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Research article

Enhancing the nitric oxide inhibitory activity using a combination of plant essential oils and mixture design approach

Mariangela Marrelli [a], Michele De Luca [a,*], Claudia-Crina Toma [b], Fedora Grande [a], Maria Antonietta Occhiuzzi [a], Rosalba Caruso [a], Filomena Conforti [a], Giancarlo Statti [a]

[a] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036, Rende, Cosenza, Italy
[b] Pharmacology Department, Faculty of Pharmacy, Vasile Goldis Western University of Arad, 87 L. Rebreanu Str., 310045, Arad, Romania

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ABSTRACT

The synergistic effects of essential oils (EOs) from three aromatic plant species, *Foeniculum vulgare* subsp. *piperitum* (C.Presl) Bég. (FV), *Origanum heracleoticum* L. (OH) and *Lavandula austroapennina* N.G.Passal., Tundis & Upson. (LA), were evaluated for their inhibitory properties on nitric oxide production in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS). We utilized a Design of Experiments (DoE) methodology to optimize a formulation by combining three Essential Oils (EOs), while simultaneously taking into account two response variables, maximization of NO inhibition with minimum cytotoxicity. The optimal blend of components was predicted, and the statistical outcome's efficacy was experimentally verified. The combination corresponding to 87.7 % FV, 12.3 % LA and 0.0 % OH showed high inhibitory effect (76.3 %) with negligible cytotoxicity (4.5 %). This research provides new information on the interactions among fennel, oregano and lavender essential oils and shows how they can synergistically inhibit in vitro LPS-induced NO production.

1. Introduction

The synergistic therapeutic actions of botanicals have gained much attention in recent years. It has been demonstrated that plant extracts, due to their multi-component nature, have a great potential for exhibiting synergistic actions [1]. These effects can be due to different mechanisms, as the constituents of a plant extract or a combination of different extracts may act on different targets or interact with each other to enhance the solubility or the bioavailability of some constituents [2]. Synergistic responses are different from simple additive responses, as the combined effect of two or more phytochemicals is greater than the sum of the potency of each specific component of the mixture. Conversely, the combination of different molecules may also produce an antagonistic effect, in which the combined potency is lower than that of the individual components [3,4]. The combined effects of botanicals have also been largely explored for plant essential oils, mainly regarding their antimicrobial properties. Indeed, the synergistic and additive effects of different EOs and their isolated chemical constituents are useful to enhance their antibacterial effects against food-borne pathogens in food systems, where the efficacy of botanicals appears to be lower than that observed in vitro [5]. The interactions between different terpenoid components of EOs may result in an increased antimicrobial efficacy. A number of studies deal with the combination of

* Corresponding author.
E-mail address: michele.deluca@unical.it (M. De Luca).

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phenolic monoterpenes, such as carvacrol or thymol, and phenylpropanoids, such as eugenol, with other classes of compounds, mainly phenylpropanoids, phenols and monoterpenes alcohols. As a result, a synergistic effect on numerous microorganisms has been observed for the combination of these molecules [6,7]. The evaluation of the synergistic effect obtained by combining different components, where effects of enhancement or reduction of a desired response may coexist, can be very complex, since the different combinations that can be used would be infinite and even the use of mixtures dictated by experience gained from years of studies and research may not be sufficient. For this purpose, it is preferable to use a multivariate method of experimental design that can guarantee the optimization of the mixture through a statistically significant and validable prediction. The design of experiments can evaluate all linear, quadratic, cubic and quartic interactions between components in a single process and with a discrete number of experiments, depending on the number of components and the type of design strategy. The design of essential oil blends has been used for different purposes, different extracts have been tested to obtain blends to exploit their antioxidant, antihyperglycemic or optimized for the treatment of diabetes [8–12].

Essential oils are produced by thousands of aromatic plant species. They are present in many plant organs, e.g., flower, leaf and fruit, but also wood, and rhizome and even seed [13]. The major components found in EO belong to two main groups: terpene hydrocarbons, particularly monoterpenes (accounting for the 80 % of EOs components) and sesquiterpenes, and oxygenated compounds. These second group comprises alcohols, phenols, aldehydes and esters [14]. In addition to their biological properties, such as antiviral, antibacterial [15], antifungal [16], anticancer [17] and immunomodulatory [18], different recent studies have investigated the anti-inflammatory activity of EOs, and, in some cases the mechanisms involved have also been explored [19]. Their anti-arthritic potential has been investigated as well [20]. It is well known that inflammation plays a key role in the pathogenesis of several diseases, including the emergence and progression of different types of cancer. This link is due to a number of mechanisms, such as the induction of genomic instability, the promotion of angiogenesis and the enhancement of cell proliferation [21]. Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and the tumor necrosis factor-α (TNF-α), promote the expression of inducible nitric oxide synthase (iNOS), thus increasing the nitric oxide (NO) production [22]. This molecule contains a nitrogen atom covalently bonded to an oxygen atom with an unpaired electron. NO is produced by nitric oxide synthases (NOSs) enzymes and it plays a role in both inflammation and a number of other biological processes including neurotransmission and immune response. While under physiological condition NO has an anti-inflammatory activity, it is considered a pro-inflammatory mediator following the overproduction occurring in abnormal situations [23]. Three NOS isoforms are known: neuronal, constitutive endothelial and inducible NOS (nNOS, eNOS and iNOS, respectively) [24]. The neuronal enzyme is responsible for the production of NO in the central nervous system, where nitric oxide acts as a neurotransmitter, while the last two isoforms (iNOS and eNOS), produce nitric oxide that acts as an inflammatory mediator [24].

In the present study, three EOs from three different species collected in Calabria (Southern Italy) were taken into account: *Foeniculum vulgare* subsp. *piperitum* (C.Presl) Bég. (FV), *Origanum heracleoticum* L. (OH) and *Lavandula austroapennina* N.G.Passal., Tundis & Upson (LA). The first species, *F. vulgare* subsp. *piperitum* (synonym of *Foeniculum vulgare* Mill.), belongs to the Apiaceae family, while the other two plants belong to the Lamiaceae.

The essential oils from these plants demonstrated very interesting in vitro inhibitory effects on the production of nitric oxide [20,25]. For this reason, we decided to assess their potential synergist effects, evaluating the ideal combination of the three essential oils.

Here, we investigated the combination of these samples in terms of their potential synergistic inhibitoryeffect on the production of the pro-inflammatory mediator nitric oxide, assessed in the murine macrophage cell line RAW 264.7 stimulated with lipopolysaccharide (LPS) from *Escherichia coli*. The presence of nitrites, NO oxidized stable end-products, was assessed in the cell culture medium by using the Griess reagent. To rule out the possibility that the NO inhibition was caused by cell death and not by the anti-inflammatory effect of the EOs mixture, a second biological effect was considered. All experiments, planned by application of the simplex-lattice design, were evaluated with two response variables: NO inhibition and cytotoxicity. Optimization was performed by targeting maximum NO inhibition and minimum cytotoxic effect.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium, fetal bovine serum, L-glutamine, penicillin/streptomycin, trypan blue, phosphate buffered saline, MTT, Griess reagent, lipopolysaccharide (LPS) from *Escherichia coli*, indomethacin and L-NAME were purchased from Sigma-Aldrich S.p.A. (Italy).

2.2. Plant materials and extraction procedures

Essential oils from the fruits of *F. vulgare* subsp. *piperitum* and the aerial parts of *O. heracleoticum* were collected in Calabria (Southern Italy), and voucher specimens are available in the Herbarium of the University of Calabria and the Ethnobotanical Conservatory of Castelluccio Superiore, Potenza, Italy. EOs were extracted from fresh plant material. Plants were cleaned and extracted by steam distillation for 2 h, using a steel extractor apparatus (Albrigi Luigi, Verona, Italy). Obtained EOs were dried over anhydrous sodium sulfate. They were stored at +4 °C until analyses. *Lavandula austroapennina* EO was kindly provided by 'Parco della Lavanda' (Morano Calabro, Cosenza, Italy).

2.3. Gas chromatography-mass spectrometry (GC-MS) analyses

Analyses were run with a Hewlett-Packard 6890 GC connected to a mass selective detector Hewlett Packard 5973. A SE-30 capillary column (30 m × 0.25 mm) with 0.25 μm film thickness was used. Analyses were run using a programmed temperature (range 60–280 °C, rate 16 °C/min). Column inlet was set at 250 °C. The MS operating parameters were set as follows: 70 eV Ion source, 230 °C ion source temperature, 34.6 μA electron current; 10^{-5} torr vacuum. Mass spectra were acquired over a 40–800 amu range at 1 scan/sec. The utilized carrier gas was helium (linear velocity 0.00167 cm/s). The mass spectra of the detected constituents were compared with the Wiley Mass Spectral Database of the GC–MS system.

2.4. Cell cultures

RAW 264.7 murine macrophages (ATCC no. TIB-71, UK) were grown in Dulbecco's Modified Eagle's Medium (DMEM). The medium was supplemented with L-glutamine, penicillin/streptomycin and fetal bovine serum (1 %, 1 % and 10 %, respectively). Cells were cultured at 37 °C in humidified air with 5 % CO_2. To perform the experiments onto 96 wells-microplates (1×10^5 cells/well), cells were removed from the culture flask by scraping.

2.5. Nitric oxide production inhibition

After 24 h from seeding, cells were treated with samples in concentration ranging from 6 to 1000 μg/mL, stimulated with lipopolysaccharide (LPS, 1 μg/mL) and incubated overnight. The day after, the cell surnatant (100 μL) and Griess reagent (100 μL) were mixed in order to assess the presence of nitrite, the nitric oxide oxidation stable end product. Absorbance was measured at 550 nm and indomethacin and L-NAME were used as positive controls [26].

2.6. Cytotoxicity

In order to verify that investigated EOs did not affect cell viability, a MTT assay was performed. Briefly, 100 μL of a a 0.5 % of MTT solution were added to each well. Four hours later, DMSO (100 μL/well) was added and absorbance was measured at 550 nm [27].

2.7. Mixture design and statistical analyses

The ternary combinations of EOs were selected by considering a simplex lattice mixture design of fourth degree [8,9,28,29]. The design was unconstrained and randomised. The different experiments were performed according to the surface of Fig. 1 were: the vertices of the triangle corresponding to the pure 100 % oils and all the other points representing the various combinations with ratios 25, 50 and 75 %. The central point of the triangle represents the mixture comprising one-third of each pure oil (Table 1). The design is

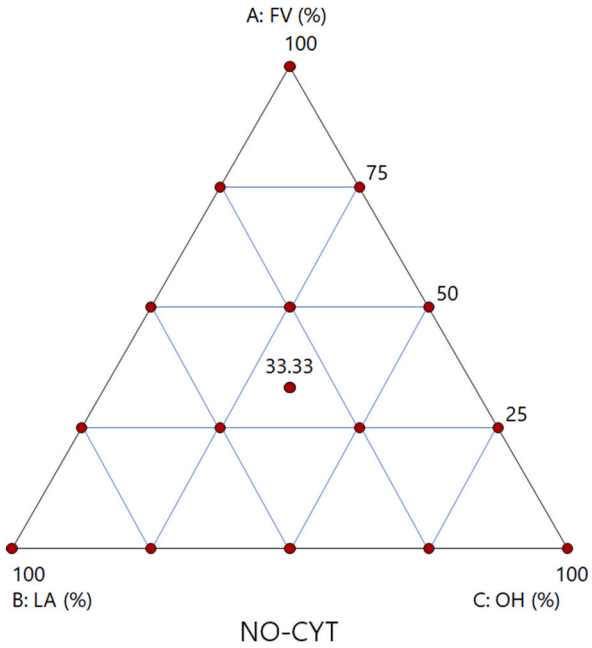

Fig. 1. The simplex lattice design for the three-component mixture and point test. NO inhibition (NO) and cytotoxic activity (CYT); FV, *Foeniculum vulgare* subsp. *piperitum* (C.Presl) Bég.; LA, *Lavandula austroapennina* N.G.Passal., Tundis & Upson; OH, *Origanum heracleoticum* L..

Table 1
EOs mixtures designed with the Simple Lattice Design method.

Experiment	EO (%) v/v		
	FV	LA	OH
SLM1	100	0	0
SLM2	75	25	0
SLM3	75	0	25
SLM4	50	50	0
SLM5	50	25	25
SLM6	50	0	50
SLM7	25	75	0
SLM8	25	50	25
SLM9	25	25	50
SLM10	25	0	75
SLM11	0	100	0
SLM12	0	75	25
SLM13	0	50	50
SLM14	0	25	75
SLM15	0	0	100
SLM16	33.33	33.33	33.33
SLM17	33.33	33.33	33.33
SLM18	33.33	33.33	33.33

FV, *F. vulgare* subsp. *piperitum* (C.Presl) Bég.; LA, *Lavandula austroapennina* N.G.Passal., Tundis & Upson; OH, *Origanum heracleoticum*.

composed of 48 experiments (16x3, three replications for all point test). The results obtained from the selected experiments were fitted to a cubic polynomial model using least squares regression to compute the equation coefficients listed in equation (1) and (2). These analyses were carried out using The Unscrambler X, version 10.3 (Camo Process As., Oslo, Norway), Matlab version R2023a (The MathWorks, Inc. Natick, MA, USA) and Origin 2019 (OriginLab Corporation, Northampton, MA).

3. Results

3.1. Extraction yields and phytochemical profile

The steam distillation of the fruits from *F. vulgare* subsp. *piperitum* allowed obtaining a yield equal to 0.7 % *w/w*, while the yield of the EO from *O. heracleoticum* aerial parts was equal to 0.8 % *w/w*. In our previous works, we already investigated the chemical profile of *F. vulgare* subsp. *piperitum* [20], *L. austroapennina* [25] and *O. heracleoticum* essential oils [30]. Here, the phytochemical composition of the EOs from these same plants was firstly verified with GC-MS analyses before assessing their potential synergistic effects. GC-MS was chosen as it is considered the main technique for the analysis of essential oils [19]. As expected, the major components *F. vulgare* subsp. *piperitum* fruit EO were estragole (32.22 %), anethole (25.10 %) and fenchone (17.84 %). Moreover, this sample was particularly rich in monoterpene hydrocarbons, with limonene (5.82 %), α-pinene (1.82 %), α-phellandrene (1.73 %) and γ-terpinene (1.20 %) being the most abundant ones (Table 2). In accordance with our previous analyses [25], linalool (24.31 %) was instead the most abundant volatile compound identified in *L. austroapennina* EO, followed by linalyl acetate and terpinen-4-ol (12.30 % and 15.11 %, respectively). As regards the *O. heracleoticum* EO, the most abundant components were confirmed to be the oxygenated monoterpenes linalyl acetate (19.00 %), lilanool (10.62 %) and linalool propionate (7.21 %). Sabinene (4.04 %) and α-ocimene (6.04 %) were the most abundant monoterpene hydrocarbons.

3.2. Combinations of the three EOS by mixture design methodology

The in vitro inhibitory properties on NO production were assessed in LPS-stimulated RAW 264.7 cells incubated in the presence of different combinations of essential oils. Nitric oxide was indirectly determined with the use of the Griess reagent. During the well-known Griess reaction, which is considered the standard method for the measurement of NO production, the NO stable end-products, nitrite and nitrate are measured colorimetrically. The autoxidation of NO or the acid-catalyzed formation of nitrous acid from nitrite generates dinitrogen trioxide (N_2O_3), which in turns reacts with sulfanilamide to produce a diazonium derivative, which is then coupled to N-(1-napthyl)ethylenediamine. This reaction produces a colored diazo product that can be measured at 540 nm [31].

We previously reported the biological potential of *L. austroapennina* [25] and *F. vulgare* subsp. *piperitum* [20] essential oils, which were proven to be very effective in inhibiting the nitric oxide production in LPS-stimulated murine macrophage cell line. In addition to verifying and confirming the biological activity of *F. vulgare* and *L. austroapennina* EOs, a very interesting activity was here observed for the first time the third species taken into account in this study, *O. heracleoticum*, for which an IC_{50} value of 182.0 ± 9.9 μg/mL was detected.

The eighteen designed experiments resulting from the application of a simplex-lattice mixture approach with three replicates, are reported in Table 1. The maximum concentration used in the experiments for each EO was chosen taking into account both their

Table 2
Chemical profile of investigated EOs.

Compound [a]	Rt [b]	RAP [c]		
		FV	LA	OH
Thuiene	6.345	0.10 ± 0.01	1.02 ± 0.10	1.44 ± 0.13
α-Pinene	6.580	1.82 ± 0.06	2.10 ± 0.12	1.00 ± 0.01
Camphene	6.814	0.13 ± 0.01	0.70 ± 0.05	–
1-Octen-3-ol	7.102	–	0.64 ± 0.04	–
3-Octanone	4.350	–	1.02 ± 0.01	–
Sabinene	7.482	0.54 ± 0.02	1.90 ± 0.18	4.04 ± 0.40
β-Pinene	7.558	0.30 ± 0.01	–	0.63 ± 0.01
Myrcene	7.700	0.43 ± 0.02	1.70 ± 0.20	2.10 ± 0.02
α-Phellandrene	8.015	1.73 ± 0.08	–	0.40 ± 0.01
α-Terpinene	8.118	–	0.74 ± 0.08	–
4-Carene	8.446	–	–	1.54 ± 0.04
Cymene	8.480	0.21 ± 0.01	–	–
Eucalyptol	8.624	0.10 ± 0.01	–	3.90 ± 0.30
β-Ocimene	8.730	–	0.83 ± 0.06	2.00 ± 0.05
Limonene	8.800	5.82 ± 0.30	–	0.41 ± 0.01
α-Ocimene	8.885	–	–	6.04 ± 0.41
γ-Terpinene	9.089	1.20 ± 0.01	1.73 ± 0.20	0.50 ± 0.01
Fenchone	9.400	17.84 ± 1.02	–	–
Linalool oxide	9.620	–	1.50 ± 0.09	–
Terpinolene	9.700	–	1.44 ± 0.10	–
Linalool	9.758	–	24.31 ± 1.60	10.62 ± 0.82
Camphor	9.980	1.52 ± 0.04	3.00 ± 0.12	–
Borneol	10.101	–	1.00 ± 0.01	–
Terpinen-4-ol	10.220	–	15.11 ± 0.98	–
α-Terpineol	10.870	–	0.94 ± 0.06	–
Terpinyl acetate	10.910	–	–	0.50 ± 0.02
Linalool propionate	11.103	–	–	7.21 ± 0.63
Estragole	11.300	32.22 ± 2.10	–	–
Linalyl acetate	12.012	–	12.30 ± 0.09	19.00 ± 1.02
Lavandulyl acetate	12.100	–	0.41 ± 0.01	–
Anethole	12.330	25.10 ± 1.15	–	–
Neryl acetate	12.880	–	0.93 ± 0.02	0.60 ± 0.01
Geranyl acetate	13.284	–	1.40 ± 0.05	0.84 ± 0.02
Copaene	13.340	–	–	0.22 ± 0.01
Bourbonene	13.427	–	0.20 ± 0.01	1.00 ± 0.01
β-Caryophyllene	13.570	–	4.04 ± 0.24	0.23 ± 0.02
α-Humulene	13.630	–	0.20 ± 0.02	–
Germacrene D	13.830	–	0.52 ± 0.03	–
β-Selinene	14.164	–	–	0.81 ± 0.05
Alloaromadendrene	14.231	–	–	0.40 ± 0.02
γ-Cadinene	14.350	–	1.30 ± 0.02	0.22 ± 0.01
β-Cubebene	14.440	–	–	0.20 ± 0.01
Bicyclosesquiphellandrene	14.480	0.11 ± 0.01	–	4.12 ± 0.22
β-Bisabolene	14.632	–	–	2.00 ± 0.12
Caryophyllene oxide	14.670	–	0.63 ± 0.03	–
Cadalene	15.120	–	0.21 ± 0.01	–

[a] Components are reported according to their elution order.
[b] Retention time (as min).
[c] Relative area percentage, peak area relative to total peak area in total ion current (TIC) %. Each value is the mean ± S.D. of three independent measurements. FV, *Foeniculum vulgare* subsp. *piperitum* (C.Presl) Bég; LA, *Lavandula austroapennina* N.G.Passal., Tundis & Upson; OH, *Origanum heracleoticum* L.

biological activity and cytotoxicity. Although a certain cytotoxicity was observed, these undesired effects were induced at the highest tested concentrations only, and they were not detectable at the lowest ones. For this reason, the maximum concentration was fixed at 500 μg/mL for *F. vulgare* (FV) species, and 250 μg/mL for the other two EOs (OH and LA), since higher concentrations were demonstrated to be toxic for treated cells. The inhibitory effects induced by these formulations are reported in Table 3. The highest inhibition percentages (>70 %) were observed for the mixtures containing 75 % v/v of *F. vulgare* (SLM2 and SLM3), the mixture half-half *F. vulgare* and *O. heracleoticum* (SLM6) and the pure *F. vulgare* or *L. austroapennina* EOs (SLM1 and SLM11). However, two of these formulations (SLM3 and SLM6) were cytotoxic, strongly affecting cell viability.

3.3. Data modelling and statistical validation

The experimental results (Table 3) were subjected to statistical analysis, the multiple linear regression procedure was applied to build the equations for the two dependent variables, the first to correlate the mixture of components with NO inhibition (NO) and the

Table 3
Inhibitory properties on NO production (%) exerted *by* EOs formulations and cell viability inhibition.

Experiment	% NO inhibition	% Cytotoxicity
SLM1	71.68 ± 1.86	8.6 ± 0.87
SLM2	78.58 ± 3.59	10.79 ± 5.23
SLM3	87.77 ± 2.15	82.36 ± 2.66
SLM4	54.80 ± 1.88	0
SLM5	59.49 ± 3.65	37.15 ± 1.25
SLM6	72.65 ± 2.21	80.92 ± 3.33
SLM7	55.49 ± 3.53	20.69 ± 3.65
SLM8	55.23 ± 3.11	5.71 ± 1.52
SLM9	30.77 ± 2.35	0
SLM10	40.98 ± 0.98	34.27 ± 3.62
SLM11	76.37 ± 0.66	15.71 ± 1.76
SLM12	23.69 ± 1.48	16.84 ± 2.19
SLM13	13.78 ± 1.28	15.32 ± 1.88
SLM14	34.88 ± 4.09	9.61 ± 0.87
SLM15	52.00 ± 0.75	25.82 ± 1.97
SLM16	39.27 ± 0.98	16.33 ± 0.74
SLM17	40.04 ± 1.11	16.44 ± 0.98
SLM18	40.18 ± 1.75	13.54 ± 1.55

Values are expressed as mean ± SD (n = 3).

second to predict and describe changes in cytotoxic activity (CYT). The data reported in Table 4 indicate that the regressions are significant, since the probability of the significance of the risk p-value is less than 0.05 for both models and the amounts of variation explained have satisfactory values with a coefficient of determination R^2 of 0.96, and 0.95 for the NO and CYT models, respectively. Considering the construction of cubic models for both NO and CYT variables, first order, quadratic, and cubic factors and regression coefficients were evaluated and listed in Table 5. A negative coefficient in the model indicates the ability of the associated factor to decrease the response, whereas a positive coefficient indicates the ability of a factor to increase the response variable. This study aimed to optimize a mixture of EOs that would at the same time be able to maximize the NO-inhibition effect without having cytotoxic activity. Thus, the most important factors for the NO response were those with a positive sign, whereas the most important factors for the CYT response had a negative sign.

With the aim of obtaining models that could best explain the responses investigated, a selection of terms for the construction of the equations was made by an automatic procedure using p-values, retaining only those terms with a p-value of less than 0.1 and necessary to generate a hierarchical model [32]. The representation of the variations in the responses considering the mixtures of the EOs and the calculated models is shown in a 3D-plot (Fig. 2). The shape of the surface plot at the interactions between the three components highlights the influence they have on the responses.

In the NO model, a significant synergistic effect was detected between the FV and OH components ($p_{AC} = 0.0389$ and $p_{AC(A-C)} = 0.0018$), and a lower synergistic effect between FV and LA ($p_{AB(A-B)} = 0.0424$), while the pair of LA and OH components decreased the effect on NO inhibition in their binary mixture. No significant ternary effect was observed. With regard to the CYT model, the FV-OH binary combination was found to have a highly enhanced cytotoxic effect with an intense synergistic effect ($p_{AC} = 0.0001$ and $p_{AC(A-C)} = 0.0004$), the FV-LA binary mixture showed no relevant enhancement in cytotoxic activity, while the LA-OH pair was found to have less cytotoxic effects than the single components. The reduction in cytotoxic activity was more pronounced in the ternary mixture with a $p_{(ABC)}$-value = 0.0014 and a negative regression coefficient of −647.33. According to the significance of each coefficient (Table 5), equations (1) and (2) are obtained for model NO and CYT, respectively:

Table 4
ANOVA for Reduced Cubic models, nitric oxide production inhibition and cytotoxicity.

Source	Sum of Squares	df	Mean Square	F-value	p-value
NO					
Model	6775.90	9	752.88	20.28	0.0001
Residual	297.06	8	37.13		
Cor Total	7072.95	17			
R^2	0.958				
Adjusted R^2	0.921				
CYT					
Model	9033.63	9	1003.74	19.88	0.0001
Residual	403.82	8	50.48		
Pure Error	5.40	2	2.70		
Cor Total	9437.45	17			
R^2	0.947				
Adjusted R^2	0.910				

Table 5
Regression coefficients and statistical significance as p-value calculated for NO and CYT models.

Model	NO		CYT	
	Coefficient Estimate	*p-value*	*Coefficient Estimate*	*p-value*
FV (A)	72.46	<0.0001[a]	8,01	0.0004[a]
LA (B)	76.11		16.83	
OH (C)	52.59		25.82	
AB	−53.79	0.0256	−10.47	0.5950
AC	20.00	0.0389	237.13	<0.0001
BC	−191.89	<0.0001	−38.73	0.2099
ABC	−12.77	0.9226	−647.33	0.0014
AB(A-B)	107.97	0.0424	−13.39	0.8185
AC(A-C)	200.53	0.0018	334.15	0.0004
BC(B–C)	−93.55	0.0489	76.39	0.2132

[a] For linear mixtures were used the sums of squares.

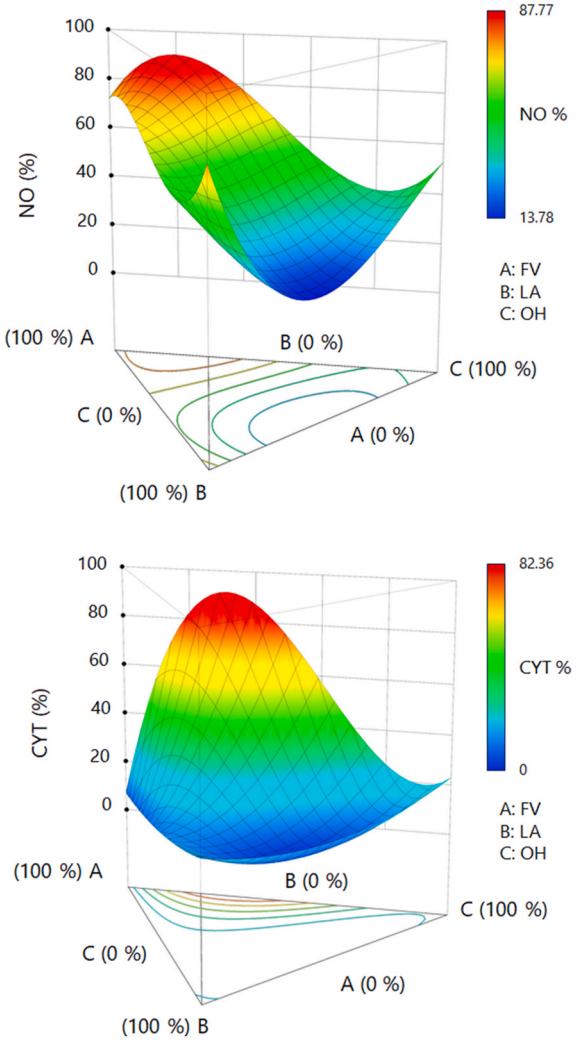

Fig. 2. 3-D surface plot for NO inhibition (NO) and cytotoxic activity (CYT) models and effect of mixture components on the responses.

$$NO = 72.60FV + 76.25LA + 52.73OH - 55.14FVLA + 18.65FVOH - 193.23LAOH + 107.97FVLA(FV\text{-}LA) + 200.53FVOH(FV\text{-}OH) - 93.55LAOH(LA\text{-}OH) \quad (1)$$

$$CYT = 7.65FV + 20.03LA + 22.97OH - 16.16FVLA + 243.55FVOH - 39.45LAOH - 647.33FVLAOH + 330.83FVOH(FV\text{-}OH) \quad (2)$$

3.4. Optimization of the EOs formulation

The aim of our research was to obtain a formulation with high biological potential starting from the combination of three EOs with demonstrated ability to inhibit the nitric oxide production in LPS-stimulated RAW 264.7 macrophages. Obtained results showed that both models were statistically significant and could be used to predict the two following optimization conditions: maximum inhibitory activity on NO production, and minimum cytotoxicity. The two criteria could be selected independently or simultaneously (Table 6), in the former case the calculated models are used individually. If an attempt were made to maximize the effect of NO inhibition without any constraint for the CYT model, the optimized mixture would give a prediction with a predicted NO inhibition potency (90.86 %) well above all those obtained with the experiments carried out in the experimental domain of the simplex design, but on the other hand the cytotoxicity (81.28 %) also reaches very high levels that do not allow this possibility to be considered valid. If cytotoxicity were to be given priority, a ternary mixture could be found that would ensure cell viability (CYT predicted = 3.31 %) but a very low NO inhibition effect (NO predicted = 22.61 %). Finally, the dual condition (NO max and CYT min) was considered simultaneously (Fig. 3). multivariate model successfully predicted the optimal mixture of EOs, and the statistical outcome's efficacy was experimentally confirmed. Based on our investigations, the optimized blend with the highest inhibitory effect and minimal cytotoxicity consisted of 87.7 % FV, 12.3 % LA, and 0 % OH (Table 6). A good correlation between the experimental and predicted values was observed. As reported in Table 6, the mixture was able to effectively inhibit the production of nitric oxide, with an inhibition percentage equal to 76.25 ± 3.88 %, with a low cytotoxic effect (4.55 ± 1.55 %) on treated cells [33]. For completeness, in order to provide a comprehensive understanding of the optimization process, the other two hypotheses were also verified (Table 6).

4. Discussion

The investigated EOs were obtained through steam distillation from fresh plants materials, with the exception of lavender sample, which was kindly provided by 'Parco della Lavanda' (Morano Calabro, Cosenza, Italy). The three aromatic species were collected in Southern Italy. *Lavandula austroapennina*, in particular, is native to Southern and Central Italy [34] and it is typical of the Pollino National Park, the largest Italian protected area, where these lavender populations are locally named "loricanda lavender" [25]. *Lavandula* genus comprises about 40 species, with *L. angustifolia* Mill, the so-called 'true' lavender, being the most commonly used. These plants are largely used in aromatherapy, cosmetics and perfumery, but also have interesting medicinal properties, such as anti-inflammatory, antimicrobial and anxiolytic effects [35]. The topical administration of *L. angustifolia* EO was demonstrated to exert a strong anti-inflammatory activity which was in part related to the involvement of nitric oxide (NO) and pro-inflammatory cytokines [36]. We also previously reported the inhibitory properties of *L. austroapennina* on nitric oxide production [25]. An in vivo anti-inflammatory potential was also reported for *F. vulgare* subsp. *piperitum* extracts and essential oil [37–39]. Fennel (*Foeniculum vulgare* Mill., Apiaceae) is a species native to theMediterranean areal and it is widely cultivated because its fruits are used as a culinary spice, being considered one of the most widespread aromatic plants [40,41]. The third plant species taken into account in this study, *Origanum heracleoticum* L., is a synonym of *Origanum vulgare* subsp. *viridulum* (Martrin-Donos) Nyman [42]. *Origanum* genus comprises the main aromatic species from the Lamiaceae family and *O. vulgare* L. and its subspecies are spread in the Mediterranean and Western Eurasia regions [43,44]. As well as plants previously discussed, some *Origanum* species also demonstrated anti-inflammatory properties. *O. ehrenbergii* essential oil was demonstrated to be effective in inhibiting the NO production in RAW 264.7 cells. Moreover, *O. vulgare* EO was able to decrease the pro-inflammatory TNF-a, IL-1b and IL-6 cytokines synthesis, and to increase the IL-10 production [45]. Our investigation demonstrated that different combinations of three studied EOs extracts can contribute differently to biological activity taken into account. The use of the experimental design method allowed us to understand which the best mixtures of the extracts are to obtain a very powerful synergistic NO inhibition effect while avoiding the concomitant cytotoxic effect. By simultaneously evaluating the two desired effects, maximum NO inhibition and minimum cytotoxicity, it was possible to optimize a mixture with good NO inhibition and minimal cytotoxicity. In conclusion, our findings demonstrated that the combination of investigated essential oils synergistically inhibited the LPS-induced NO production in murine macrophages, thus providing new insights regarding the synergisms between these different botanicals.

5. Conclusion

The application of multivariate experimental design allowed the optimization of a mixture of essential oils from three different medicinal plants. The extracts of *Foeniculum vulgare* subsp. *piperitum* (C.Presl) Bég. (FV), *Origanum heracleoticum* L. (OH) and *Lavandula austroapennina* N.G.Passal., Tundis & Upson. (LA) were mixed with the aim of exploting the synergistic effect of their metabolites with a double result, to find a mixture that had inhibitory properties for NO production without having the adverse effect of cytotoxicity. The use of a simplex lattice design allowed the calculation of two statistically significant equations that predicted an optimal mixture with high NO inhibition and minimum cytotoxicity. The results of our research represent a promising starting point for the design of new formulations with anti-inflammatory effects by using aromatic plants.

Data availability

No data associated with this study has been deposited into a publicly available repository. Data will be made available upon request to the Corresponding Author.

Table 6
EOs predicted optimized formulation using the Simplex Lattice Design method and validation test results.

Criteria selection	EO (%) v/v			Prediction		Validation	
	FV	LA	OH	NO (%)	CYT (%)	NO (%)[a]	CYT (%)[a]
NO max	80.1	0.0	19.9	90.86	81.28	91.40 ± 4.02	80.32 ± 2.50
CYT min	14.7	45.6	38.7	22.61	3.31	21.78 ± 1.02	2.40 ± 0.38
NO max and CYT min	**87.7**	**12.3**	**0.0**	**75.89**	**7.43**	**76.25 ± 3.88**	**4.55 ± 1.55**

FV, *Foeniculum vulgare* subsp. *piperitum* (C.Presl) Bég.; LA, *Lavandula austroapennina* N.G.Passal., Tundis & Upson; OH, *Origanum heracleoticum. L.*; NO, nitric oxide inhibition; CYT, cytotoxic activity.
[a] Values are expressed as mean ± SD (n = 3).

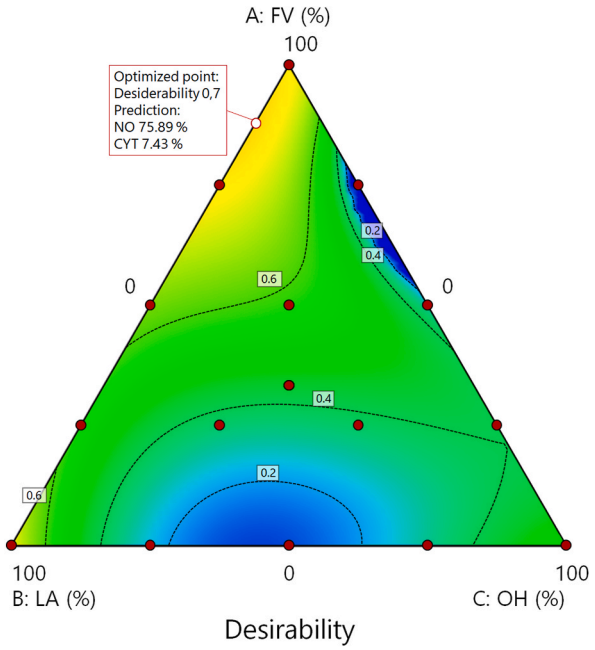

Fig. 3. Optimal design regions using desirability function and the best combination prediction.

CRediT authorship contribution statement

Mariangela Marrelli: Writing – original draft, Methodology, Data curation, Conceptualization, Investigation. **Michele De Luca:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Claudia-Crina Toma:** Writing – original draft, Investigation. **Fedora Grande:** Writing – original draft, Investigation. **Maria Antonietta Occhiuzzi:** Writing – original draft, Investigation. **Rosalba Caruso:** Writing – original draft, Investigation. **Filomena Conforti:** Writing – review & editing, Writing – original draft, Investigation. **Giancarlo Statti:** Writing – review & editing, Writing – original draft, Supervision, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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