the volumetric flask was shaken and allow to stand for 30 min at 37 °C in a RY-DHG-9030A electric heat laboratory oven. No phase separation was observed since the sample completely reacted with FRAP reagent (Seo et al., 2015). The calibration curve was assembled based on TROLOX pure standard in the range 10^{-4} – 10^{-3} mg L^{-1}. The spectrophotometric measurements at the wavelength of 593 nm were performed using a Thermo Scientific GENESYS 10S UV-Vis Spectrophotometer.

2.4.2. ABTS assay

Antioxidant activity was determined by means of the ABTS method (Baananou et al., 2013). For the radical formation, 3.3 mg of sodium persulfate was placed together with 19.4 mg of $ABTS^{\bullet+}$ in a dark bottle. 5 mL of distilled water were added. The mixture was left to stand at room

Table 1
Experimental conditions of mixture design. Antioxidant experimental activity and synergistic effect calculated from individual ingredients.

Mixture[a]	Ingredient proportions			Measured antioxidant						Synergistic effect (%)	
				FRAP (g Eq. Trolox L^{-1}) (Y_1)			ABTS (% Inhibition) (Y_2)				
	A. graveolens (X_1)	*T. vulgaris* (X_2)	*C. sativum* (X_3)	Actual value	Predicted value	Residual	Actual value	Predicted value	Residual	FRAP	ABTS
1	1/6	1/6	2/3	61.5	61.31	0.26	19.0	18.69	0.35	10.1	8.0
2	1/2	1/2	0	169.2	163.49	5.72	52.4	51.77	0.7	8.9	4.9
3	1	0	0	19.9	23.24	−3.33	3.2	3.89	−0.68	0	0
4	0	1/2	1/2	169.5	156.98	12.57	51.9	50.9	1.02	13.2	5.7
5	0	1	0	288.2	303.74	−15.51	96.5	99.64	−3.12	0	0
6	1/6	2/3	1/6	226.5	208.07	18.5	72.1	67.44	4.7	13.3	9.7
7	0	0	1	5.9	10.22	−4.26	1.3	2.16	−0.83	0	0
8	0	1	0	298.0	303.74	−5.72	98.5	99.64	−1.12	0	0
9	1	0	0	21.0	23.24	−2.18	3.5	3.89	−0.38	0	0
10	2/3	1/6	1/6	69.3	67.82	1.5	19.5	19.56	0.023	10.1	5.7
11	0	0	1	6.1	10.22	−4.06	1.6	2.16	−0.54	0	0
12	1/2	0	1/2	14.8	16.73	−1.87	2.3	3.03	−0.66	12.9	4.2
13	1/3	1/3	1/3	110.7	112.4	−1.61	35.7	35.23	0.52	5.5	5.7

[a] non-randomized sequence.

temperature for 16 hours. After that, the solution was diluted in a 1:10 ratio with ethanol in order to get an absorbance value near to 0.8730 (A_i) at 754 nm against ethanol blank (Thermo Scientific GENESYS 10S UV-Vis Spectrophotometer). Then, 40 μL of each EO mixture, (obtained according to the SL-MD), was added, shaken, and, after 7 minutes, the final absorbance (A_f) was determined. The antioxidant activity of the EOs was expressed using the following equation:

$$Inhibition(\%) = \left(\frac{A_i - A_f}{A_i}\right) \times 100 \quad (1)$$

2.5. Simple lattice mixture design to determine antioxidant response

A Simple Lattice Mixture Design was applied to find out the interactive effects of antioxidant activity from the EOs extracted from *A. graveolens*, *T. vulgaris* and *C. sativum*. The response variable was determined by FRAP and ABTS antioxidant activity assays. Mixture design experiments were conceived and analyzed by means of Design-Expert 10 software (Trial key: 2182-0638-8336-EVAL). The entire 13 mixtures are shown in Table 1.

It is important to encourage that, although not all possible combinations were made with replicates and not all possible mixtures or combinations were represented in the study, precisely the advantage of this type of design is that the data can be processed statistically with a minimum number of points (Cornell, 2011).

The following polynomial equation of function *Xi* was fitted for each factor evaluated at each experimental point, where *Y* is the variable response and β_1, β_2, β_3, β_{12}, β_{13}, and β_{23} are constant regression coefficients (Eq. 2):

$$Y = \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=1+1}^{3} \beta_{ij} X_i X_j (i < j) = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

The information was analyzed using a general linear model. Values of antioxidant properties were explained by the three-dimensional response surface analysis of the independent variable (X_i) and dependent variable (Y) representing the measured antioxidant activity.

2.6. Selection and validation of SL-MD model

The statistical attributes of the models were considered in selection. The adjusted and predicted R^2 coefficient values were determined. The weight for each experimental run was obtained, and the appropriateness of the model was calculated by analysis of variance (ANOVA) (Nazir et al., 2017; Pompeu et al., 2009).

Finally, the 'synergistic effect' was calculated as the percentage of increase shown in relation to the algebraic sum of the proportion, taking into consideration the individual contribution of each pure oil.

3. Results and discussions

3.1. Antioxidant activity

The highest antioxidant activity was observed using pure EOs, for *T. vulgaris* (Table 1). Pure EO of *A. graveolens* presented much lower activity, while *C. sativum* showed the lowest antioxidant activity. This is in accordance with other studies of these EOs. Al-Asmari et al. (2017) also found *T. vulgaris* rich in pinene, thymol and caryophyllene, to be a strong antioxidant. *A. graveolens*, as previously described, has free radical suppression activity which could be attributed to its major component 4-chloro-4,4-dimethyl-3-(1-imidazolyl)-valerophenone (Nagella et al., 2012), whereas the same attributes found in *C. sativum* could be attributed to the action of (E)-2-Decenal and linalool (Chahal et al., 2018). Lower antioxidant activity is typically seen in EOs rich in monoterpenes (pinenes) (Miller et al., 1993) and *C. sativum* is no exception (Prachayasittikul et al., 2018).

All the experimental mixtures obtained had higher antioxidant activity than *A. graveolens* and *C. sativum* alone. The addition of *T. vulgaris* to the mixtures resulted in higher antioxidant activity as well. It is obvious that antioxidant activity depends on the *T. vulgaris* content in any given mixture. If the proportional contribution of the individual EOs to the mixture is compared according to the actual antioxidant activity shown, a synergistic effect is evidenced (see Table 1). Obtained results are in accordance with those found by other authors in mixture design studies for optimum antioxidant activity of mixtures of essential oils (Baj et al., 2018), and avocado byproducts (Calderón-Oliver et al., 2016).

3.2. SL-MD and antioxidant response

Response Surface Methodology is a compilation of statistical and mathematical techniques that are established on the fit of polynomial equation to the experimental data (Mansouri et al., 2018). It describes well the behavior of data set aiming to make statistical previsions. It is more favorable than the traditionally used single parameter optimization since it reduces time, space and raw material usage (Anderson and Whitcomb, 2016a).

The SL-MD allowed for the finding of a combination of the three EOs studied that guarantees a synergic potentiation of the antioxidant activity. This could have a practical use in the food industry for food preservation and conservation. Using mixtures of different essential oils, in addition to potentiating antioxidant activity, guarantees other important organoleptic properties for consumers, for example increased smell and taste.

Using a Simplex Lattice Mixture Design, a linear model was obtained for both methods with an R^2 value of nearly 0.99. The expected R^2 was in agreement with the adjusted R^2; the difference was lower than 0.2

Table 2
Analysis of variance (ANOVA) for linear model.

FRAP	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model Linear	15561.76	2	7780.88	2100.33	<0.0001	significant
Residual	37.05	10	3.7			
Lack of Fit	34.96	7	4.99	7.18	0,0664	not significant
Pure Error	2.09	3	0.7			
Cor Total	15598.8	12				
ABTS	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model Linear	1.38E+05	2	68752.6	794.55	<0.0001	significant
Residual	865.3	10	86.53			
Lack of Fit	816.69	7	116.67	7.2	0,0667	not significant
Pure Error	48.6	3	16.2			
Cor Total	1,38E+05	12				

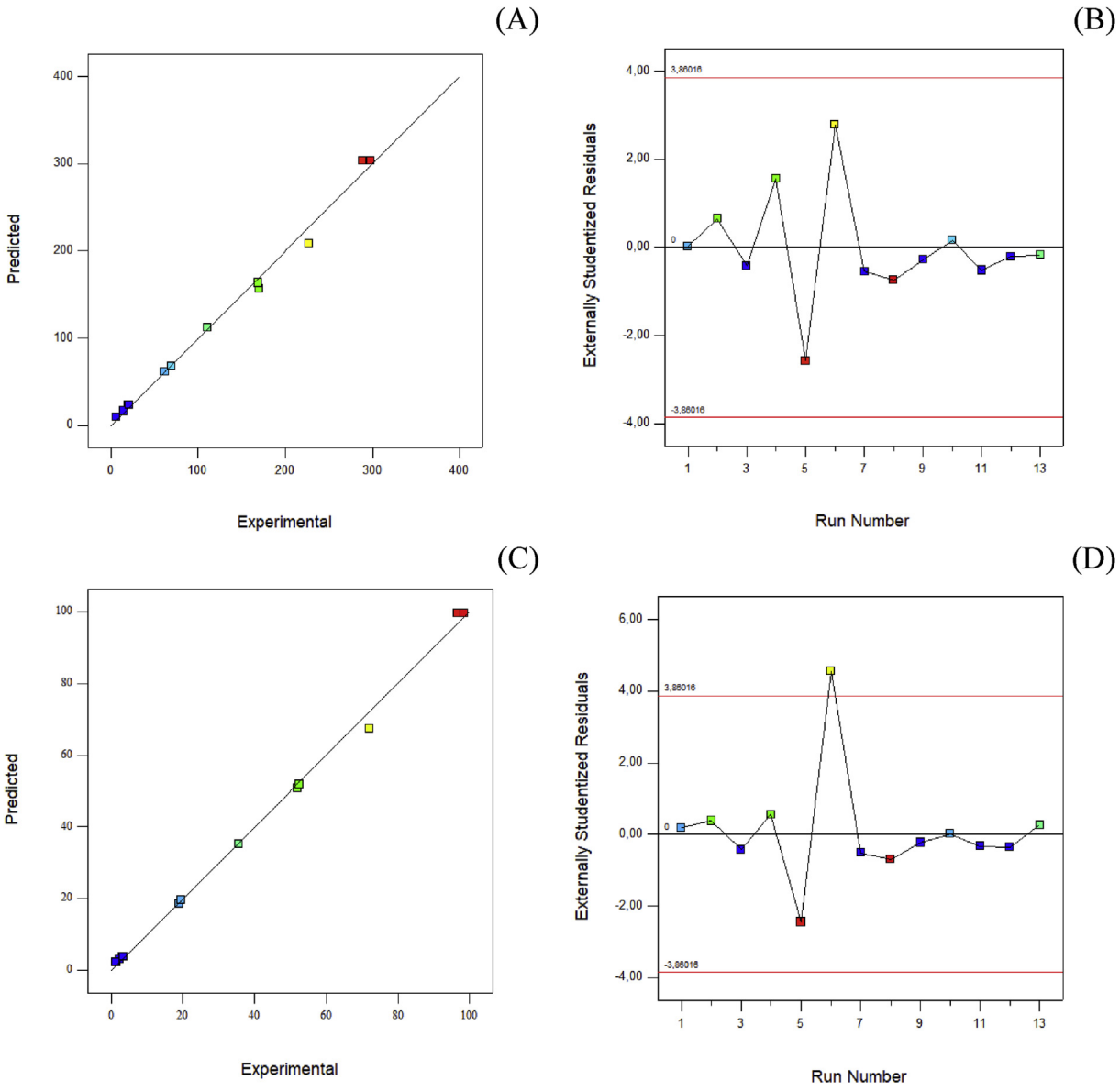

Fig. 1. Relationship between experimental and predicted values and residues vs. run number (A and B, FRAP method; C and D, ABTS method).

(Anderson and Whitcomb, 2016b). With respect to the ANOVA (Table 2), the lack of fit (F value) was not significant. Additionally, a non-significant lack of fit indicated that the calculated model was suitable for an experimental run. The model was deemed satisfactory because it generated results higher than four (Crespo et al., 2017).

The antioxidant response values expected for the linear model and the real ones obtained from the experiment are shown in Fig. 1. The scatter plot confirms the accuracy of the model in reflecting the entire range of experiments conducted.

It is important to note that the linear equation found may be used to predict the response for several levels of each factor considered. A coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. The regression model for the experiment is shown in Eq. 3 and Eq. 4:

$$FRAP = 303.74 \times X_1 + 10.22 \times X_2 + 23.24 \times X_3 \quad (3)$$

$$ABTS = 99.64 \times X_1 + 2.16 \times X_2 + 3.89 \times X_3 \quad (4)$$

X_1, X_2, X_3 – single components in mixtures.

The regression analysis results showed that *T. vulgaris* had greater weight than the rest of the EOs, where *C. sativum* < *A. graveolens* < *T. vulgaris*. In order to establish the validity of the regression, a residue analysis was carried out. A normal distribution was achieved (Fig. 1 B and D).

In the prepared mixtures where the *T. vulgaris* was in 66.7% proportion, the activity was higher, regardless of the second ingredient in the mixture (exp. 6). However, the obtained activity was always higher for the mixtures than for the pure *T. vulgaris* essential oil. This could be indicative of the potentiated properties of the mixtures compared to the those of the essential oils individually (see Table 1). The following mixture proportions showed high values of antioxidant activity and also greater synergism: 66.7% *T. vulgaris*, 16.7% *C. sativum* and 16.7% *A. graveolens*.

By using the SL-MD, every mixture was analyzed, and in each case, a higher antioxidant property was obtained when *T. vulgaris* was added (Fig. 2). An optimization procedure was conducted in order to find the mixture having the highest antioxidant activity.

Previous studies have used a mixture design in order to achieve the best antioxidant mixture. Higher synergistic results have recently been

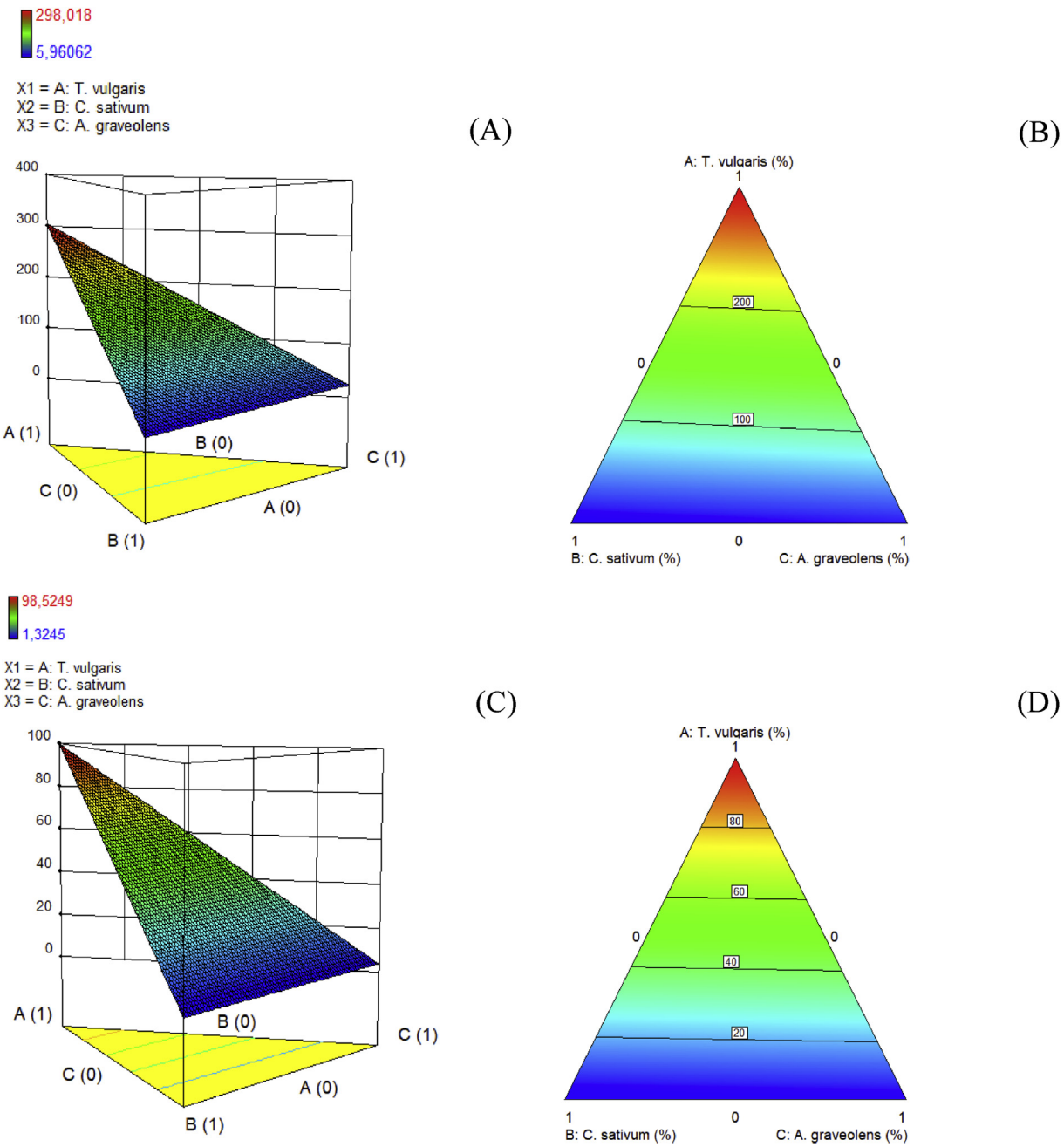

Fig. 2. Antioxidant responses of the mixture of *A. graveolens*, *T. vulgaris* and *C. sativum* (A and B, FRAP method; C and D, ABTS method).

published by Calderón-Oliver et al. (2016); the authors found synergistic effects in the range: 16.5–45.8%. A study on the application of a mixture design for optimal antioxidant activity of EO mixtures has recently been published, which analyzed *Ocimum basilicum* L., *Origanum majorana* L. and *Rosmarinus officinalis* L. (Baj et al., 2018). The percentages of synergy found were in the range of 6.6–57.2%. Additional effects of EO mixtures have been reported, including the synergistic antibacterial effects of EO mixtures using *Origanum compactum* L., *O. majorana* and *Thymus serpyllum* L. (Ouedrhiri et al., 2016).

3.3. Correlation between FRAP and ABTS assays

Both of these spectrophotometric analytical methods are based on different chemical mechanisms. The measurements were taken in order to evaluate the antioxidant biological activity of the EO mixture, and were achieved by employing a linear regression model (Fig. 3).

4. Conclusions

The spectrophotometric measurements based on different chemical mechanisms allowed evaluation of the antioxidant activity of the EO mixtures.

The highest antioxidant activity was observed for pure *T. vulgaris* with intermediate activity for *A. graveolens*, and the lowest antioxidant activity for *C. sativum*.

The mixture proportions of 66.7% *T. vulgaris*, 16.7% *C. sativum* and 16.7% *A. graveolens* showed higher values of antioxidant activity. A mild synergistic relationship between EOs with regard to their antioxidant activity was predicted by a linear regression model using SL-MD and was observed at various proportions.

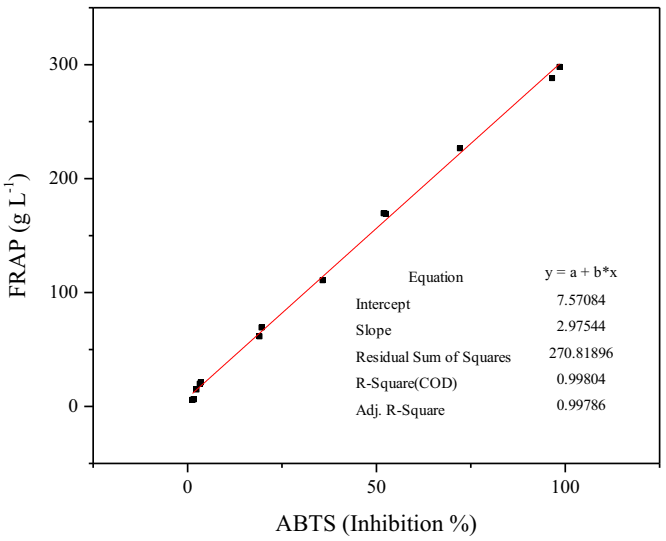

Fig. 3. Relationship between FRAP and ABTS assays.

Declarations

Author contribution statement

Yasiel Arteaga Crespo, Luis Ramón Bravo Sánchez: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yudel García Quintana, Andrea Silvana Tapuy Cabrera: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Abdel Bermúdez del Sol, Doris Magaly Guzmán Mayancha: Performed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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