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Synergistic Antioxidant Activity in Brazilian Propolis Extract Blends: An *in Vitro* Study

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Abstract

A simplex-lattice mixture design with response surface methodology was used to evaluate *in vitro* synergistic antioxidant activity of red, green, and brown Brazilian propolis extract blends. The *in vitro* antioxidant capacity of propolis extract blends was measured using the fluorine method of oxygen radical absorption capacity assay (ORAC assay). A synergistic antioxidant interaction was identified between green and brown propolis extracts, and the predictive model accused a binary mixture composed of 59% green and 41% brown propolis extracts with increased antioxidant activity of about 54%. Our findings suggest a possible reduction in the dosages of these natural antioxidants in their various potential applications, including health and food, thereby proving to be a highly promising alternative for the rational use and valorization of propolis.

Keywords

Green Propolis, Red Propolis, Natural Antioxidants, Food Additives, Mixture Modeling

1. Introduction

Propolis is a bee product from resinous, gummy, and balsamic substances collected from buds, flowers, and plant exudates, added with salivary secretions, wax, and pollen, and deposited in colonies as a physical and chemical colony defense [1] [2] [3] [4]. These functions are performed by bioactive molecules belonging to different classes, including phenolic compounds, the main group of these substances [1]-[6]. Among the bioactive properties, propolis has antioxidant activi-

ty, expressed in the colony and in plants, animals, and foods, presenting potential applicability as a functional ingredient and natural preservative [3] [5] [7] [8] [9].

An outstanding characteristic of propolis is the variability of its composition and multiple factors such as seasonality, climate, geographic location, and vegetation [1] [6] [7], making propolis extract production with standardized properties and their industrial exploitation quite challenging [8] [9]. In addition, the low solubility of propolis extracts in water can change the flavor and odor when incorporated into foods, leading to higher costs than conventional ingredients and making their application in foods even more strenuous [8] [10].

The growing demand for natural products (e.g. food ingredients) supports new investigations for technological solutions to overcome these obstacles, including developing propolis extract blends with enhanced action to reduce the doses required to achieve the desired effects [8] [9]. Combining propolis extracts from different origins is one strategy whose phytochemical patterns can interact synergistically and optimize antioxidant systems [9] [11] [12]. Evidence presented by Peixoto *et al.* [9] [13] instigates the prospection of new synergistic mixtures of propolis extracts while considering other phytochemical patterns of propolis produced worldwide, including Brazilian propolis.

Brazilian propolis is classified into 13 groups in terms of their botanical origin, chemical composition, and antioxidant and antimicrobial activities. The varieties of Groups 12 and 13, popularly known as green and red, respectively, have great commercial importance due to their chemical compositions and bioactive properties [14] [15]. Brazilian green propolis and red propolis are known for their unprecedented composition in bioactive compounds, granting them the "Denomination of Origin" seals for some producing regions of these classes [16]. Their differentiated composition is due to the main vegetable sources collected by the bees to produce them, *Baccharis dracunculifolia* and *Dalbergia ecastophyllum*, both native to Brazil. The former is found in southeastern Brazil in the Cerrado biome, while the latter is found in coastal regions in the mangrove biome [14] [17] [18] [19] [20].

Brazilian green propolis, produced mainly in southeastern Brazil, has the native plant *B. dracunculifolia* as its primary botanical source, which is rich in prenylated coumaric acid derivatives [2] [18] [19]. As a result, these compounds are found in high concentrations in green propolis and primarily include drupanin, baccharin, and artepillin C [18] [19] [21]. The green tone is due to the high chlorophyll levels [3] [22] [23]. Red propolis, characteristic of the coast of northeastern Brazil, mainly contains flavonoids and prenylated benzophenones, including vestitol, neovestitol, medicarpin, formononetin, biochanin A, daidzein, retusapurpurin A and B, guttiferone E, and xanthochymol and oblogifolin B [2] [14] [17] [20]. The flavonoids retusapurpurin A and B are the pigments responsible for this propolis red color [2] [24].

Brown Brazilian propolis is attributed to the class with this color found throughout the country, although its composition is still little known given the territorial

extent [25]. Brazil is a country of continental proportions and one of the most megadiverse places in the world, with over 40,000 different plant propolis, representing 20% of the world's flora [26] [27]. Over half of the plants in Brazil are endemic and only occur in this country [27]. Hence, the number of brown propolis chemotypes is not only expected to be proportional to this diversity, but also reveals new phytochemical profiles [25] [28] [29]. As studies on the composition of Brazilian brown propolis advance, new chemotypes have been found, such as propolis with high prenylated benzophenone content (Amazon region, northern Brazil) [30], propolis with high acidic diterpene content (Araucaria forest, southern Brazil) [31], and propolis with high benzyl benzoate and benzyl salicylate content in the essential oil fraction (Mata Atlantica, southeastern Brazil) [32]. New research aimed at evaluating the bioactive properties, composition, and botanical origin of brown propolis can help strengthen knowledge about its identity [25].

Evidence of antioxidant synergism in blends of propolis extracts from different origins is scarce in the literature [9] [13], therefore, little is known about how the antioxidant activity of extracts from different patterns of Brazilian propolis is affected when they are combined. Given the above, this study sought to evaluate the antioxidant interactions of mixtures of red, green, and brown propolis extracts (PE) using a simplex-lattice mixture design (SLMD) with response surface methodology (RSM).

2. Materials and Methods

2.1. Propolis Extracts Origin and Characterization

Three commercial extracts of Brazilian propolis (brown propolis, red propolis and green propolis) were kindly provided by Beeva Indústria Comércio e Exportação de Mel e Derivados S/A (Marechal Deodoro, AL, Brazil). The green and brown propolis used in the manufacture of the extracts were collected in February 2020 and April 2020, respectively, in Alterosa, MG, Brazil (21˚15'56.2"S 46˚08'17.7"W for green propolis; 20˚48'30.0"S 45˚18'42.3"W for brown propolis). Red propolis was collected in April 2020 in Porto de Pedras, AL, Brazil (9˚09'33.6"S 35˚18'18.7"W), and Canavieiras, BA, Brazil (15˚39'55.6"S 38˚57'01.6"W). According to Beeva S/A, these extracts were produced in their commercial manufacturing plant by maceration using hydroalcoholic solution (70% food grade ethanol) as the extracting solution for 7 days. In Brazil, a comprehensive list of solvents used in propolis extract production is regulated, including acetone, ethanol, isopropanol, hexane, methanol, and hydroethanolic solutions [33] [34], and solvent selection depends on prior analysis of multiple factors, such as propolis composition, yield, price, and technological trends [35] [36] [37]. Hydroethanolic solutions of ethanol:water of 70:30 (v/v) are conventionally used at a commercial scale to manufacture propolis extracts [35] [36] [38] due to the low toxicity, obtaining a wide range of bioactive compounds [36]. Ethanol is a readily available, low-cost solvent that is generally recognized as safe (GRAS) [37].

Other green solvents, such as water, limonene, and vegetable oils, have been seen as alternatives to replace ethanol-based extracts and other organic solvents, even though they have lower extraction performance [39] [40] [41].

The extracts were characterized (**Table 1**) for the content of total phenolic compounds and total flavonoids, by high-performance liquid chromatography-diode array detection (HPLC-DAD), and the separation was performed on a reverse-phase C-18 hypersil gold column (5-µm particle size, 150 mm, 4.6 mm; Thermo Fisher Scientific, Massachusetts, USA) at 38˚C [42], and dried extract by drying in an oven with air circulation at 102˚C ± 2˚C to constant weight [43].

2.2. Experimental Design, Data Analysis, Modeling of Experimental Data and Statistical Analyses

A SLMD was used to find out the interactive effects of antioxidant activity from the red, green and brown PE in the blend extracts [44] [45]. This system has symmetrically distributed experimental points and a well-chosen polynomial equation to represent the surface response over the entire SLMD region. The distribution of the independent variables (x_1, x_2 and x_3) in the simplex space (equilateral triangle), **Figure 1**, the sum of all must be 100% ($\sum X_n = 1$). The distribution of mixtures within this space is known as lattice, which a mixture can be pure element (triangle vertex) or binary interactions (triangle edges) and ternary (triangle center) between them. In this trial, the SLMD for 3 factors and a 3rd degree polynomial will consist of ${}^5C_4 = 9$ points plus one central point. Therefore, the design points are: (1, 0, 0), (2/3, 1/3, 0), (1/3, 2/3, 0), (0, 1, 0), (0, 2/3, 1/3), (0, 1/3, 2/3), (0, 0, 1), (1/3, 0, 2/3), (2/3, 0, 1/3) and the central point with equal parts of all components of the mixture (1/3, 1/3, 1/3). Therefore, 3 points of pure PE (vertex), 6 points of blend PE (edges) and one central point (inside of the triangle) with 4 replications are obtained (**Figure 1**). The design of experiment (DOE) is shown in **Table 2**. The cubic polynomial RSM in mixture design model is presented in Equation (1).

$$Y_{ijk} = \sum_{i=1}^{3} \beta_i X_i + \sum_{i \le j}^{3} \sum_{j}^{3} \beta_{ij} X_i X_j + \sum_{i \le j}^{3} \sum_{j \le k}^{3} \sum_{k}^{3} \beta_{ijk} X_i X_j X_k + \varepsilon_{ijk} \quad (1)$$

where the Y_{ijk} is observed value in the *i-j-k*-th propolis extract level; X_i, X_j and X_k are coded independent variables; β_i, β_{ij} and β_{ijk} are linear, quadratic (component pairs) and cubic (3-component sets) regression coefficients of the model, respectively, whose quadratic and cubic parameters may be either

Table 1. Characterization of PE in terms of dry extract (g/100g), total phenolics (mg/100mg dry weight) and total flavonoids (mg/100mg dry weight).

Parameters	Red PE	Green PE	Brown PE
Dry extract	10.30	9.38	9.97
Total phenolics	21.29	18.46	14.04
Total flavonoids	6.96	1.71	3.49

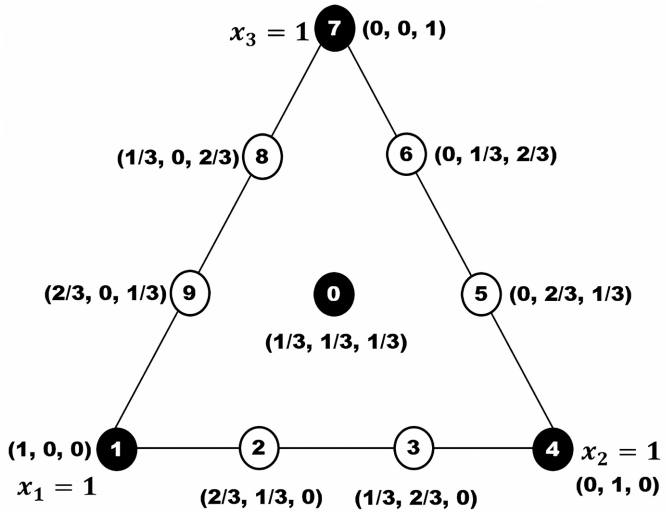

Figure 1. Space plot of the pseudo-components in SLMD for ternary mixture and third degree polynomial model.

Table 2. Pseudo-components (coded) and original components for SLMD with three independent variables (red, green and brown PE), as well as experimental responses.

Space point[1] (run)	Coded values			Original values (%)			Response (μM TEAC/mg dry weight)
	Red [3]PE (x_1)	Green PE (x_2)	Brown PE (x_3)	Red PE (x_1)	Green PE (x_2)	Brown PE (x_3)	
1	1	0	0	100	0	0	4.10
2	2/3	1/3	0	66.667	33.333	0	4.50
3	1/3	2/3	0	33.333	66.667	0	5.06
4	0	1	0	0	100	0	6.16
5	0	2/3	1/3	0	66.667	33.333	7.51
6	0	1/3	2/3	0	33.333	66.667	6.81
7	0	0	1	0	0	100	4.07
8	1/3	0	2/3	33.333	0	66.667	4.03
9	2/3	0	1/3	66.667	0	33.333	3.19
0 (CP[2])	1/3	1/3	1/3	33.333	33.333	33.333	5.70
0 (CP)	1/3	1/3	1/3	33.333	33.333	33.333	7.64
0 (CP)	1/3	1/3	1/3	33.333	33.333	33.333	6.24
0 (CP)	1/3	1/3	1/3	33.333	33.333	33.333	6.88

[1]Treatments; [2]CP = central point; [3]PE = propolis extract.

synergistic or antagonistic blending; and ε_{ijk} is the error associated to the Y_{ijk} observation. Note that the intercept term is not included due to the correlation between all the components (their sum is 100%). The effect of blends PE on the antioxidant activity (trolox equivalent antioxidant capacity, TEAC/mg dry weight) was analyzed using the least square method. For this trial, typical multiple regression models were used: linear model, Equation (2), quadratic model, Equation

(3), and special cubic model, Equation (4).

$$Y_{ijk} = \beta_i X_i + \beta_j X_j + \beta_k X_k + \varepsilon_{ijk} \quad (2)$$

$$Y_{ijk} = \beta_i X_i + \beta_j X_j + \beta_k X_k + \beta_{ij} X_i X_j + \beta_{ik} X_i X_k + \beta_{jk} X_j X_k + \varepsilon_{ijk} \quad (3)$$

$$Y_{ijk} = \beta_i X_i + \beta_j X_j + \beta_k X_k + \beta_{ij} X_i X_j + \beta_{ik} X_i X_k + \beta_{jk} X_j X_k + \beta_{ijk} X_i X_j X_k + \varepsilon_{ijk} \quad (4)$$

where the β's is the model parameter coefficient; X's are the independent variables (input points); Y_{ijk} and ε_{ijk} as previously defined. The selection model was based on ANOVA and significance test and, for the lack of fit and evaluation of the coefficient of determination R^2. Antioxidant capacity values were explained by the three-dimensional response surface analysis of the independent variables. From the predictive model, a mixture of propolis extracts with optimal antioxidant activity was established. The adjusted validation model was determined comparing the predict antioxidant activity from the optimized mixture with experimental data by student's t-test. Statistical analyzes were carried out using the Statistica version 9.1 software (StatSoft Inc., Tulsa, OK, USA) at 5% significance level.

2.3. Antioxidant Capacity Determination

The antioxidant capacity of propolis extracts was evaluated through oxygen radical removal capacity (ORAC) fluorimetric method using fluorescein as a molecular probe and 96-well black microplate [46]. The ORAC method is based on the elimination of peroxyl radicals (ROO•) by the antioxidant, by the hydrogen atom transfer mechanism (HAT), under controlled temperature, pH and time (**Figure 2**). The radical ROO• is produced during the assay by the thermal degradation of a radical generator, usually an azo compound, including *α,α*-azobis-isobutyronitrile (AIBN), 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), 2,2'-azobis(2-amidinopropane) (ABAP), and the hydrophilic 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH). Since ROO• is one of the main free radicals produced during the oxidation of biological systems, the data obtained through this method are considered representative for food systems [47] [48].

The propolis extracts and blends were diluted with ethanol 99.8% (1:50, v/v) and 75 mM phosphate buffer, pH 7.4 (1:400, v/v). The total dilution factor was 1:20.000 (v/v). A blank sample (70%, v/v, hydroalcoholic ethanol solution) was diluted as previously described for the propolis extracts. Based on the reference method, 25 µL of diluted extracts, blends or blank and 150 µL of fluorescein solution (81 nM in 75 mM phosphate buffer, pH 7.4) were pipetted into each well of the microplate. After incubation (10 min at 37˚C) under constant agitation, 25 µL of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) solution (152 mM in 75 mM phosphate buffer, pH 7.4) were added to each well of the microplate. The decay of fluorescence was followed every minute of reaction at 37˚C between 0 and 90 min, under excitation wavelength of 485 nm and emission wavelength of 528 nm. The result was expressed as TEAC/mg dry weight, based

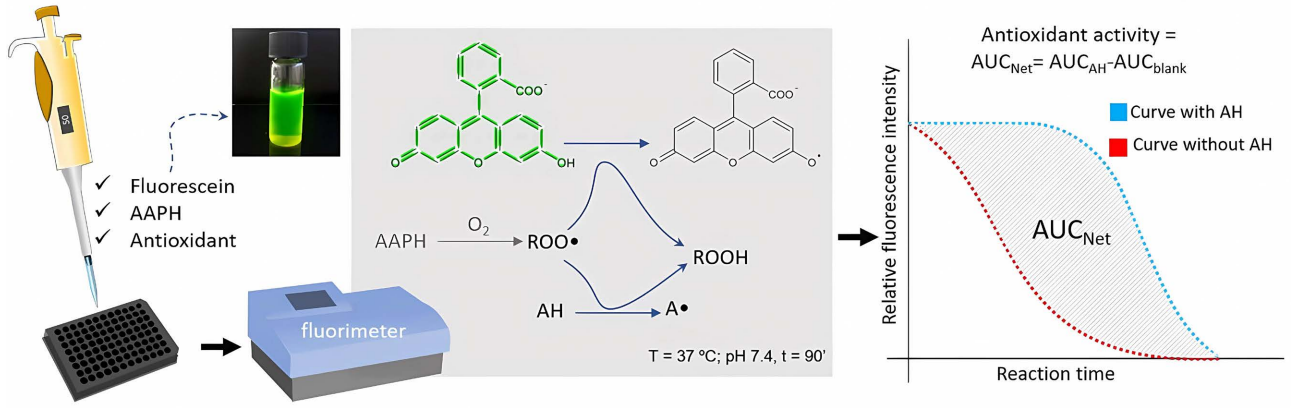

Figure 2. Illustration of the ORAC method considered in this research; legend: AAPH = 2,2'-azobis(2-amidinopropane); ROO• = peroxyl radical; AH = antioxidant; AUC_{AH} = area under the curve of the reaction with antioxidant; AUC_{blank} = area under the curve of the reaction without antioxidant; AUC_{net} = net area under the curve (between the samples and the blank), corresponding to the antioxidant activity. Source: the authors based on Ou *et al.* [46] and Echegaray *et al.* [48].

on a calibration curve prepared using trolox solutions with concentrations between 2 to 128 μM, diluting the standard trolox solution in 75 mM phosphate buffer, pH 7.4 [46].

2.4. Synergism Evaluation

Regression model obtained through the SLMD was used to find out the interactive effects of antioxidant activity from the red, green and brown PE in the blend extracts. Predictive model signs coefficients are evaluated for identification of favoring (synergism) or compromise (antagonism) in the relationship between two or more predictor variables: coefficients with positive signs indicate synergistic effect between the predictors, while negative signs indicate antagonism. The absence of polynomials of degree 2 or higher in the predictive model indicates an additive relationship [12] [49]. Synergism values (SV, %) were calculated as Equation (5). Theoretical ORAC was calculated as the weighted average of the observed antioxidant activities for each component extract of the mixture. The obtained difference values (%) indicate that there are potential synergistic (positive values) or antagonistic (negative values) and additive (null values) effects [50].

$$\mathrm{SV}(\%) = \left[\frac{\text{experimental ORAC}}{\text{theoretical ORAC}} \times 100\right] - 100 \tag{5}$$

3. Results and Discussions

3.1. Antioxidant Activity Model

An adequate model must be significant ($p \leq 0.05$) and with non-significant lack of fit ($p > 0.05$) [45] [51]. The coefficient of determination informs about the model predictive capacity. When the value is closer to 1.00, better is its capability. R^2 values ≥ 0.70 indicate high correlation and it is considered sufficient to demonstrate the adequacy of the model [52] [53]. The quadratic model was the

unique model that met the three criteria (Table 3), and then was selected. However, the model was reparametrized, eliminating the non-significant coefficients ($p > 0.05$) (Table 3), obtaining the following adjusted equation for antioxidant activity, Equation (6):

$$\hat{y}_{\mathrm{ORAC(TEAC/mg)}} = 4.0500x_1 + 6.0593x_2 + 3.9917x_3 + 12.1394x_2x_3 \left(R^2 = 0.81\right) \quad (6)$$

A ternary response surface diagram was obtained (Figure 3). It is observed that the response for antioxidant activity was greater around the edges x_2x_3, represented by the binary mixture of green PE (x_2) and brown PE (x_3) and intensified as the proportion of each extract in blend approached 1:1 (midpoint of the vertex). In fact, the adjusted equation revealed a binary mixture formed by 59% of green PE and 41% of brown PE as maximum point of antioxidant activity, whose predicted value was 8.15 μmol TEAC/mg dry weight.

Table 3. *p*-values, coefficients of determination (R^2) and regression coefficients (β) for the tested polynomial models.

Item	*p*-values and R^2 of the tested polynomial models			Terms or predictors						
	Model *p*-value	Lack of fit *p*-value	R^2	x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	$x_1x_2x_3$
Linear	0.0626^{ns}	0.232^{ns}	0.43	3.8010*	7.4289***	5.3613***	-	-	-	-
Quadratic	0.0328*	0.564^{ns}	0.82	3.7098*	5.8522***	3.9040*	1.9614^{ns}	1.0660^{ns}	12.2074*	-
Special cubic	0.0624^{ns}	0.9142^{ns}	0.91	3.9987***	6.1411***	4.1929***	−1.2882^{ns}	−2.1836^{ns}	8.9578*	33.1461^{ns}

[1]red, [2]green and [3]brown propolis extracts, x_1 x_2 and x_3 are coefficients of linear terms; x_1x_2, x_1x_3 and x_2x_3 are the coefficients of quadratic terms; and $x_1x_2x_3$ is the coefficient of special cubic term. ^{ns}Not significant ($p > 0.05$); *Significant ($p \leq 0.05$); **Significant ($p \leq 0.01$); ***Significant ($p \leq 0.001$).

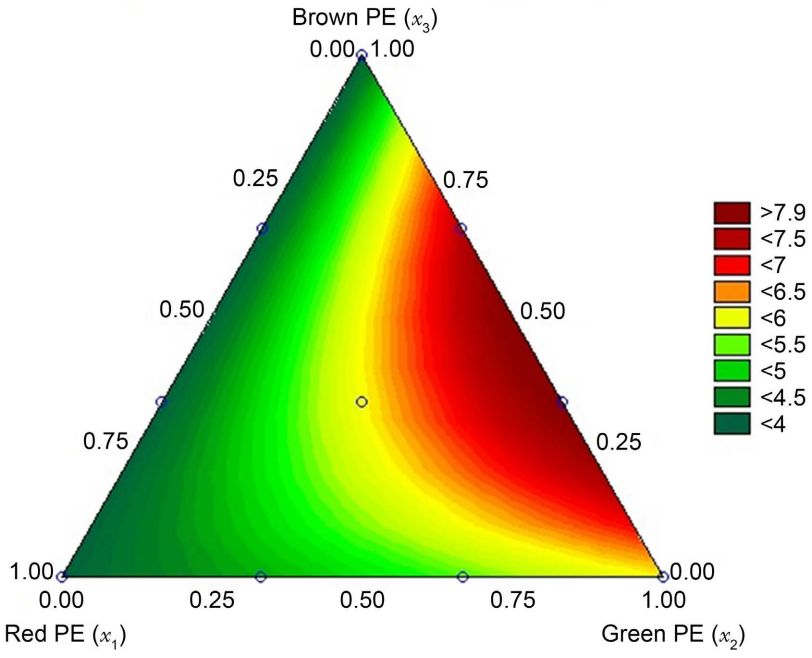

Figure 3. Ternary diagram of the response surface of the model.

The response observed experimentally for the optimized mixture (8.22 ± 0.75 μmol TEAC/mg dry weight; $n = 5$) did not differ ($p > 0.05$) from the predicted value (8.15 μmol TEAC/mg dry weight), ensuring the suitability of the adjusted predicted model for the antioxidant activity of the mixtures.

3.2. Synergism Study

Synergism studies are important to understand how the combination of antioxidants affects the activity of each component and the overall consequence for the mixture in terms of efficiency. It is known that these mixtures of ingredients can result in more or less efficiency, leading for possible synergistic or antagonistic relationships. In synergistic relations, the effect is greater in the mixture than the sum of the isolated effects. Although, when the response in the mixture is smaller than the sum of each isolated effect, the effect is antagonistic. In the absence of these effects, it can be said as additive when the result is the same as the sum of each isolated effect [11] [12].

The occurrence of term x_2x_3 (+ $12.1394x_2x_3$) in regression model indicates that there was a synergistic interaction between the green and brown propolis extracts, improving 54% (SV) the efficiency of the 59% of green PE and 41% of brown PE blend (8.15 μmol TEAC/mg dry weight) when compared with the equivalent sum of isolated effects (5.30 TEAC/mg dry weight). This association is visualized on the vertex x_2x_3 (**Figure 3**), an increment of antioxidant activity was observed in the center of the edge (blends 1:1 of green PE and brown PE extracts) red region, in relation with the vertex (pure extracts) green/yellow region. The absence of the terms x_1x_2 e x_1x_3 evidences the inexistence of synergy or antagonism between red PE (x_1) with green PE (x_2) or brown PE (x_3), which means that among the other extracts, in any proportions, the relationship was additive. When mixing red PE with green PE or red PE with brown PE, the antioxidant properties are preserved.

Evidence on antioxidant synergism in propolis extract blends is scarce in the literature and available data are based on *in vitro* studies. To the best of our knowledge, this is the first study that shows a synergistic relationship between Brazilians green PE and brown PE. Mixtures of Portuguese propolis extracts collected over five years (2011-2015) in Gerês, Portugal (41˚45'41.62"N; 7˚58'03.34"W), were evaluated in terms of its antioxidant activity by Peixoto *et al.* [9]. The authors concluded that the mixtures presented greater antioxidant activity than the individual extracts, and attributed this improvement to the synergism between the different compounds present in extracts, not only limited to phenolic compounds and flavonoids [9]. In addition, Oliveira [54] observed synergistic and antagonistic relationships combining propolis extracts commercialized in different regions of Portugal.

It is believed that the synergistic effects between propolis extracts on antioxidant activity are a function of their complex phytochemical composition, which contribution of minority compounds would be as important as of majority com-

pounds [4] [9] [55]. The structures of phenolic compounds, the main class of bioactive compounds found in propolis, including some markers found in Brazilian propolis [2] [14] [17] [19] [24] [56], are presented in **Figure 4**. Flavonoids are one of subclasses of phenolic compounds mostly found in propolis extracts [2] [4] [57]. Other groups of bioactive substances were identified including terpenoids [1], benzophenones [55] and chalcones [56].

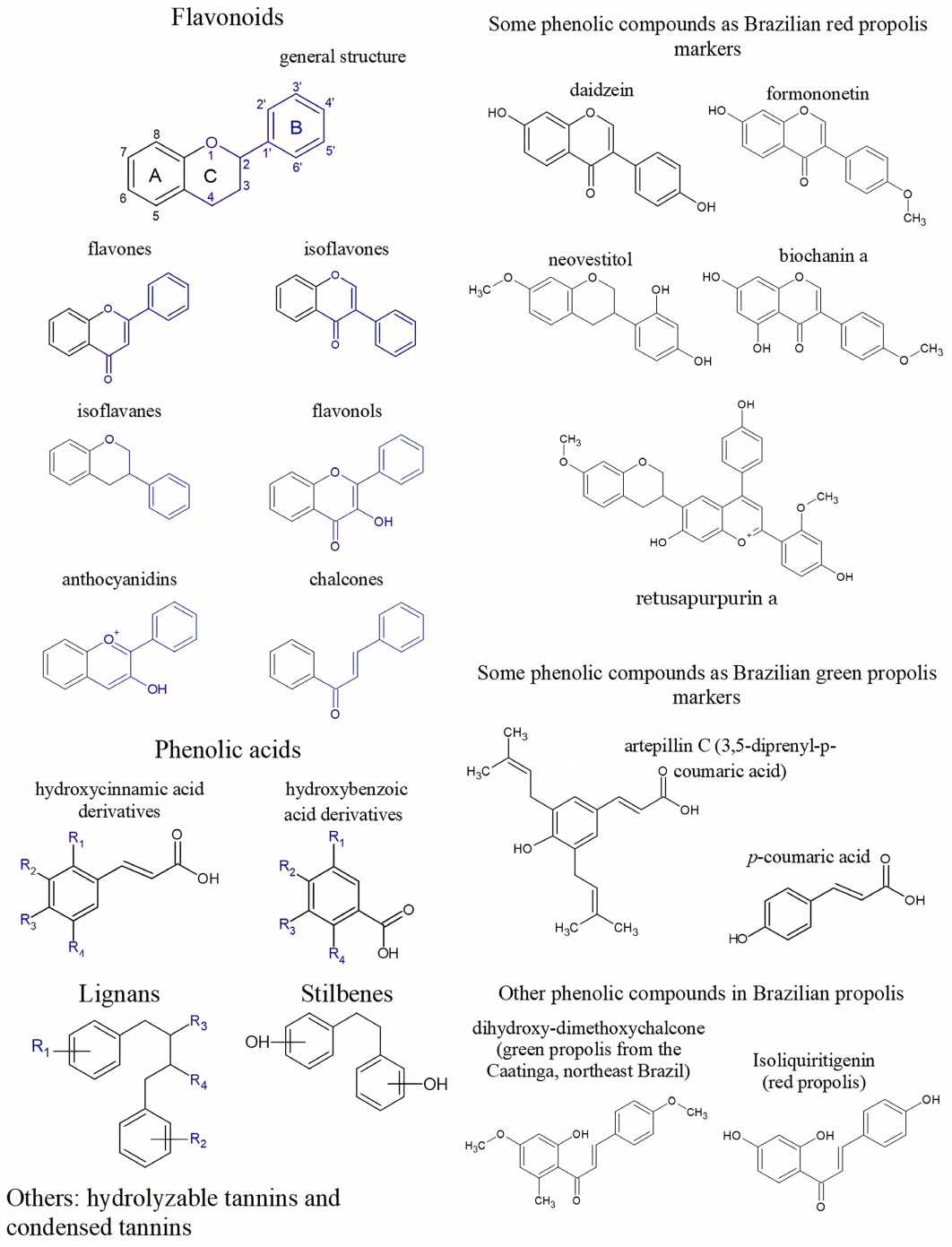

Figure 4. Phenolic classes and some chemical markers found in Brazilian propolis. R1 - 4 are different substituents.

Souza *et al.* [57] purified samples of Brazilian brown propolis, obtaining fractions enriched in phenolic compounds and flavonoids. In opposite of expectations, the antioxidant activity of the enriched fractions was lower than the original extract, indicating that there is synergism among different compounds. When investigating the effect of seasonality on the composition and antioxidant activity of Brazilian red propolis, do Nascimento *et al.* [55] attributed the obtained synergistic effect to interactions between flavonoids and gutiferones and between total phenolic compounds and gutiferones.

Osés *et al.* [4] compared the antioxidant activity of propolis extracts with analytical standards blend composed of the same majority of phenolic compounds identified in the extracts at equivalent concentrations. The authors reported that the antioxidant activity of the extracts was superior to the analytical standards blend, attributing to the synergistic effect of the minority compounds for better antioxidant activity in the propolis extracts.

Synergism between different classes of compounds present in propolis extracts was observed such as between flavonoids and benzophenones [55], terpenes and polyphenols [58] and phenolic acids and flavonoids [59], which may give an idea of the possible interactions that lead to the enhancement of antioxidant activity in the studied propolis extracts blends. The explanation of the compounds and mechanisms involved in these interactions will complement our findings and guide future research for the rational development of new antioxidant mixtures based on these natural resources, with better applicability as natural additives in food, among other commercial applications.

4. Conclusion

Synergistic antioxidant interaction in an *in vitro* model was identified between green and brown Brazilian propolis extracts. The predictive model accused a binary mixture composed of 59% green and 41% brown propolis extracts with increased antioxidant activity by about 54%. This is the first known study to show an antioxidant synergistic relationship between green and brown Brazilian propolis extracts. From a practical point of view, synergism will make it possible to reduce the dosage of these extracts as antioxidants and preservatives in foods, whose applicability is often limited due to low efficiency and high commercial value. Hence, it is highly recommended to verify the performance of these mixtures *in situ* (food models) to obtain more information about these interactions.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Elnakady, Y.A., Rushdi, A.I., Franke, R., Abutaha, N., Ebaid, H., Baabbad, M., Omar,

M.O.M. and Al Ghamdi, A.A. (2017) Characteristics, Chemical Compositions and Biological Activities of Propolis from Al-Bahah, Saudi Arabia. *Scientific Reports*, **7**, Article No. 41453. https://doi.org/10.1038/srep41453

[2] Rufatto, L.C., dos Santos, D.A., Marinho, F., Henriques, J.A.P., Ely, M.R. and Moura, S. (2017) Red Propolis: Chemical Composition and Pharmacological Activity. *Asian Pacific Journal of Tropical Biomedicine*, **7**, 591-598. https://doi.org/10.1016/j.apjtb.2017.06.009

[3] Veiga, R.S., De Mendonça, S., Mendes, P.B., Paulino, N., Mimica, M.J., Lagareiro Netto, A.A., Lira, I.S., López, B.G., Negrão, V. and Marcucci, M.C. (2017) Artepillin C and Phenolic Compounds Responsible for Antimicrobial and Antioxidant Activity of Green Propolis and *Baccharis dracunculifolia* DC. *Journal of Applied Microbiology*, **122**, 911-920. https://doi.org/10.1111/jam.13400

[4] Osés, S.M., Marcos, P., Azofra, P., de Pablo, A., Fernández-Muíño, M.Á. and Sancho, M.T. (2020) Phenolic Profile, Antioxidant Capacities and Enzymatic Inhibitory Activities of Propolis from Different Geographical Areas: Needs for Analytical Harmonization. *Antioxidants*, **9**, Article 75. https://doi.org/10.3390/antiox9010075

[5] Alvareda, E., Iribarne, F., Espinosa, V., Miranda, P., Santi, D., Aguilera, S., Bustos, S. and Zunini, M.P. (-2019) *In Silico* and *in Vitro* Approach for the Understanding of the Xanthine Oxidase Inhibitory Activity of Uruguayan Tannat Grape Pomace and Propolis Poliphenols. *Journal of Biophysical Chemistry*, **10**, 1-14. https://doi.org/10.4236/jbpc.2019.101001

[6] Fabris, F., Bertelle, M., Astafyeva, O., Gregoris, E., Zangrando, R., Gambaro, A., Lima, G.P.P. and Stevanato, R. (2013) Antioxidant Properties and Chemical Composition Relationship of Europeans and Brazilians Propolis. *Pharmacology & Pharmacy*, **4**, 46-51. https://doi.org/10.4236/pp.2013.41006

[7] Huang, S.A., Zhang, C.P., Wang, K., Li, G.Q. and Hu, F.L. (2014) Recent Advances in the Chemical Composition of Propolis. *Molecules*, **19**, 19610-19632. https://doi.org/10.3390/molecules191219610

[8] Irigoiti, Y., Navarro, A., Yamul, D., Libonatti, C., Tabera, A. and Basualdo, M. (2021) The Use of Propolis as a Functional Food Ingredient: A Review. *Trends in Food Science & Technology*, **115**, 297-306. https://doi.org/10.1016/j.tifs.2021.06.041

[9] Peixoto, M., Freitas, A.S., Cunha, A., Oliveira, R. and Almeida-Aguiar C. (2021) Antioxidant and Antimicrobial Activity of Blends of Propolis Samples Collected in Different Years. *LWT*, **145**, Article ID: 111311. https://doi.org/10.1016/j.lwt.2021.111311

[10] Kinasih, I., Julita, U., Suryani, Y., Cahyanto, T., Khoirunnisa, E.M. and Putra, R.E. (2021) Comparing the Effect of Two Coating Methods of *Tetragonula laeviceps* Propolis Water Extraction on Physical Quality and Shelf Life of Eggskept in Room Temperature. *Malaysian Applied Biology*, **49**, 75-79. https://doi.org/10.55230/mabjournal.v49i2.1526

[11] Huang, R.Y., Pei, L.L., Liu, Q.J., Chen, S.Q., Dou, H.B., Shu, G., Yuan, Z.X., Lin, J.C., Peng, G.N., Zhang, W. and Fu, H.L. (2019) Isobologram Analysis: A Comprehensive Review of Methodology and Current Research. *Frontiers in Pharmacology*, **10**, Article 1222. https://doi.org/10.3389/fphar.2019.01222

[12] Velásquez, P., Giordano, A., Valenzuela, L.M. and Montenegro, G. (2022) Combined Antioxidant Capacity of Chilean Bee Hive Products Using Mixture Design Methodology. *LWT*, **155**, Article ID: 112982. https://doi.org/10.1016/j.lwt.2021.112982

[13] Peixoto, M., Freitas, A. S., Cunha, A., Oliveira, R., and Almeida-Aguiar, C. (2022). Mixing Propolis from Different Apiaries and Harvesting Years: Towards Propolis

Standardization? *Antibiotics*, **11**, Article 1181. https://doi.org/10.3390/antibiotics11091181

[14] Silva, B.B., Rosalen, P.L., Cury, J.A., Ikegaki, M., Souza, V.C., Esteves, A. and Alencar, S.M. (2008) Chemical Composition and Botanical Origin of Red Propolis, a New Type of Brazilian Propolis. *Evidence-Based Complementary and Alternative Medicine*, **5**, 313-316. https://doi.org/10.1093/ecam/nem059

[15] Bhargava, P., Mahanta, D., Kaul, A., Ishida, Y., Terao, K., Wadhwa, R. and Kaul, S.C. (2021) Experimental Evidence for Therapeutic Potentials of Propolis. *Nutrients*, **13**, Article 2528. https://doi.org/10.3390/nu13082528

[16] Brasil (2022) Lista de IGs Nacionais e Internacionais Registradas. Ministério da Agricultura e Pecuária, Brasília. https://www.gov.br/agricultura/pt-br/assuntos/sustentabilidade/indicacao-geografica/listaigs

[17] Rufatto, L.C., Luchtenberg, P., Garcia, C., Thomassigny, C., Bouttier, S., Henriques, J., Roesch-Ely, M., Dumas, F. and Moura, S. (2018) Brazilian Red Propolis: Chemical Composition and Antibacterial Activity Determined Using Bioguided Fractionation. *Microbiological Research*, **214**, 74-82. https://doi.org/10.1016/j.micres.2018.05.003

[18] Jorge, R., Furtado, N.A.J.C., Sousa, J.P.B., da Silva Filho, A.A., Gregório Junior, L.E., Martins, C.H.G., Soares, A.E.E., Bastos, J.K., Cunha, W.R. and Silva, M.L.A. (2008) Brazilian Propolis: Seasonal Variation of the Prenylated *p*-Coumaric Acids and Antimicrobial Activity. *Pharmaceutical Biology*, **46**, 889-893. https://doi.org/10.1080/13880200802370373

[19] Moise, A.R. and Bobiş, O. (2020). *Baccharis dracunculifolia* and *Dalbergia ecastophyllum*, Main Plant Sources for Bioactive Properties in Green and Red Brazilian Propolis. *Plants*, **9**, Article 1619. https://doi.org/10.3390/plants9111619

[20] Aldana-Mejía, J.A., Ccana-Ccapatinta, G.V., Squarisi, I.S., Nascimento, S., Tanimoto, M.H., Ribeiro, V.P., Arruda, C., Nicolella, H., Esperandim, T., Ribeiro, A.B., de Freitas, K.S., da Silva, L.H.D., Ozelin, S.D., Oliveira, L.T.S., Melo, A.L.A., Tavares, D.C. and Bastos, J.K. (2021) Nonclinical Toxicological Studies of Brazilian Red Propolis and Its Primary Botanical Source *Dalbergia ecastaphyllum*. *Chemical Research in Toxicology*, **34**, 1024-1033. https://doi.org/10.1021/acs.chemrestox.0c00356

[21] Ferreira, J.C., Reis, M.B., Coelho, G.D.P., Gastaldello, G.H., Peti, A.P.F., Rodrigues, D.M., Bastos, J.K., Campo, V.L., Sorgi, C.A., Faccioli, L.H., Gardinassi, L.G., Tefé-Silva, C. and Zoccal, K.F. (2021) Baccharin and *p*-Coumaric Acid from Green Propolis Mitigate Inflammation by Modulating the Production of Cytokines and Eicosanoids. *Journal of Ethnopharmacology*, **278**, Article ID: 114255. https://doi.org/10.1016/j.jep.2021.114255

[22] Park, Y.K., Paredes-Guzman, J.F., Aguiar, C.L., Alencar, S.M. and Fujiwara, F.Y. (2004) Chemical Constituents in *Baccharis dracunculifolia* as the Main Botanical Origin of Southeastern Brazilian Propolis. *Journal of Agricultural and Food Chemistry*, **52**, 1100-1103. https://doi.org/10.1021/jf021060m

[23] Salatino, A., Teixeira, E.W., Negri, G. and Message, D. (2005) Origin and Chemical Variation of Brazilian Propolis. *Evidence-Based Complementary and Alternative Medicine*, **2**, 33-38. https://doi.org/10.1093/ecam/neh060

[24] Salatino, A., Salatino, M.L.F. and Negri, G. (2021) How Diverse Is the Chemistry and Plant Origin of Brazilian Propolis? *Apidologie*, **52**, 1075-1097. https://doi.org/10.1007/s13592-021-00889-z

[25] Ribeiro, V.P., Mejia, J.A.A., Rodrigues, D.M., Alves, G.R., de Freitas Pinheiro, A.M.,

Tanimoto, M.H., Bastos, J.K. and Ambrósio, S.R. (2023) Brazilian Brown Propolis: An Overview about Its Chemical Composition, Botanical Sources, Quality Control, and Pharmacological Properties. *Revista Brasileira de Farmacognosia*: *Orgao Oficial da Sociedade Brasileira de Farmacognosia*, **33**, 288-299. https://doi.org/10.1007/s43450-023-00374-x

[26] Oliveira, V.B., Yamada, L.T., Fagg, C.W. and Brandão, M.G.L. (2012) Native Foods from Brazilian Biodiversity as a Source of Bioactive Compounds. *Food Research International*, **48**, 170-179. https://doi.org/10.1016/j.foodres.2012.03.011

[27] Brasil (2020) Flora do Brasil. Jardim Botânico do Rio de Janeiro, Rio de Janeiro. https://dspace.jbrj.gov.br/jspui/bitstream/doc/118/5/Flora%202020%20digital.pdf

[28] Park, Y.K., Ikegaki, M. and Alencar, S.M. (2000) Classificação das própolis brasileira a partir de suas características fisico-químicas e propriedades biológicas. *Mensagem Doce*, **58**. https://www.apacame.org.br/mensagemdoce/58/artigo.htm

[29] Costa, A.G., Yoshida, N.C., Garcez, W.S., Perdomo, R.T., Matos, M.F.C. and Garcez, F.R. (2020) Metabolomics Approach Expands the Classification of Propolis Samples from Midwest Brazil. *Journal of Natural Products*, **83**, 333-343. https://doi.org/10.1021/acs.jnatprod.9b00783

[30] Ishida, V.F.D., Negri, G., Salatino, A. and Bandeira, M.F.C.L. (2011) A New Type of Brazilian Propolis: Prenylated Benzophenones in Propolis from Amazon and Effects against Cariogenic Bactéria. *Food Chemistry*, **125**, 966-972. https://doi.org/10.1016/j.foodchem.2010.09.089

[31] Santos, M.F.C., Oliveira, L.C., Ribeiro, V.P., Soares, M.G., Morae, G.O.I., Sartori, A.G.O., Rosalen, P.L., Bastos, J.K., de Alencar, S.M., Veneziani, R.C.S. and Ambrósio, S.R. (2021) Isolation of Diterpenes from Araucaria sp Brazilian Brown Propolis and Development of a Validated High-Performance Liquid Chromatography Method for Its Analysis. *Journal of Separation Science*, **44**, 3089-3097. https://doi.org/10.1002/jssc.202100374

[32] Fernandes-Silva, C.C., Teixeira, E.W., Alves, M.L.T.M.F., Negri, G., Salatino, M.L.F. and Salatino, A. (2021) Propolis Obtained in a Clearing inside the Atlantic Forest in Ubatuba (São Paulo state, Brazil): Essential Oil and Possible Botanical Origin. *Journal of Apicultural Research*, **60**, 853-861. https://doi.org/10.1080/00218839.2020.1797994

[33] Brasil (2001) Instrução normativa SDA n° 03, de 19 de janeiro de 2001. Diário Oficial da União, Brasília. https://pesquisa.in.gov.br/imprensa/jsp/visualiza/index.jsp?jornal=1&pagina=46&data=23/01/2001

[34] Brasil (2021) Resolução RDC Nº 466, de 10 de fevereiro de 2021. Diário Oficial da União, Brasília. https://www.in.gov.br/en/web/dou/-/resolucao-rdc-n-466-de-10-de-fevereiro-de-2021-303765551

[35] Bankova, V., Trusheva, B. and Popova, M. (2021) Propolis Extraction Methods: A Review. *Journal of Apicultural Research*, **60**, 734-743. https://doi.org/10.1080/00218839.2021.1901426

[36] Oroian, M., Dranca, F. and Ursachi, F. (2020) Comparative Evaluation of Maceration, Microwave and Ultrasonic-Assisted Extraction of Phenolic Compounds from Propolis. *Journal of Food Science and Technology*, **57**, 70-78. https://doi.org/10.1007/s13197-019-04031-x

[37] Santos, T.R.J. and Santana, L. (2022) Conventional and Emerging Techniques for Extraction of Bioactive Compounds from Fruit Waste. *Brazilian Journal of Food Tech-*

nology, **25**, 1-18. https://doi.org/10.1590/1981-6723.13021

[38] FAO (2020) How to Process Raw Propolis into Propolis Extracts. FAO, Rome. https://teca.apps.fao.org/teca/fr/technologies/8580

[39] Contieri, L.S., de Souza Mesquita, L.M., Sanches, V L., Viganó, J., Martinez, J., da Cunha, D.T. and Rostagno, M.A. (2022) Standardization Proposal to Quality Control of Propolis Extracts Commercialized in Brazil: A Fingerprinting Methodology Using a UHPLC-PDA-MS/MS Approach. *Food Research International*, **161**, Article ID: 111846. https://doi.org/10.1016/j.foodres.2022.111846

[40] Janani, D., Lad, S.S., Rawson, A., Sivanandham, V. and Rajamani, M. (2022) Effect of Microwave and Ultrasound-Assisted Extraction Methods on Phytochemical Extraction of Bee Propolis of Indian Origin and Its Antibacterial Activity. *International Journal of Food Science and Technology*, **57**, 7205-7213. https://doi.org/10.1111/ijfs.16066

[41] Keskin, S. (2020) Orange Peel Volatile Oil: A Green Solvent for Propolis Extraction, Enhanced β-Amylase Inhibition Activity. *Flavour and Fragrance Journal*, **35**, 411-416. https://doi.org/10.1002/ffj.3576

[42] Quatrin, A., Pauletto, R., Maurer, L.H., Minuzzi, N., Nichelle, S.M., Carvalho, J.F. C., Maróstica, M.R., Rodrigues, E., Bochi, V.C. and Emanuelli, T. (2019) Characterization and Quantification of Tannins, Flavonols, Anthocyanins and Matrix-Bound Polyphenols from Jaboticaba Fruit Peel: A Comparison between *M. trunciflora* and *M. jaboticaba*. *Journal of Food Composition and Analysis*, **78**, 59-74. https://doi.org/10.1016/j.jfca.2019.01.018

[43] Brazil (2019) Manual de Métodos Oficiais para Análise de Alimentos de Origem Animal. 2nd Edition, MAPA, Brasília, 73-74. https://www.gov.br/agricultura/pt-br/assuntos/laboratorios/credenciamento-e-laboratorios-credenciados/legislacao-metodos-credenciados/produtos-de-origem-animal

[44] Neto, B.B., Scarminio, I.S. and Bruns, R.E. (2003) Como fazer experimentos: Pesquisa e desenvolvimento na ciência e na indústria. Editora da Unicamp, Campinas.

[45] Baj, T., Baryluk, A. and Sieniawska, E. (2018) Application of Mixture Design for Optimum Antioxidant Activity of Mixtures of Essential Oils from *Ocimum basilicum* L., *Origanum majorana* L. and *Rosmarinus officinalis* L. *Industrial Crops and Products*, **115**, 52-61. https://doi.org/10.1016/j.indcrop.2018.02.006

[46] Ou, B., Hampsch-Woodill, M. and Prior, R.L. (2001) Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescente. *Journal of Agricultural and Food Chemistry*, **49**, 4619-4629. https://doi.org/10.1021/jf010586o

[47] Abeyrathne, E.D.N.S., Nam, K. and Ahn, D.U. (2021) Analytical Methods for Lipid Oxidation and Antioxidant Capacity in Food Systems. *Antioxidants*, **10**, Article 1587. https://www.mdpi.com/2076-3921/10/10/1587 https://doi.org/10.3390/antiox10101587

[48] Echegaray, N., Pateiro, M., Munekata, P.E.S., Lorenzo, J.M., Chabani, Z., Farag, M.A. and Domínguez, R. (2021) Measurement of Antioxidant Capacity of Meat and Meat Products: Methods and Applications. *Molecules*, **26**, Article 3880. https://doi.org/10.3390/molecules26133880

[49] Saoudi, S., Chammem, N., Sifaoui, I., Jiménez, I.A., Lorenzo-Morales, J., Piñero, J.E., Bouassida-Beji, M., Hamdi, M. and Bazzocchi, I. (2017) Combined Effect of Carnosol, Rosmarinic Acid and Thymol on the Oxidative Stability of Soybean Oil Using a Simplex Centroid Mixture Design. *Journal of the Science of Food and Agriculture*, **97**, 3300-3311. https://doi.org/10.1002/jsfa.8179

[50] Skroza, D., Mekinić, I.G., Svilović, S., Šimat, V. and Katalinić, V. (2015) Investigation of the Potential Synergistic Effect of Resveratrol with Other Phenolic Compounds: A Case of Binary Phenolic Mixtures. *Journal of Food Composition and Analysis*, **38**, 13-18. https://doi.org/10.1016/j.jfca.2014.06.013

[51] Crespo, Y.A., Sanchez, L.R.B., Quintana, Y.G., Cabrera, A.S.T., del Sol, A.B. and Mayancha, D.M.G. (2019) 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. *Heliyon*, **5**, E01942. https://doi.org/10.1016/j.heliyon.2019.e01942

[52] Fakayode, O.A. and Abobi, K.E. (2018) Optimization of Oil and Pectin Extraction from Orange (*Citrus sinensis*) Peels: A Response Surface Approach. *Journal of Analytical Science and Technology*, **9**, Article No. 20. https://doi.org/10.1186/s40543-018-0151-3

[53] Mahzir, K.A.M., Gani, S.S.A., Zaidan, U.H. and Halmi, M.I.E. (2018) Development of *Phaleria macrocarpa* (Scheff.) Boerl Fruits Using Response Surface Methodology Focused on Phenolics, Flavonoids and Antioxidant Properties. *Molecules*, **23**, Article 724. https://doi.org/10.3390/molecules23040724

[54] Oliveira, T.I.F. (2015) Efeitos tóxicos de amostras de propolis Português: Potencial antioxidante e atividades biológicas de extratos e misturas. Mestrado, Universidade do Minho, Braga. http://repositorium.sdum.uminho.pt/handle/1822/37479

[55] Nascimento, T.G., Arruda, R.E.D., Almeida, E.T.D., Oliveira, J.M.D., Basilio, I.D., Porto, I., Sabino, A.R., Tonholo, J., Gray, A., Ebel, R.E., Clements, C., Zhang, T. and Watson, D.G. (2019) Comprehensive Multivariate Correlations between Climatic Effect, Metabolite-Profile, Antioxidant Capacity and Antibacterial Activity of Brazilian Red Propolis Metabolites during Seasonal Study. *Scientific Reports*, **9**, Article No. 18293. https://doi.org/10.1038/s41598-019-54591-3

[56] Ferreira, J.M., Fernandes-Silva, C.C., Salatino, A., Negri, G. and Message, D. (2017) New Propolis Type from North-East Brazil: Chemical Composition, Antioxidant Activity and Botanical Origin. *Journal of the Science of Food and Agriculture*, **97**, 3552-3558. https://doi.org/10.1002/jsfa.8210

[57] Souza, R.M., de Souza, M.C., Patitucci, M.L. and Silva, J.F.M. (2007) Evaluation of Antioxidant and Antimicrobial Activities and Characterization of Bioactive Components of Two Brazilian Propolis Samples Using a pK_a-Guided Fractionation. *Journal of Biosciences: Zeitschrift für Naturforschung*, **62**, 801-807. https://doi.org/10.1515/znc-2007-11-1205

[58] Guo, Y., Baschieri, A., Amorati, R. and Valgimgli, L. (2021) Synergic Antioxidant Activity of γ-Terpinene with Phenols and Polyphenols Enabled by Hydroperoxyl Radicals. *Food Chemistry*, **345**, Article ID: 128468. https://doi.org/10.1016/j.foodchem.2020.128468

[59] Liu, H., Wang, D., Ren, Y., Wang, L., Weng, T., Liu, J., Wu, Y., Ding, Z. and Liu, M. (2022) Multispectroscopic and Synergistic Antioxidant Study on the Combined Binding of Caffeic Acid and (-)-Epicatechin Gallate to Lysozyme. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **272**, Article ID: 120986. https://doi.org/10.1016/j.saa.2022.120986