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Herbicidal Potential of Combined Essential Oil Nanoemulsions against *Megathyrsus maximus* (Jacq.) B.K. Simon & S.W.L. Jacobs and *Lactuca sativa* L.

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HIGHLIGHTS

- Formulations of combined essential oil nanoemulsions exhibit significant herbicidal activity.
- *In vitro* assays confirm strong inhibitory effects on seed germination and seedling growth.
- A specific three-oil mixture demonstrated superior control of *Megathyrsus maximus* germination.

Abstract: The development of sustainable alternatives to synthetic herbicides has become increasingly important due to environmental and health concerns. This study assessed the herbicidal activity of nanoemulsion (NE) mixtures, emphasizing the combination of distinct essential-oil nanoemulsions under constant surfactant load and multivariate statistical assessment of interaction effects. Nanoemulsions were prepared from seven commercial essential oils (EOs): *Eucalyptus globulus, Melaleuca alternifolia, Mentha piperita, Cymbopogon winterianus, Cymbopogon flexuosus, Zingiber officinale*, and *Eucalyptus staigeriana.* The experiments were conducted in two stages to assess effects on *Megathyrsus maximus* and *Lactuca sativa*. A structured experimental approach combining Plackett–Burman screening and full factorial optimization was employed to detect and quantify additive interaction effects under controlled formulation. The results for the first stage, all seven NEs were screened using a Plackett–Burman design at 0.03% (w/v), and four formulations were excluded based on lower phytotoxic performance. In the second stage, the three most active NEs were evaluated and optimized using a 3^3 full factorial design, individually and in combination at 0.02% and 0.04% with the statistical evaluation of the interaction effects. Germination percentage, germination speed index, shoot length, root length and dry mass were analyzed. The NEs of *C. flexuosus, E. globulus, and E. staigeriana* showed the most pronounced herbicidal effects. The *C. flexuosus* NE inhibited germination and early growth, but its combination with the other two oils produced stronger inhibition, suggesting an additive interaction, with combined formulations outperforming individual treatments under identical surfactant conditions. These findings indicate that combined NEs are a viable and environmentally sound option for pre-emergent weed control. This work contributes to the area of agricultural technologies, biotechnology and environment by developing and optimizing an essential-oil nanoemulsion-based pre-emergent bioherbicide as a sustainable alternative.

Keywords: essential oils; nanoemulsion; weed control; *Megathyrsus maximus*; bioherbicides; factorial design.

INTRODUCTION

Weeds are a major obstacle in agricultural systems, as they compete with crops for essential resources such as light, water, and nutrients, and frequently act as reservoirs for pests and pathogens. Merfield [1] defined a weed as “a plant, or a population of plants, at a specific time and place, causing significant damage, either immediately or in the long term, based on a holistic analysis of its positive and negative attributes.” Global annual losses linked to weed infestations are estimated at approximately USD 95 billion [2]. In many regions, particularly in developing countries, weed control strategies still rely heavily on synthetic herbicides [3].

One of the most invasive and economically important species is *Megathyrsus maximus* (guinea grass, family Poaceae). Native to Africa, this perennial grass is characterized by rapid growth, broad ecological tolerance, and strong colonization capacity. Although it serves as an important forage species, it frequently invades agricultural areas and ecological restoration sites and is considered one of the most troublesome weeds in sugarcane cultivation [4–6].

Brazil is among the world’s largest pesticide consumers, applying over 800,000 tons of active substances, both chemical and biochemical, in 2022 [7]. Glyphosate and its salts dominate the herbicide market, acting as nonselective, systemic agents that inhibit aromatic amino acid biosynthesis by blocking specific enzymatic pathways [8]. While its effectiveness is well established, extensive use raises concerns about environmental persistence, toxicological risks, and potential carcinogenicity. Some countries have restricted or banned glyphosate; in Brazil, however, it remains widely used and is officially classified as having a “low probability of causing acute harm” [9]. Experimental evidence also demonstrates phytotoxic and cytogenotoxic effects on nontarget plants, including *Lactuca sativa* [8]. The known sensitivity of *L. sativa* to various phytotoxins, including monoterpenes like citral and eucalyptol which are abundant in the EOs used in this study, reinforces its suitability as an indicator species for assessing the herbicidal potential of these natural compounds [3].

The widespread dependence on chemical herbicides, coupled with the emergence of resistant weed biotypes and the risk of soil, water, and air contamination, highlights the urgency of developing environmentally sound and effective control methods. Bioherbicides, derived from plants, microorganisms, or insects, represent a promising alternative, as they offer biodegradability, potential selectivity, and reduced ecological impact [10,11].

Essential oils (EOs), complex mixtures of volatile secondary metabolites such as monoterpenes, sesquiterpenes, and phenylpropanoids [12,13], are recognized for their ability to interfere with seed germination and seedling development [14-16]. These natural compounds have attracted increasing attention as potential substitutes for synthetic herbicides. However, their practical application is limited by low aqueous solubility, high volatility, and chemical instability. To overcome these limitations, nanoemulsions (NEs) have been proposed as suitable formulations. NEs are colloidal systems stabilized by emulsifiers, with droplet sizes typically between 20 and 200 nm [17]. Such systems improve solubility, stability, dispersion, surface wetting, and controlled release of active constituents, thereby enhancing biological performance while minimizing volatilization and degradation at the target site [18,19].

The combination of different essential oils can also be an effective strategy to achieve stronger biological effects. Although research in this area remains limited, available studies indicate that combinations may produce additive or synergistic interactions, enhancing phytotoxic, antibacterial, and antifungal activities [20-22]. Most previous investigations have focused on mixtures of free essential oils, whereas considerably fewer studies have evaluated mixtures of pre-formulated nanoemulsions, in which physicochemical stability, droplet size distribution, and surfactant load may influence biological outcomes. Moreover, the statistical detection of interaction effects in structured nanoemulsion systems remains underexplored. This knowledge gap reinforces the need to investigate more effective and sustainable alternatives for weed management.

In this context, experimental design is a key methodological tool for evaluating bioherbicidal activity. Because biological systems involve multiple factors and interactions, traditional one-factor-at-a-time approaches are inefficient and often fail to detect synergistic or antagonistic effects. Designs such as Plackett - Burman and full factorial models allow for the simultaneous assessment of multiple variables while reducing the total number of experiments. This strategy increases statistical robustness, enhances reproducibility, and provides more reliable insights into the complex interactions among treatments. For these reasons, the use of structured experimental design is not only methodological but also strategic in studies with natural product formulations. When applied to nanoemulsion systems under constant surfactant load, such structured designs allow clearer attribution of biological effects to the active components rather than formulation artifacts. [23-25].

Therefore, the development of nanoemulsion-based bioherbicides fits within the scope of agricultural technologies, biotechnology and environment, aimed at sustainable weed management. The novelty of the present study lies not in the isolated evaluation of essential oils, but in the systematic screening and statistical optimization of combinations of distinct nanoemulsions under controlled formulation conditions. In this study, we propose and optimize nanoemulsion formulations as a pre-emergent bioherbicide targeting *Megathyrsus maximus*. The phytotoxic and herbicidal effects of NE formulations containing seven commercial EOs - *Eucalyptus globulus* Labill., *Melaleuca alternifolia* (Maiden & Betche) Cheel, *Mentha piperita* L., *Cymbopogon winterianus* Jowitt ex Bor, *Cymbopogon flexuosus* (Nees) W. Watson, *Zingiber officinale* Roscoe, and *Eucalyptus staigeriana* F. Muell. ex Bailey - were evaluated on *L. sativa* and *M. maximus*.

MATERIAL AND METHODS

Plant Material

Seeds of *Megathyrsus maximus* (guinea grass) were supplied by Sementes Plante Forte Ltda. (Auriflama, São Paulo, Brazil; Batch 05/2023; Harvest 2023; Exp. 10/2024). Seeds of *Lactuca sativa* 'Mimosa' were purchased from ISLA Sementes Ltda. (Porto Alegre, Brazil; Batch 156225-000S2; Exp. 08/2024). All seeds were stored in sealed containers under cool, dry conditions until use.

Essential Oils

Seven commercially sourced essential oils (EOs) were evaluated: *Eucalyptus globulus*, *Melaleuca alternifolia*, *Mentha piperita*, *Cymbopogon winterianus*, *Cymbopogon flexuosus*, *Zingiber officinale*, and *Eucalyptus staigeriana*. All EOs were obtained from Ferquima Indústria e Comércio Ltda. (Vargem Grande Paulista, São Paulo, Brazil) and stored in amber vials at 4 °C to minimize oxidation and volatilization.

The chemical characterization of the essential oils was performed using an Agilent Gas Chromatograph coupled to a Mass Spectrometer (7890 B) and a mass detector model 5977A MSD with an electron impact of 70 eV. An HP-5 column measuring 30 m x 250 μm x 0.25 μm was used for the separation process. The injector and the detector were set at 290°C and 310°C, respectively. The elution system operated with a heating ramp starting at 40°C with a heating rate of 5°C/min up to 280°C, followed by a rate of 15°C/min up to 310°C. For the characterization of the chemical compounds, an alkane standard from C10 to C40 was used to calculate the Kovats index (KI). The substances were identified by comparing the mass spectra from the NIST library with the retention indices and similarities of the mass spectra to data from the literature [26].

Nanoemulsion preparation and characterization

Nanoemulsions (NEs) were prepared as nanostructured colloidal systems (NCSs) via the low-energy phase inversion method, employing a Vortex mixer (Mod. VX-38) [17]. The oil phase (OP) consisted of each EO at a fixed concentration of 5% (w/w), while the emulsifier content ranged from 5% to 20% (w/w) relative to the OP. The aqueous phase (AQ) was added to complete the formulation to 100% (w/w). The general formula was:

$$\%\ \text{total (2g final mass)} = 5\%\ \text{EO} + \text{X\% emulsifiers} + \text{AQ}\ (95\% - \text{X\% emulsifiers}),$$

where X = 20%, 15%, 10%, and 5%.

Emulsifiers were selected and combined based on their hydrophilic–lipophilic balance (HLB) values: polysorbate 20 (HLB 16.7), polysorbate 80 (HLB 15), sorbitan monooleate (HLB 4.3), and sorbitan trioleate (HLB 1.8). The target HLB (HLB_f) for binary mixtures was calculated as:

$$HLBf = (HLBa\,.ma) + (HLBb\,.mb)\,/\,(ma + mb).$$

Where HLB*a* and HLB*b* are the HLB values of individual emulsifiers, and *ma* and *mb* are their respective masses.

Physical stability was assessed visually; and formulations exhibiting bluish opalescence (Tyndall effect) without phase separation or creaming were selected for further testing. Stable NEs were diluted to EO concentrations of 2.5%, 1.0%, and 0.5% (w/w, relative to formulation mass), and droplet size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Litesizer 500 (Anton Paar, Austria). Measurements were performed in disposable cuvettes at 25 °C, with the instrument's refractive index and viscosity parameters set according to water at 25 °C. Each sample was analyzed in triplicate (n = 3), and results are presented as the z-average diameter (nm) and PDI. The stability of mixed NEs during Stage 1 and Stage 2 was monitored visually throughout the bioassay period to detect potential phase separation or coalescence. No macroscopic instability was observed under the experimental conditions.

Experimental design

Stage 1 - Screening of nanoemulsions (Plackett - Burman)

A Plackett - Burman (PB) design with 8 runs was used to screen the bioherbicidal potential of seven essential-oil nanoemulsions (factors: essential oils) at two levels (present/absent). For each run, the NEs indicated as "present" were combined to reach a fixed total EO concentration of 0.03% (w/v in the final Petri dish volume). When multiple NEs were present, their contributions were equally apportioned. A control emulsion (emulsifier system without EO) was added to ensure that the final surfactant concentration and total application volume (3 mL per Petri dish) were identical across all treatments and controls. (Table 1). [23,24]

All screening mixtures were prepared at a fixed total application volume per Petri dish (3 mL). The final total essential-oil concentration in the dish was kept constant at 0.03% (w/v); when a run contained more than one NE, the 0.03% was equally apportioned among the present NEs, and blank emulsion (surfactant without EO) was used to keep the surfactant content identical across treatments.

Table 1. Plackett - Burman design (Stage 1) for screening essential oil nanoemulsions (0.03%) against *Lactuca sativa* and *Megathyrsus maximus*.

Tests	Nanoemulsion of essential oils (0.03%*)						
	EG	*MA*	*MP*	*CW*	*CF*	*ZO*	*ES*
1	X			X		X	X
2	X	X			X		X
3	X	X	X			X	
4		X	X	X			X
5	X		X	X	X		
6		X		X	X	X	
7			X	-	X	X	X
8	Control						

EG: *Eucalyptus globulus; MA: Melaleuca alternifolia; MP: Menta piperita; CW: Cymbopogon winterianus; CF: Cymbopogon flexuosus; ZO: Zingiber officinalis; ES: Eucalyptus staigeriana.*

* In all tests, the total EO in the dish was fixed at 0.03% (w/v). When multiple NEs were present, equal volumes of each NE were combined to reach the target, complementing with blank emulsion to maintain a constant surfactant concentration. "X" represents the presence of a given NE (+1 level), whereas empty cells correspond to its absence (−1 level)

Stage 2 – Factorial Optimization

Based on Stage 1, three NEs showing the strongest phytotoxic responses were selected for further evaluation using a 3-factor, 3-level full factorial (3^3 = 27 treatments), with factor levels defined as EO concentration of 0.00%, 0.02%, and 0.04%, in the dish, for each NE. This structure enables estimation of main effects and two- and three-way interactions. The selected concentrations (0.02% and 0.04%) were chosen based on the effective range observed during screening and to enable evaluation of concentration-dependent responses within a low-dose interval relevant to pre-emergent laboratory assays.

Bioassays

Seeds were surface-sterilized with 2% sodium hypochlorite for 15 min, rinsed thoroughly with sterile distilled water, and placed in 7 cm Petri dishes lined with double-layer filter paper. For each replicate, 20 seeds were treated with 3 mL of either the NE formulation or emulsifier solution (negative control) at the same surfactant concentration. Plates were incubated in germination chambers (BOD) under species-specific conditions: *L. sativa* at 20 °C and *M. maximus* under alternating 35–15 °C, both with a 12 h photoperiod [27,28]. Germination (radicle ≥ 1 mm) was recorded daily for 7 days (*L. sativa*) or 28 days (*M. maximus*) [29].

The following parameters were measured:

- Germination percentage (%G) = (N/A) × 100, where N = germinated seeds and A = total seeds [30].
- Germination speed Index (GSI) = Σ (Gi / Ni), where Gi = seeds germinated at the i-th count; Ni = days elapsed since sowing [31].
- Shoot length (SL) and radicle length (RL) measured with a digital caliper (cm/seedling) [32].
- Dry mass (DM) of shoots and radicles, after drying at 60 °C for 72 h (mg/seedling) [33].

RESULTS

In the GC/MS analysis, the retention time of each peak was observed in the chromatograms, indicating the substances present in the essential oils and the relative area of each peak (Table S1). GC/MS analysis revealed distinct chemical profiles for the essential oils evaluated. *Eucalyptus globulus* was dominated by 1,8-cineole (RT 10.726 min; ~90.4%), with minor contributions of *α*-pinene and *o*-cymene. *Melaleuca alternifolia* showed terpinen-4-ol (RT 17.289 min; ~36.7%) as the major constituent, followed by *o*-cymene and *α*-terpineol. *Mentha piperita* was characterized by high levels of isomenthol (RT 17.198 min; ~42.1%) and menthone (RT 16.139 min; ~25.3%). *Cymbopogon winterianus* exhibited citronellal (RT 16.225 min; ~28.6%), geraniol (RT 20.774 min; ~18.3%), and citronellol (RT 19.521 min; ~14.5%) as major components. *Cymbopogon flexuosus* was rich in citral, represented by geranial (RT 21.592 min; ~38.3%) and neral (RT 20.150 min; ~30.8%). *Zingiber officinale* presented a sesquiterpene-rich profile, dominated by *ar*-curcumene (RT 30.284 min; ~59.6%) and *α*-zingiberene (RT 30.730 min; ~35.4%). Finally, *Eucalyptus staigeriana* showed geranial (RT 21.592 min; ~9.4%), neral (RT 20.150 min; ~6.8%), methyl geranate, and geranyl acetate as its main constituents.

The nanoemulsions selected as the most stable are summarized in Table 2 and Figure S1. For each essential oil, we report the concentration associated with the highest physical stability, the emulsifiers used, the mean droplet size (DLS; hydrodynamic diameter), and the polydispersity index (PDI)

Table 2. Dynamic light scattering (DLS) characterization of essential oil nanoemulsions (NEs)

NEs	Essential oil (0.5% w/w)	Emulsifiers used	Particle size (nm)	Polydispersity index (PdI)
1	*Eucalyptus globulus*	polysorbate 20 + sorbitan trioleate	27.30	0.277
2	*Melaleuca alternifolia*	polysorbate 20 + sorbitan monooleate	63.99	0.081
3	*Mentha piperita*	polysorbate 20 + sorbitan monooleate	64.46	0.095
4	*Cymbopogon winterianus*	polysorbate 20	78.18	0.257
5	*Cymbopogon flexuosus*	polysorbate 20	95.90	0.110
6	*Zingiber officinale*	polysorbate 80	60.22	0.255
7	*Eucalyptus staigeriana*	polysorbate 20 + sorbitan trioleate	27.77	0.242

The smallest droplets were observed for *E. globulus* and *E. staigeriana* (~27 - 28 nm) with polysorbate 20 + sorbitan trioleate, whereas *C. flexuosus* displayed the largest droplet size (~96 nm) with polysorbate 20. The lowest PDIs (≤0.10) were obtained for *M. alternifolia* and *M. piperita* (~64 nm), indicating narrower size distributions. All nanoemulsion systems exhibited PDI < 0.30, consistent with stable nanoemulsions suitable for subsequent bioassays.

Plackett - Burman screening ANOVA using pure error (df = 32; within-replicate variability used as the denominator of the F test) showed that the nanoemulsions significantly ($p < 0.05$) affected germination percentage (%G), shoot length (SL) and root length (RL) of *Lactuca sativa* (Table 3). The largest F-ratios were observed for *C. winterianus* (CW), *C. flexuosus* (CF) and *E. staigeriana* (ES), indicating that these factors accounted for the greatest share of response variation; the Pareto plots (Figure S2A - C) reproduce the same ranking.

In *Megathyrsus maximus* (Table 4), CF and ES produced the strongest effects on %G and RL and remained significant for SL, consistent with the corresponding Pareto plots (Figure S2 D - F). The estimated main-effect coefficients for CF and ES were negative, meaning that their presence reduced %G, SL and RL.

Table 3. Results of the analysis of variance for Experimental Design 1, according to the Plackett - Burman method, for the seven nanoemulsions tested at a concentration of 0.03% in *Lactuca sativa*.

Treatments		Germination percentage (%G)				Shoot length (SL)				Root length (RL)			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
EG	1	525.63	525.63	8.2049	0.007319	0.48620	0.486203	59.0052	0.000000	3.66025	3.66025	62.2968	0.000000
MA	1	525.63	525.63	8.2049	0.007319	0.48620	0.486203	59.0052	0.000000	3.66025	3.66025	62.2968	0.000000
MP	1	525.63	525.63	8.2049	0.007319	0.48620	0.486203	59.0052	0.000000	3.66025	3.66025	62.2968	0.000000
CW	1	15405.63	15405.63	240.4780	0.000000	3.96270	3.962703	480.9105	0.000000	13.24801	13.24801	225.4789	0.000000
CF	1	15405.63	15405.63	240.4780	0.000000	3.96270	3.962703	480.9105	0.000000	13.24801	13.24801	225.4789	0.000000
ZO	1	525.63	525.63	8.2049	0.007319	0.48620	0.486203	59.0052	0.000000	3.66025	3.66025	62.2968	0.000000
ES	1	15405.63	15405.63	240.4780	0.000000	3.96270	3.962703	480.9105	0.000000	13.24801	13.24801	225.4789	0.000000
Pure Error	32	2050.00	64.06			0.26368	0.008240			1.88016	0.05876		
Total SS	39	50369.38				14.09660				56.26519			

EG: *Eucalyptus globulus*; MA: *Melaleuca arternifolia*; MP: *Menta piperita*; CW: *Cymbopogon winterianus*; CF: *Cymbopogon flexuosus*; ZO: *Zingiber officinale*; ES: *Eucalyptus staigeriana*. df, degrees of freedom; SS, sum of squares; MS, mean square; F, F statistic; p, p-value. Note on F-ratios and p-values: The identical F-ratios and p-values observed for some treatments are a consequence of the orthogonal nature of the saturated Plackett-Burman design, where each factor has the same pattern of level combinations relative to the others. This is an expected statistical outcome and not an error

Table 4. Results of the analysis of variance for Experimental Design 1, according to the Plackett - Burman method, for the seven nanoemulsions tested at a concentration of 0.03% in *Megathyrsus maximus*.

Treatments		Germination percentage (%G)				Shoot length (SL)				Root length (RL)			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
EG	1	2402.50	2402.50	71.1852	0.000000	6.13872	6.13872	6.06586	0.019348	27.95584	27.95584	15.11086	0.000480
MA	1	1102.50	1102.50	32.6667	0.000002	3.18660	3.18660	3.14878	0.085499	0.00001	0.00001	0.00001	0.998159
MP	1	1822.50	1822.50	54.0000	0.000000	1.12560	1.12560	1.11224	0.299493	17.45041	17.45041	9.43240	0.004327
CW	1	2402.50	2402.50	71.1852	0.000000	1.13232	1.13232	1.11888	0.298079	16.92601	16.92601	9.14895	0.004876
CF	1	3802.50	3802.50	112.6667	0.000000	13.32870	13.32870	13.17049	0.000980	50.71504	50.71504	27.41279	0.000010
ZO	1	2102.50	2102.50	62.2963	0.000000	0.64262	0.64262	0.63499	0.431402	8.87364	8.87364	4.79643	0.035922
ES	1	2722.50	2722.50	80.6667	0.000000	7.50822	7.50822	7.41910	0.010366	41.24961	41.24961	22.29648	0.000045
Pure Error	32	1080.00	33.75			32.38440	1.01201			59.20160	1.85005		
Total SS	39	17437.50				65.44720				222.372216			

EG: *Eucalyptus globulus*; MA: *Melaleuca arternifolia*; MP: *Menta piperita*; CW: *Cymbopogon winterianus*; CF: *Cymbopogon flexuosus*; ZO: *Zingiber officinale*; ES: *Eucalyptus staigeriana*. df, degrees of freedom; SS, sum of squares; MS, mean square; F, F statistic; p, p-value. The identical F-ratios and p-values observed for some treatments are a consequence of the orthogonal nature of the saturated Plackett-Burman design, where each factor has the same pattern of level combinations relative to the others. This is an expected statistical outcome and not an error.

The Plackett - Burman screening at 0.03% (w/v) identified three influential nanoemulsions - EG (*Eucalyptus globulus*), CF (*Cymbopogon flexuosus*), and ES (*Eucalyptus staigeriana*). Accordingly, Experimental Design 2 was a full three-level factorial (3^3) used to resolve dose–response and mixtures. Each factor was tested at 0.00, 0.02, and 0.04% (w/v), yielding 27 runs with a fixed application volume and constant surfactant burden (blank emulsion when a factor = 0) (Table S2). The linear term (L) measures the average change in the endpoint as concentration increases across levels. The quadratic term (Q) captures curvature, i.e., whether the mid dose departs from the mean of the extremes. First-order interactions (EG×CF, EG×ES, CF×ES) quantify non-additivity between nanoemulsions. Endpoints were germination percentage (%G), shoot length (SL), and root length (RL). This structure separates main concentration effects from curvature and interaction effects (Figure S3).

ANOVA terms in Tables 5 - 6 are standard: df (degrees of freedom) represents the information supporting each effect (L+Q for a factor gives df = 2; a two-factor interaction has df = 4). SS (sum of squares) is th variation explained; MS (mean square) = SS/df. F compares the MS of an effect to the residual MS; larger F means a stronger effect relative to noise. The p-values is the probability of such an F if the true effect were zero (significant if $p<0.05$). Pure Error is replicate variability; Lack of Fit tests whether the model (L, Q, and first-order interactions) fits the data. In *Lactuca sativa* (Table 5), CF(L+Q) and ES(L+Q) dominate the variation in %G, with EG(L+Q) smaller; CF×ES and EG×ES are significant. Lack of fit is non-significant for %G (adequate) but significant for SL.

Table 5. Analysis of variance results for the 3^3 factorial design evaluating the three nanoemulsions and their combinations tested at concentrations of 0.04% and 0.02% in *Lactuca sativa*.

Treatments	Germination percentage (%G)					Shoot length (SL)				
	df	SS	MS	F	p	df	SS	MS	F	p
EG (L+Q)	2	3934.4	1967.22	7.8923	0.000632	2	0.70401	0.352003	3.706443	0.028992
CF (L+Q)	2	74013.3	37006.67	148.4666	0.000000	2	0.86872	0.434361	4.573639	0.013237
ES (L+Q)	2	26914.4	13457.22	53.9889	0.000000	2	1.50010	0.750052	7.897732	0.000753
EG+CF	4	1148.9	287.22	1.1523	0.336073	3	0.62204	0.207348	2.183288	0.096648
EG+ES	4	1527.8	381.94	1.5323	0.197930	4	1.02585	0.256462	2.700439	0.036514
CF+ES	4	9212.2	2303.06	9.2396	0.000002	4	1.25508	0.313769	3.303856	0.014855
Lack of Fit	8	3288.9	411.11	1.6493	0.119330	6	1.48123	0.246871	2.599449	0.023839
Pure Error	108	26920.0	249.26			78	7.40771	0.094971		
Total SS	134	146960.0				101	19.46624			

EG: *Eucalyptus globulus*; CF: *Cymbopogon flexuosus*; ES: *Eucalyptus staigeriana*; L: linear term; Q: quadratic term. df, degrees of freedom; SS, sum of squares; MS, mean square; F, F statistic; p, p-value.

In *Megathyrsus maximus* (Table 6), EG(L+Q), CF(L+Q), and ES(L+Q) are all highly significant for %G, and all first-order interactions are significant, evidencing mixture effects. Lack of fit is significant, implying additional structure beyond the quadratic form; inspection of the response-surface is therefore informative. Pareto C ranks CF(L) and ES(L) as dominant, with several interactions above the significance threshold. Model coefficients show negative main effects for CF and ES, meaning higher concentrations reduce %G (and, in *L. sativa*, SL). Biologically, these reductions indicate herbicidal activity within the range 0.00 - 0.04% (w/v), both individually and in combination. Quadratic terms confirm curvature, so 0.02% is not merely the average of 0 and 0.04%. Overall, the 3^3 design confirms CF and ES as principal inhibitors and quantifies their additive behavior with EG for optimization.

Table 6. Analysis of variance results for Experimental Design 2, based on the full three-level (3^3) factorial, for the three nanoemulsions and their combinations tested at concentrations of 0.04% and 0.02% in *Megathyrsus maximus*.

Treatments	Germination percentage (%G)				
	df	SS	MS	F	p
EG (L+Q)	2	5295.93	2647.963	77.9237	0.000000
CF (L+Q)	2	9507.04	4753.519	139.8856	0.000000
ES (L+Q)	2	4410.37	2205.185	64.8937	0.000000
EG+CF	4	690.74	172.685	5.0817	0.000863
EG+ES	4	1320.74	330.185	9.7166	0.000001
CF+ES	4	5046.30	1261.574	37.1253	0.000000
Lack of Fit	8	2289.26	286.157	8.4210	0.000000
Pure Error	108	3670.00	33.981		
Total SS	134	32230.37			

EG: *Eucalyptus globulus*; CF: *Cymbopogon flexuosus*; ES: *Eucalyptus staigeriana*.; L: linear term; Q: quadratic term. df, degrees of freedom; SS, sum of squares; MS, mean square; F, F statistic; p, p-value.

In *Lactuca sativa*, linear (L) terms for CF (-28.7), ES (-17.1), and EG (-6.6) are negative and significant ($p \ll 0.05$), showing that increasing concentration decreases %G; CF has the largest magnitude. The CF(L)×ES(L) interaction is positive (+11.75; $p<0.001$), indicating partial non-additivity/antagonism (the mixture reduces %G slightly less than the sum of individual linear effects). The intercept (38.7%) is the predicted germination at the center point (all at 0.02%). In *Megathyrsus maximus*, EG(L) (-7.67), CF(L) (-10.0) and ES(L) (-7.0) are likewise negative and highly significant; CF(Q) (-2.06) evidences curvature, i.e., 0.02% is not the simple average of 0 and 0.04%. Several interactions are significant: CF(L)×ES(L) is positive (+7.25), again suggesting partial antagonism of their linear components, whereas some mixed terms with Q negative (e.g., EG(Q)×ES(L)) indicate nonlinearities that shift minima. Overall, the coefficients confirm CF and ES as the main drivers of inhibition in both species, with EG acting as a modulator and mixtures showing additive behavior.

The response-surface plots (Figures 1 and 2) were generated from these models by fixing one factor at the center level (0.02%) and varying the other two within 0.00 - 0.04%. The mesh shows the model prediction, and blue dots are observations; the color scale encodes %G (green = lower). In *L. sativa*, the CF - ES, EG - ES, and EG - CF surfaces display downward slopes along CF and ES, consistent with the negative linear coefficients; a shallow valley at high CF+ES agrees with the positive interaction (mild antagonism). In *M. maximus*, %G decreases steeply with increasing CF and ES, curvature is more evident along CF (negative Q term), and saddle-like shapes appear where interactions dominate, producing low-%G regions near the upper boundary of CF and ES within the design space. The close alignment of points and mesh indicates good in-domain fit, although the significant lack-of-fit for *M. maximus* suggests additional structure beyond the quadratic model. Thus, the surfaces visualize findings and guide the identification of operational regions of minimal %G under constant surfactant load and application volume (Table S3)

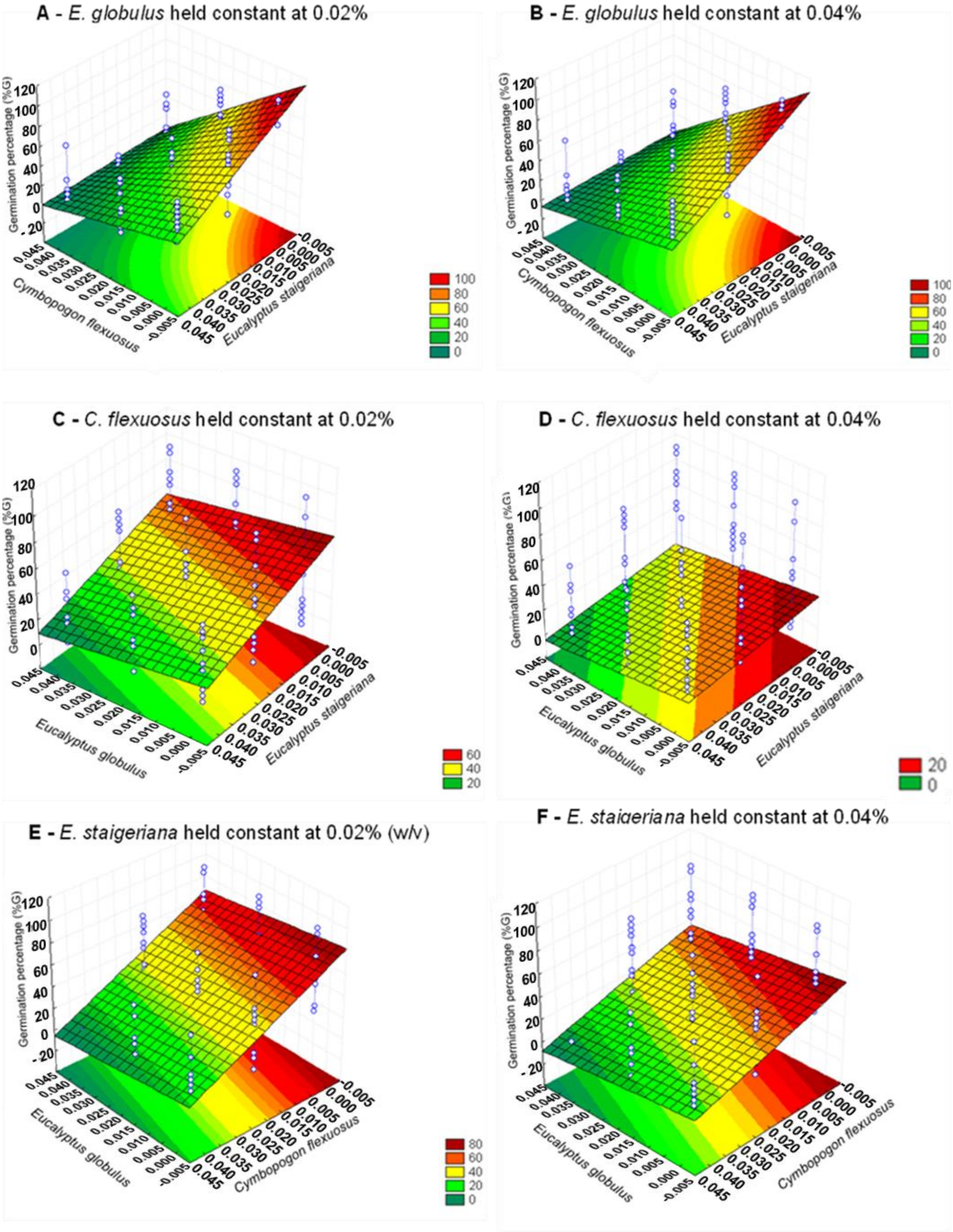

Figure 1. Germination percentage (%G) of *Lactuca sativa* as a function of combinations of *Eucalyptus globulus, Cymbopogon flexuosus*, and *Eucalyptus staigeriana* under different conditions.

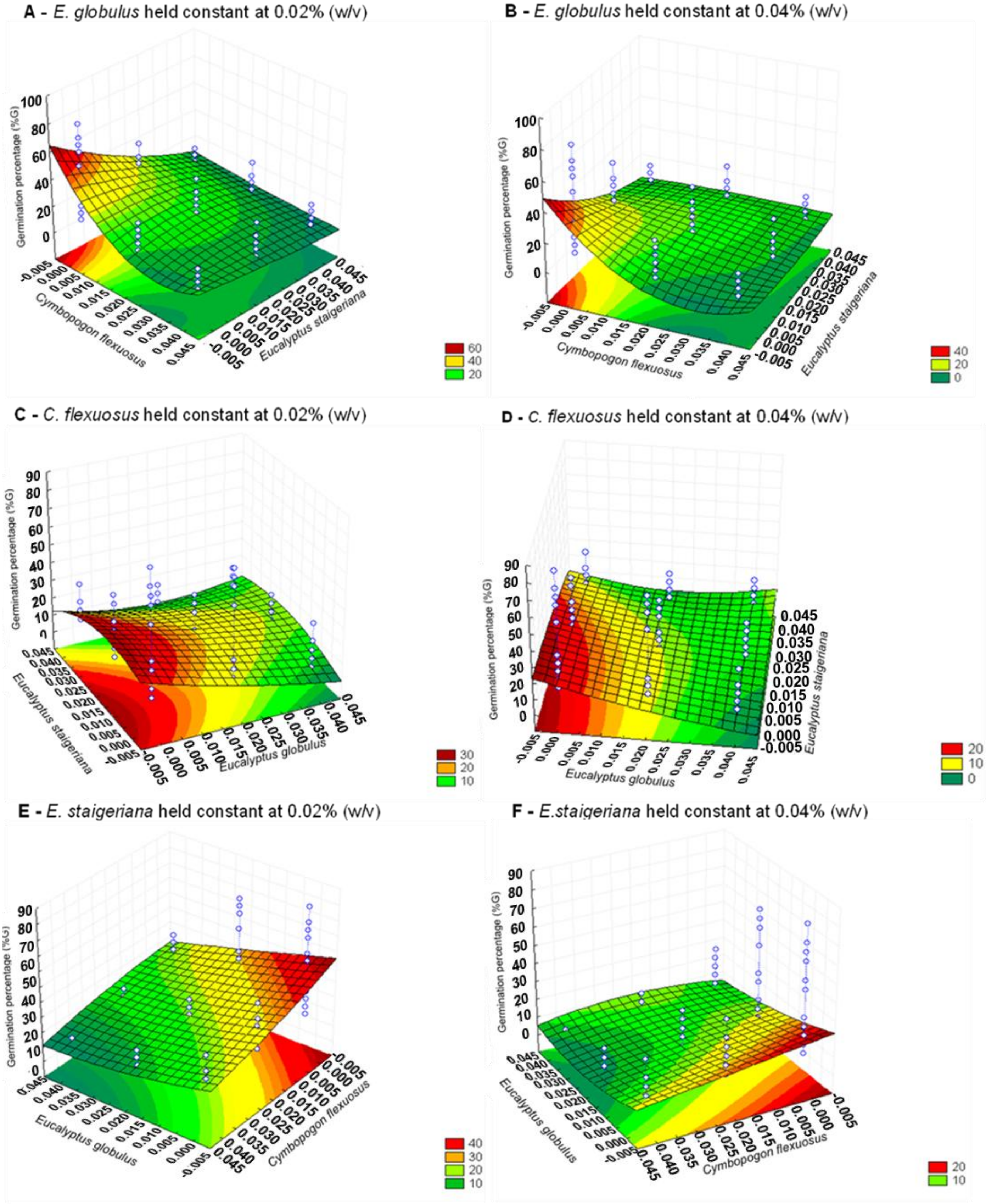

Figure 2. Germination percentage (%G) of *Megathyrsus maximus* as a function of combinations of *Eucalyptus globulus, Cymbopogon flexuosus*, and *Eucalyptus staigeriana* under different conditions.

DISCUSSION

The experimental design proved to be an effective tool for reducing experimental effort, as it provided robust data with a limited number of experiments. By combining Plackett - Burman screening and factorial optimization, it was possible to identify and rank the most promising nanoemulsions, whether used alone or in combination, for the development of a bioherbicide targeting *Megathyrsus maximus* as a potential option for replacing synthetic herbicides. Furthermore, the meticulous planning facilitated the establishment of a mathematical model that enabled the prediction of the germination percentage of *M. maximus* and *L. sativa* as a function of the concentrations of *C. flexuosus*, *E. globulus*, and *E. staigeriana* nanoemulsions used in the formulation of the three-NE combination.

Megathyrsus maximus (colonião-grass) is a significant weed species, and *Lactuca sativa* (lettuce) is an indicator plant that is frequently used in studies of this nature due to its sensitivity to phytotoxins in general [33]. The present study demonstrated the effectiveness of nanoemulsion combinations in inhibiting germination and initial growth of the two species tested. Among the nanoemulsions that were analyzed, the NE of *Cymbopogon flexuosus* caused greater inhibitions in the percentage of germination and initial growth. However, the combination of the NEs of *E. globulus* and *E. staigeriana* proved to be more effective, indicating statistically significant non-additive interaction effects within the tested concentration range, rather than direct evidence of classical biochemical synergy.

The factorial design comprising three factors at three levels confirmed that *C. flexuosus* was the most active individual NE. However, the combined formulation consistently outperformed the individual components, demonstrating interaction effects that deviated from strict additivity within the statistical model, although not necessarily fulfilling formal criteria for pharmacological or biochemical synergy. According to the mathematical model, the ideal condition is the combination of the three NEs at a concentration of 0.04%, which can predict complete inhibition of germination within the design space. Additionally, it was evident that the effect of each individual NE did not exceed the effect observed when they were combined.

A study that combined eight EOs in pairs and evaluated their influence on *L. sativa* seeds found that the interactions between the EO pairs were predominantly reported as additive or synergistic according to checkerboard-based frameworks, with only a few antagonistic interactions [20]. In a separate study, it was observed that a combination of *Rosmarinus officinalis* and *Corymbia citriodora* essential oils exhibited enhanced antifungal properties [22]. Enhanced antimicrobial effects were also reported with the combination of *Drypetes gossweileri* with four other essential oils against the bacteria *Bacillus cereus*, *B. megaterium*, *B. subtilis*, and *Geobacillus stearothermophilus* [21].

The interaction effects between essential oils can be characterized through *in vitro* experiments using the checkerboard assay. This assay is primarily used to evaluate interactions in antibacterial activity, but it can also be used with plant species [20,34]. A comprehensive understanding of these interactions is essential to elucidate the behavior of EOs in formulated combinations.

The biological activity of *C. flexuosus* EO has been the subject of extensive research, particularly with regard to its antimicrobial properties [35]. However, its potential as an herbicide has received comparatively less attention. The findings of this study underscore the considerable potential of its nanoemulsion, both as a standalone entity and in conjunction with other agents. The essential oil (EO) of *Cymbopogon flexuosus* are predominantly composed of citral and geraniol [35]. These monoterpenes have been shown to induce alterations in membrane composition and depolarization of microtubules, thereby affecting cell division [15]. While the *C. flexuosus* NE had the largest droplet size (96 nm), its pronounced bioactivity suggests that the chemical composition, particularly the high concentration of citral, may be the dominant factor driving its phytotoxicity, potentially overriding the influence of particle size in this context. In contrast, the smaller droplets of *E. globulus* and *E. staigeriana* (~27-28 nm) may facilitate faster penetration and action, contributing to their own efficacy and their role in the observed mixture effects. Additionally, geraniol has been reported to influence germination and root elongation [2], a property that may contribute, at least in part, to the observed outcomes.

The NE of *Eucalyptus globulus* produced considerable variation in the analyzed variables. Its EO has been the focus of numerous studies, and its biological activity is well-documented [36]. However, the development of formulations designed to increase its bioavailability remains in its early stages. The phytotoxic activity of *E. globulus* essential oil has been demonstrated in *Lactuca sativa* and in the weeds *Avena fatua* and *Amaranthus hybridus*, corroborating the results of the present study [37]. The essential oil (EO) of *E. globulus* is predominantly composed of 1,8-cineole, which is also referred to as eucalyptol. This monoterpene has been reported to induce a delay in the germination and growth of seedlings, as well as to inhibit cell proliferation, DNA synthesis, and cellular respiration in various plant species [37,15,2].

Eucalyptus staigeriana has been the subject of extensive research, as it is a commonly studied species in the evaluation of biological activities in various studies. However, the herbicidal and phytotoxic potential of *E. staigeriana* remains to be thoroughly explored, representing a significant gap in our understanding of its ecological impacts. The essential oil's primary components include citral, 1,8-cineole, and limonene [38]. In the present study, GC/MS analysis revealed a compositional profile dominated by citral-related compounds, with lower contributions of other monoterpenes, reflecting the known chemical plasticity of *Eucalyptus* essential oils. Citral-rich profiles have been associated with inhibition of seed germination and early seedling development, mainly through interference with auxin transport and disruption of meristematic organization, whereas eucalyptol has been linked to effects on mitochondrial respiration and cellular metabolism [14,15]. Therefore, despite quantitative differences in composition between the present GC-MS results and previous reports, the dominance of citral-related compounds in the analyzed oil provides a mechanistic basis compatible with the phytotoxic effects observed in this study.

The greater impact observed in combination treatments may be attributed to the combined concentrations of certain compounds, such as citral and eucalyptol. This hypothesis suggests that the combination treatments may exhibit a greater toxic effect, potentially resulting from the accumulation of these compounds.

The phytotoxic activity of the combinations observed in the present study is attributable to the chemical composition of the EOs in the nanoemulsions, as they are composed of terpenes with known phytotoxic activity that may be interacting statistically detectable non-additive effects. Importantly, the positive interaction terms observed in the factorial model should not be interpreted automatically as classical synergy, but rather as deviations from expected additive responses under the applied statistical framework. However, this interaction requires further investigation.

Furthermore, the employment of nanoemulsions proved instrumental in the suppression of germination, a consequence of their ability to enhance solubility and, most notably, stability. This ensures the effective delivery of the active components of essential oils the target site [18,19]. An important challenge encountered in the formulation of essential oil-based bioherbicide preparations pertains to the instability of these formulations and the volatilization of their components, a phenomenon with the potential to compromise their efficacy [39].

From a technological perspective, the present findings represent an early stage of product development, corresponding to a low technology readiness level. The demonstrated *in vitro* bioherbicidal activity provides proof of concept but does not constitute agronomic validation. Advancement toward a commercially relevant TRL will require soil-based and greenhouse studies, evaluation of selectivity toward crop species of economic relevance, assessment of potential effects on non-target organisms and soil microbiota, and investigation of formulation stability, scalability, and compatibility with existing weed management practices. In this context, the present study establishes a scientific foundation for future research aimed at translating nanoemulsion-based essential oil formulations into practical bioherbicidal applications.

CONCLUSION

The Plackett - Burman experimental design proved to be a useful tool for the initial screening and prioritization of nanoemulsions and their combinations, providing relevant results with reduced experimental effort. The comprehensive three-level factorial design proved to be effective in assessing the bioherbicidal effects of the combination of the three nanoemulsions initially selected and in elucidating their interactions, as well as the influence of concentration on the response variables. The mixture exhibited greater efficiency in inhibiting germination compared to individual nanoemulsions. The combination nanoemulsions of *C. flexuosus*, *E. globulus*, and *E. staigeriana* demonstrated consistent pre-emergent phytotoxic effects on *M. maximus* and *L. sativa* under controlled *in vitro* conditions, supporting its potential as a bioherbicidal formulation. While the *C. flexuosus* nanoemulsion has been demonstrated to be the most effective in terms of herbicidal activity, the present study indicates that the combination is more effective overall. However, further studies are needed to better understand the interactions among the combined nanoemulsions and their mechanisms of action. From a technological standpoint, the present findings represent an early-stage proof of concept. The bioherbicidal activity demonstrated here does not yet constitute agronomic validation, and advancement toward a commercially relevant technology readiness level will require additional soil-based and greenhouse studies, assessment of crop selectivity, evaluation of potential non-target and environmental effects, and investigation of formulation stability and scalability. Thus, this study provides a scientific foundation for future research aimed at translating essential oil-based nanoemulsions into practical and sustainable bioherbicidal applications.

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Use of Generative Artificial Intelligence

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