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Optimization by mixture design of the antimicrobial activities of five selected essential oils

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The individual antimicrobial activities of essential oils have been reported by many authors. However, there is little information about the effects of their mixtures in order to maximize their effect and reduce the growing resistance of pathogens to existing medicines. So, the aim of this work is to optimize the antibacterial and antifungal activities of essential oils from *Plectranthus glandulosus*, *Ocimum gratissimum*, *Cymbopogon citratus*, *Cymbopogon nardus* and *Eucalyptus* PF1. The mixtures of these essential oils were tested on seven bacterial and one fungal strain by employing the Mueller Hinton disc diffusion method. The diameters of the inhibition zones were measured after 24 h of incubation at 37°C. The results showed significant effects and regressions due to pure and composite mixtures on the response. The highest diameters of 30 and 27 mm were observed respectively with the pure essence of *C. citratus* on *Candida albicans* and the composite mixture. The binary mixtures showed more significant effects than the pure ones with the highest positive coefficient of regression 17.20 due to the *Plectranthus glandulosus* and *Eucalyptus* PF1 mixture on *Pseudomonas aeruginosa*. The growth inhibition data fitted the quadratic models for all individual strains except those of *Staphylococcus aureus* that better fitted the special cubic model. Some regression models of individual and combined microorganism responses have been proposed, as well as optimizations to maximize the inhibition zone diameters.

Key words: Essential oils, mixtures, modeling, optimization, anti-microbial.

INTRODUCTION

The use of plant derivatives to treat diseases is universal and dates back to immemorial times (Van Wyk and Wink, 2018). Plants contain active substances capable of inactivating pathogenic microorganisms and parasites and correcting physiological dysfunctions (Daniel, 2006). They treat cancers (Mukherjee et al., 2001; Cragg and Newman, 2005; Mohan et al., 2013), malaria (Krettli et

al., 2001; Andrade-Neto et al., 2003; Negi et al., 2014), influenza (Phillipson and Wright, 1991; Wang et al., 2006; Mukhtar et al., 2008), diarrhea (Barbosa et al., 2007; Dubreuil, 2013) and many other diseases that rage around the world. All the underground and above ground parts of fresh or dried plants as well as sap are used in various forms, most often as a decoction or infusion

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in water or alcoholic beverage, sometimes in powder form. Plant products are ingested, inhaled or applied to the skin.

Research has allowed identifying numerous bioactive plants molecules, some of which are isolated and consumed directly as drugs and others serve as models for the synthesis of more active and inexpensive analogs (Dewick, 2002; Evidente and Kornienko, 2009; Roleira et al., 2018).

Among these plants are aromatic plants that produce essential oils, many of these plants belong to the families of Lamiaceae, Graminae and Myrtaceae. The essential oils produced by these plants are used in perfumery, cosmetics, phytopharmacy and medicine. Research has revealed the antimicrobial activities of essential oils (Seow et al., 2014). This is the case for oils of the genera *Cymbopogon* (Gonçalves et al., 2010; Hindumathy, 2011; Nyarko et al., 2012; Falcao et al., 2012; Gharbia et al., 2015; Singh et al., 2016; Abu-; Kačániová et al., 2017; Nyamath and Karthikeyan, 2018), *Ocimum* (Nakamura et al., 1999; Iwalokun et al., 2001; Adebolu and Oladimeji, 2005; Junaid et al., 2006; Ait-Ouazzou et al., 2011; Aneke et al., 2019) and *Eucalyptus* (Damjanović-Vratnica et al., 2011). The reviews of these medicinal plant uses and antimicrobial activities can be found in Lukhoba et al. (2006); Prabhu et al. (2009); Manvitha and Bidya (2014) and Paton et al. (2018).

The microorganisms' strains that are the subject of this study are responsible for toxiifections which can cause death in individuals of all ages (Morrison and Wenzel, 1984; Cross, 1985; Griffin and Tauxe, 1991; Kotiranta et al., 2000; Mavor et al., 2005; Skippen et al., 2006). These microorganisms, like other pathogens, develop resistance to existing drugs. This constitutes a risk with sometimes fatal consequences for patients in addition to unnecessary financial expenses. One way to overcome this obstacle is to design remedies that integrate active principles with complementary mechanisms. To this end, mixture design can help optimize the efficacy of these products. The aim of this work was to check whether there are significant interactions between essential oils from *Plecthrantus glandulosus*, *Ocimum gratissimum*, *Cymbopogon citratus*, *Cumbopogon nardus* and *Eucalyptus* PF1 to improve their antimicrobial activity.

MATERIALS AND METHODS

Essential oils extraction

The leaves of *P. glandulosus*, *O. gratissimum*, *C. citratus*, *C. nardus* were harvested in May 2019 in an experimental culture carried out in Douakani village. The specimens of this plant were identified at the National Herbarium, in Brazzaville. The *Eucalyptus* PF1 leaves were provided by the National Reforestation Service (SNR) based in Dolisie between January and February 2019. The specimens were identified by the engineers in this service. *Eucalyptus* PF1, is a natural hybrid between *E. urophylla* and two or three individuals of *Eucalyptus alba* (mother tree) and one group of poorly identified hybrid *Eucalyptus* (parent trees) that come from a Brazilian arboretum.

The essential oils were extracted by hydro-distillation using a man-made distiller. Three kilograms of dried leaves of *P. glandulosus*, *O. gratissimum*, *C. citratus*, *C. nardus* and *Eucalyptus* PF1 were placed in a 10 l boiler and water was added to 2/3 of the boiler that was then heated to boiling with firewood. Running water from a waterfall was used to cool water and oil vapors. Water was eliminated from the resulting two-phase liquid mixture by opening the tap of the separator funnel. The essential oil was then collected in a shade bottle, mixed with anhydrous sodium sulphate and stored at 4°C until required.

Mixtures design

The experiment was carried out in 3 replicates by applying a mixture design of experiment of extreme peaks of degree 1, with 5 components corresponding to the essential oils of *P. glandulosus* (X_1), *O. gratissimum* (X_2), *C. nardus* (X_3), *C. citratus* (X_4) and *Eucalyptus* PF1 (X_5). The inferior and superior levels of the oil proportions have been fixed at 0 and 2.5 respectively, with a unique total of 2.5 ml. The plan was increased with the central point and the points on the axes and designed using Minitab 3.17.1. The mixtures were prepared in 5 ml shaded flasks. The matrix of this experiment is shown in Table 1.

Strains identification

Different selective culture media were used for the isolation and identification of the strains: 1) *Salmonella-Shigella* Agar (Bio RAD) for isolation of bacteria of the genus *Salmonella* whose colonies are black-centered (H_2S+) and lactose-positive and the genus *Shigella* whose colonies were colorless (H_2S-) and lactose-negative; 2) Cetrimide Agar (BIO RAD) for *Pseudomonas aeruginosa*, 3) Mannitol Salt Agar (Bio RAD) for isolation of bacteria of the genus *Staphylococcus* including mannitol positive *Staphylococcus* (*Staphylococcus aureus*); 4) Mossel Agar medium for *Bacillus*; 5) Sabouraud chloramphenicol (Bio RAD) for *Candida albicans* and 6) Methylene Blue Eosin Agar (Bio RAD) for the isolation of enterobacteria in which the major species is *Escherichia coli* that is characterized by a metallic sheen. Strains of *Klebsiella* spp and *E. coli* were identified by the Enterobacteri Sytem gallery.

Anti-microbial test

Microbial strain cultures

The strains used were isolated from the household wastewaters in Brazzaville neighborhoods. They were stored at 4°C in cryotubes containing Luria Bertani (LB) broth added with 20% glycerol. These strains were composed of: *Klebsiella* spp (Kl), *P. aeruginosa* (Pa), *P. aeruginosa_1* (Pa_1), *E. coli* (Ec), *Bacillus subtilis* (Bs), *Salmonella* spp (Sal), *S. aureus* (Sa) and *C. albicans* (Ca). These strains were sub-cultured onto PCA agar to obtain a young culture (Mabika et al., 2020).

Essential oils antimicrobial activity evidence

Antimicrobial activity was assessed by the Mueller Hinton diffusion method (Prats et al., 2000; Matasyoh et al., 2007). An inoculum containing the strain to be tested was prepared from a pure and young colony. Its optical density was adjusted to 0.1 at a wavelength of 625 nm with a spectrophotometer which is equivalent to the cell density of 0.5 (Mac Farland No 0.5) (Boukhatem, 2013). The inhibition test was performed in Petri dish containing solid and sterile Mossel Agar (Bin RAD) medium for the growth of *Bacillus*, Mueller-Hinton agar (MHA) medium for the other bacteria, and Sabouraud dextrose agar (SDA) for fungi, previously inoculated with 0.1 ml of microbial suspension. Using forceps, the oil-soaked

Table 1. The mixture design of experiment matrix for one replicate (treatments in standard order).

Mixture	Essential oil proportions (ml)				
	X1	X2	X3	X4	X5
E1	2.50	0.00	0.00	0.00	0.00
E2	0.00	2.50	0.00	0.00	0.00
E3	0.00	0.00	2.50	0.00	0.00
E4	0.00	0.00	0.00	2.50	0.00
E5	0.00	0.00	0.00	0.00	2.50
E6	1.50	0.25	0.25	0.25	0.25
E7	0.25	1.50	0.25	0.25	0.25
E8	0.25	0.25	1.50	0.25	0.25
E9	0.25	0.25	0.25	1.50	0.25
E10	1.50	0.25	0.25	0.25	1.50
E11	0.50	0.50	0.50	0.50	0.50

Wattman paper discs were placed on the medium. After 24 h of incubation at 37°C., the diameters of the observable zone of inhibition were measured.

Data analysis

Details of statistical methods used for data processing were found in Box and Draper (2007) and ReliaSoft (2015). The computing was done using the Minitab 3.17.1 software. The null hypotheses of equality of the means were tested by the analysis of variance (ANOVA) and the P-values at the significant level α of 0.05. The null hypothesis was rejected for a P-value < 0.05. The P-value was used in order to know if the test statistic was just into the critical region or was far out into the region.

The data shown in Tables 3 to 6 result from the ANOVA test of the inhibition zone diameters of the raw data from Table 2 followed by a two by two comparison test of Tukey. The aim was to know if there were significant differences from the diameter means due to the mixtures, on one side, and to the strains on the other side. Tables 7 and 8 show the results of ANOVA used in order to assess the main effects and interactions of the mixtures as well as the regressions of the inhibition diameters as a function of the essential oil proportions for each of the strains. The issue was to test whether the coefficients were equal to 0 or not. So, some statistics such as the coefficients of regression and determination were calculated to estimate and validate the models. For this purpose, the diameters were transformed into natural logarithms (ln) in order to obtain the highest coefficient of determination values (R^2), as well as knowing that inhibiting the growth of microorganisms involves biochemical processes that generally follow logarithmic distributions. These results allowed us to suggest the models showed below. The combined and individual optimizations were carried out by selecting the response variables to be optimized. The goal was to maximize the response; the lower level and the target being fixed respectively at $\ln(Y) = 2.7$ ($Y = 14.88$ mm) and $\ln(Y) = 3$ ($Y = 20.08$ mm). Results showed in Table 9 were calculated automatically using the Minitab software.

RESULTS

Inhibition of the pathogens studied as a function of the mixtures

The inhibition zone diameters of the eight pathogen

strains by essential oils are shown in Table 2. The maximum means from 25 to 29 mm were reached with the mixtures E4, E6, E8, E9 and E10 on *P. aeruginosa_1* and *C. albicans*. According to the results of the analysis of variance, Table 3 ($P = 0.000$), there was at least one significant difference between the means at the significance level of alpha equal to 5%. The results of Tukey's two-by-two comparisons of the inhibition diameters (Table 4) classify the diameters obtained in three groups, A, B and C. The E6 and E10 mixtures yielded means of 14.46 and 14.83 mm, respectively higher than the others.

Studied strains sensitivity

In the ANOVA table (Table 5), the p-value (0,000) for strain indicates that at the 5% significance level, not all means are equal. Tukey's test provides grouping information and two sets of confidence intervals with multiple comparisons. The grouping table (Table 6) shows that group A includes the most sensitive strains overall, namely: *P. aeruginosa_1*, *C. albicans* and *Bacillus subtilis* while group B contains the less susceptible strains: *E. coli*, *Salmonella*, *P. aeruginosa* *S. aureus* and *Klebsiella* spp.

Modeling inhibition zone diameters

The estimates of the regression coefficients are shown in Table 7. They explain how well some mixtures have significant effects on the microorganism growth inhibition. The positive coefficients obtained from binary mixtures mean that the two components act in synergy or are complementary (Table 8). In other words, the inhibition zone diameter is greater than that obtained by calculating the mean of each pure mixture diameters. The synergism

Table 2. Diameters of the inhibition zones of 8 strains of pathogens in mm per mixture of essential oils (E1 to E11).

Strains	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11
Kl	10.0 ±0.5	8.7 ±0.3	10.3 ±0.3	6.3 ±0.3	11.3 ±0.3	6.0 ±0.0	6.0 ±0.0	6.7 ±0.3	8.3 ±0.3	11.0 ±0.6	7.0 ±0.6
Sa	11.0 ±0.6	7.0 ±0.6	11.0 ±0.6	7.3 ±0.3	7.3 ±0.6	7.7 ±0.3	8.3 ±0.7	6.0 ±0.0	10 ±0.6	7.3 ±0.3	10.7 ±0.3
Sal	14.7 ±0.3	6.0 ±0.0	7.3 ±0.3	12.3 ±0.9	11.7 ±0.3	10.7 ±0.3	6.7 ±0.3	6.7 ±0.3	8.0 ±0.6	10.0 ±0.6	9.3 ±0.3
Pa	9.3 ±0.3	6.7 ±0.3	6.7 ±0.3	13.3 ±0.8	6.0 ±0.0	11.7 ±0.9	9.3 ±0.3	9.0 ±0.6	6.3 ±0.3	6.7 ±0.3	8.0 ±0.6
Pa_1	19.3 ±0.3	9.0 ±0.6	21.3 ±0.8	22.7 ±0.9	6.0 ±0.0	22.3 ±0.3	15.7 ±0.3	25.0 ±0.6	25.0 ±0.6	26.0 ±0.6	21.7 ±0.3
Bs	17.3 ±1.4	15.3 ±0.3	15.0 ±0.6	11.0 ±0.6	11.3 ±0.8	20.3 ±0.3	10.7 ±0.3	21.0 ±0.6	21.0 ±0.6	21.7 ±0.3	19.7 ±0.3
Ec	16.0 ±0.6	6.0 ±0.0	13.0 ±0.6	13.7 ±0.9	6.3 ±0.3	12.3 ±0.9	6.7 ±0.3	10.0 ±0.6	11.3 ±0.9	10.7 ±0.3	11.3 ±0.3
Ca	6.0 ±0.0	13.7 ±0.3	24.0 ±0.6	29.6 ±0.6	6.0 ±0.0	26.0 ±0.6	18.3 ±0.3	21.0 ±0.6	21.0 ±0.6	25.3 ±0.9	18.3 ±0.3

Table 3. Analysis of variance for E1; E2; E3; E4; E5; E6; E7; E8; E9; E10; E11.

Source	DF	SS	MS	F	P-Value
Mixture	10	1279	127.92	3.57	0.000
Error	253	9076	35.87		
Total	263	10355			

Table 4. Grouping information using Tukey's method and 95% confidence level for essential oils.

Factor	N	Means	Group
E10	24	14.83	A
E6	24	14.63	A
E4	24	14.46	A, B
E9	24	13.88	A, B
E3	24	13.58	A, B, C
E11	24	13.25	A, B, C
E8	24	13.17	A, B, C
E1	24	12.958	A, B, C
E7	24	10.208	A, B, C
E2	24	9.042	B, C
E5	24	8.250	C

Means not sharing any letters are significantly different.

of the mixture $X_1 \times X_5$ is the most important on 4 out of 8 strains (*B. subtilis*, *E. coli*, *P. aeruginosa_1* and *C. albicans*). Negative coefficients mean that the two components are antagonistic. Thus, the mean of the inhibition zone diameter is less than the one obtained by calculating the mean of each pure mixture diameters. This was observed particularly for interactions $X_1 \times X_2$ and $X_1 \times X_3$ on 5 out of 8 strains followed by $X_1 \times X_4$. The mixtures $X_1 \times X_2$, $X_1 \times X_3$, $X_1 \times X_4$, $X_1 \times X_5$ and, $X_2 \times X_3$ are the only mixtures with two components and $X_1 \times X_2 \times X_3$ with three components giving significant results ($p < 0.05$). The following terms could not be estimated; they were deleted: $X_2 \times X_4$, $X_2 \times X_5$, $X_3 \times X_4$, $X_3 \times X_5$ and $X_4 \times X_5$.

Values of $p < 0.05$ were obtained for the special cubic regression model for *S. aureus* and quadratic for the other seven strains of pathogens, indicating that the models estimated by the regression procedure are significant at the 0.05 alpha level. This means that at least one coefficient is different from zero. The importance of the coefficients in the five pure mixtures indicates that the essential oils of *P. glandulosus* (1.810-2.970), *O. gratissimum* (1.785-2.633), *C. nardus* (1.900-3.030), *C. citratus* (1.842-3.385) and of *Eucalyptus* PF1 (1.842-3.385) give significant inhibition zone diameters (Table 7).

The R^2 values in Table 8 indicate that the predictors explain 79.51 to 95.10% of the variance in the diameter of the inhibition zone; those of R^2 (prev) from 64.64 to 89.09% indicate that the quadratic regression models predict correctly the responses of the new observations for all the strains, except for the special cubic regression of the data obtained with *S. aureus* that gave a slightly lower value of R^2 (prev) equal to 53.8%. The R^2 (fitted) values varying between 70.19 and 92.51%, showing that the theoretical data actually fit the models. All the quadratic models are written as:

$$\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3$$

For example, using the coefficients of the terms shown in Table 8, the quadratic regression model equation for the diameter of the *Klebsiella* inhibition zone is:

$$Y = \ln(d) = 0.92X_1 + 0.86X_2 + 0.91X_3 + 0.74X_4 + 0.97X_5 - 1.21X_1X_2 - 1.055X_1X_3 + 0.53X_1X_4 + 0.48X_1X_5 + 0.19X_2X_3,$$

where d is the diameter in mm.

Inhibition zone diameters optimization

The combined optimizations allowed examining the combinations of essential oil proportions that simultaneously optimize multiple responses to meet the requirements for all responses in the set, with the individual and composite desirabilities (d) of the response

variables equal to 1.0. To simultaneously inhibit *P. aeruginosa_1*, *B. subtilis* and *C. albicans*, a combination of X_1 (1.22 ml), X_2 (0 ml), X_3 (0 ml), X_4 (0 ml) and X_5 (1.24 ml), yields the following responses: 6.7115 (821.80 mm) for *P. aeruginosa_1*, 4.9116 (135.86 mm) for *B. subtilis* and 5.9191 (372.08 mm). A combination of X_1 (1.6162 ml), X_2 (0.2946 ml), X_3 (0 ml), X_4 (0.2503 ml) and X_5 (0.33389 ml) provides the following responses for *Klebsiella* spp: 2, 1601 (8.7 mm), *Salmonella* spp: 2.4531 (11.6 mm), *P. aeruginosa*: 2.0250 (7.6 mm), *P. aeruginosa_1*: 3.6168 (37.2 mm), *B. subtilis*: 3.0176 (20.4 mm), *C. albicans*: 3.4926 (32.87 mm) and *S. aureus*: 2.1819 (8.86 mm). The optimizations of the individual responses are shown in Table 9. It is observed that the binary mixtures make it possible to obtain inhibition diameters of the germs studied from 19 to 410 mm. That is, the growth of certain strains such as *P. aeruginosa_1*, *B. subtilis* and *C. albicans* should be completely inhibited.

DISCUSSION

It appears from this work that some pure and composite mixtures of tested essential oils are capable of inhibiting the growth of the microbial strains studied. This is justified by the average inhibition zone diameters observed ranging from 10 to 19 mm. The maximum diameters up to 30 mm were reached with the essential oils of *C. nardus* and *C. citratus* on *Candida albicans*. There were significant differences between the means of the inhibition zone diameters for the mixtures and the strains. The mean highest diameter of inhibition zone was given by the mixtures and E10. There was also significant difference in the sensitivity of the strains to essential oils. Three strains were more sensitive namely: *P. aeruginosa_1*, *B. subtilis* and *C. albicans*; the others being less sensitive. These diameters of inhibition are close to those reported by Leopold et al. (2002) and Naik et al. (2010) and Leopold et al. (2002). However, Kon and Rai (2012) obtained the inhibition zone diameter reaching 64 mm with *Cinnamomum zeylonicum* essential oil.

The results of the regression analyses showed that the effects of certain essential oil mixtures were significant. Some synergistic and antagonistic effects were observed. The inhibition was greater with binary mixtures which gave the highest regression coefficients. The $X_1 \times X_5$ combination of *P. glandulosus* and *Eucalyptus* PF1 gave coefficients of 17.20 and 16.43 mm on *P. aeruginosa_1* and *C. albicans*, respectively, showing the existence of a synergistic or additive effect. This mixture is therefore better indicated for treating pathologies caused by these two germs. The results reveal that the most efficient mixtures of essential oils to use for designing medicines or antiseptics should be those with two ingredients, as the active ingredient will probably be diluted with the increasing of the mixture components number. The

Table 8. Estimates of regression coefficients (component quantity)

Strains	Modèle	P value	R ² (%)	R ² (prev) (%)	R ² (ajust) (%)	Coefficients of the terms in component quantities										
						X1	X ₂	X ₃	X ₄	X ₅	X ₁ X ₂	X ₁ X ₃	X ₁ X ₄	X ₁ X ₅	X ₂ X ₃	X ₁ X ₂ X ₃
<i>Klebsiella sp</i>	Quadratic	0.000	94.61	88.17	92.51	0.92	0.86	0.91	0.74	0.97	-1.21	-1.05	0.53	0.48	0.19	
<i>Salmonella spp</i>	Quadratic	0.006	91.05	82.57	87.55	1.07	0.71	0.77	1.00	0.98	0.41	0.18	-0.74	0.06	-0.58	
<i>P. aeruginosa</i>	Quadratic	0.000	89.50	78.65	85.39	0.89	0.81	0.76	1.04	0.72	1.32	1.41	-1.66	-0.22	-0.84	
<i>P. aeruginosa_1</i>	Quadratic	0.000	89.77	78.21	85.76	1.19	0.88	1.09	1.25	0.72	-1.77	-1.10	0.51	2.76	2.27	
<i>B. subtilis</i>	Quadratic	0.000	95.10	89.21	93.19	1.14	1.09	1.07	0.96	0.97	-2.26	-0.02	1.40	1.46	0.95	
<i>E. coli</i>	Quadratic	0.000	83.75	64.64	77.40	1.14	0.71	0.92	1.04	0.73	-0.8	-0.32	-0.03	1.02	0.39	
<i>C. albicans</i>	Quadratic	0.000	93.91	89.09	91.53	0.72	1.05	1.21	1.35	0.72	0.39	0.19	-0.49	2.63	-0.11	
<i>S. aureus</i>	Cubic spécial	0.000	79.51	53.89	70.19	0.96	0.78	0.9	0.8	0.79	-1.02	-2.56	1.00	0.03	-0.55	8.22

Table 9. Results of the individual optimizations of the responses for each of the germ strains

Strains	X1	X2	X3	X4	X5	Diameter (mm)	Desirability
<i>Klebsiella spp</i>	1.19	0.00	0.00	0.0	1.31	22.66	1.00
<i>S. aureus</i>	0.72	0.98	0.80	0.0	0.00	65.90	1.00
<i>Salmonella spp</i>	1.68	0.82	0.00	0.0	0.00	19.10	0.83
<i>P. aeruginosa</i>	1.29	0.00	1.21	0.0	0.00	71.74	1.00
<i>P. aeruginosa_1</i>	0.00	1.21	1.29	0.0	0.00	410.51	1.00
<i>B. subtilis</i>	1.31	0.00	0.00	0.0	1.19	136.26	1.00
<i>E. coli</i>	1.45	0.00	0.00	0.0	1.05	53.02	1.00
<i>C. albicans</i>	1.25	0.00	0.00	0.0	1.25	372.22	1.00

synergistic effect of binary essential oil mixtures was reported by Ouedhiri et al. (2016) on *B. subtilis* and *S. aureus*, Fadil et al. (2018) on *Salmonella typhimurium*, Falleh et al. (2019) on *Escherichia coli*. The synergism of antibacterial activities of essential crude oils or their compounds was also reported by authors such as Wang et al. (2006), Bassolé and Juliani (2012), Zengin and Baysal (2014), Semeniuc et al. (2017) and Gadisa et al. (2019). A single ternary mixture gave significant results on *S. aureus*. Most of the available reports deal with binary mixtures. The

authors reporting results of the tests of more than two component mixtures like Baj et al. (2018) are rare.

The most antagonistic effect was observed on the X₁*X₃ mixture composed of *P. glandulosus* and *C. nardus* on *S. aureus*, giving a regression coefficient of -16.01. The combinations X₁*X₂, X₃*X₃ and X₁*X₄ that showed antagonistic effects on 4-5 strains out of 8 should be avoided for inhibiting most of the microorganisms studied. What is important in these results is not the size of the inhibition zone diameter, but the regression

coefficient and the degree of correlation between the input variables and the responses allowed to explain the variability of the response and to predict responses of untested factor levels. In fact, the size of the inhibition diameter depends both on the concentration of the active principle in the ingredient and on its efficacy. However, the degree of correlation between the variable and the response depends on the effectiveness of the growth inhibition. Thus, an essential oil that will give higher coefficients and values of R² will probably contain the most effective molecule to

inhibit the growth of the tested germ even if the diameters values are low.

The tests of the regression validation led to select the quadratic model for all the strains except one, *S. aureus*, the data of which better fitted the special cubic model. Optimization was done to maximize the diameter of individual or group of germs. Individual or combined optimizations yielded combinations of essential oils that would significantly inhibit the growth of the tested strains. The combination of essential oils of *P. glandulosus* and *Eucalyptus* PF1 was the one that gave the best results on *P. aeruginosa_1*, *B. subtilis* and *C. albicans*. Forecast diameters of the order of 400 mm simply mean that microorganisms can be completely inhibited, depending on the proportion and diffusivity of the ingredient.

Conclusion

The blending design allowed 88 combinations of essential oils and microbial strains to be tested in one experiment. Statistical analyses of the obtained results made it possible to identify both the most effective combinations of essential oils and the most sensitive microbial strains. It should be noted, as revealed by other authors, that it is the binary mixtures that indicate the most important positive and negative interactions. The most significant positive and negative interactions were observed respectively with the combinations, X1*X5 (*P. glandulosus* + *Eucalyptus* PF1) and X1*X2 (*P. glandulosus* and *O. gratissimum*).

Analyses of the obtained data allowed the selection of the mathematical models best adapted to the variation of the diameter of the inhibition zones of the microbial strains according to the proportions of essential oils in the mixtures. Data from all strains were more suitable for quadratic regression except for *Staphylococcus* that was better adapted to the special cubic model. This led to optimizations of the individual and combined responses of the essential oils to obtain the proportions of essential oils that would maximize the growth inhibition diameters of the microbial strains. Finally, this is the first time that the antimicrobial activity of the essential oil of the PF1 clone of *Eucalyptus* has been demonstrated.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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