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ORIGINAL ARTICLE

The antibacterial, antioxidant, and insecticidal activities of essential oils from *Thymus vulgaris* L., *Salvia officinalis* L., and *Ocimum basilicum* L.

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Abstract

The essential oils from *Thymus vulgaris*, *Salvia officinalis*, and *Ocimum basilicum* were extracted by hydrodistillation, characterized by gas chromatography/mass spectrometry, and quantified by gas chromatography/flame ionization detector. The principal constituents were thymol, *ρ*-cymene and carvacrol (*T. vulgaris*); camphor, β-pinene, and 1,8-cineole (*S. officinalis*); and (*E*)-anethole, linalool, and 1,8-cineole (*O. basilicum*). The essential oil from *T. vulgaris* was the most effective, forming inhibition halos of 46.16 ± 0.16 and 26.38 ± 0.33 mm, respectively, for *Salmonella choleraesuis* and *Listeria monocytogenes*. This essential oil was also more effective against *S. choleraesuis*, with a minimum inhibitory concentration of 8.85 mg mL^{-1}, and a minimum inhibitory concentration of 17.71 mg mL^{-1} for *L. monocytogenes*. No bactericidal activity against *S. choleraesuis* and *L. monocytogenes* was observed for the essential oils from *S. officinalis*, and *O. basilicum*. Scanning electron micrographs showed that the addition of essential oils left the bacterial cells damaged and deformed. Significant 2,2-diphenyl-1-picrylhydrazyl free radical scavenging capacity and lipid substrate protection were observed in the β-carotene bleaching assay for the essential oil from *T. vulgaris*, with IC_{50} of 231.13 ± 0.53 and 15.25 ± 0.38 μg mL^{-1}, respectively. A dose-dependent relationship between antioxidant activity and concentrations was observed in the tests. Toxicities of $LC_{50} = 1.24$, 3.51 and 1.19 mg mL^{-1} against *Drosophila suzukii* flies, respectively, were observed for the essential oils from *T. vulgaris*, *S. officinalis*, and *O. basilicum*. Results suggest that essential oils can be promising antioxidant agents, insecticides, and inhibitors of pathogenic bacteria.

KEYWORDS
DPPH, *Drosophila suzukii*, *Listeria monocytogenes*, *Salmonella choleraesuis*, secondary metabolites, β-carotene

1 | INTRODUCTION

Bacterial infections can result from the consumption of food contaminated by pathogenic bacteria, such as *Listeria monocytogenes* and *Salmonella* spp, which can cause morbidity and mortality. Antibiotics are widely used to treat bacterial infections; however, because of their indiscriminate use, some bacteria have become resistant to these drugs (Indrotristanto et al., 2023; Ricci et al., 2022).

In addition to the presence of microorganisms in food, the existence of free radicals can also influence the quality of these products. Free radicals are reactive species responsible for triggering undesirable reactions. In food, these free radicals can be controlled by synthetic antioxidants (butylhydroxyanisole [BHA], 2,6-di-*tert*-butyl-4-hydroxytoluene [BHT], *tert*-butylhydroquinone, and propyl gallate) that can neutralize them through the donation of hydrogen atoms or electrons, thereby delaying chain reactions and inhibiting the attack of free radicals on lipids and proteins (Orlo et al., 2023; Teixeira et al., 2022).

Other factors, such as insect attacks, are responsible for generating economic losses for the food industry. Among these insect pests, *Drosophila suzukii* (Diptera: Drosophilidae) flies are known to attack several red fruit crops. Using a serrated ovipositor, the females of this phytophagous pest lay their eggs in healthy ripe fruits, cause wounds, and make the fruits susceptible to secondary infections by other insects, fungi, and bacteria, thus making the fruits unsuitable for commercialization (De Souza et al., 2022; Pineda et al., 2023). The control of insects such as *D. suzukii* is generally accomplished by the application of synthetic insecticides. Therefore, the search for alternative methods, mainly natural ones, is of great interest (Finetti et al., 2020; Pineda et al., 2023).

As an alternative to the use of synthetic products, essential oils have been extensively studied because they have proven biological properties (De Souza et al., 2022; Rezende et al., 2022). Taking into account the fact that the bioeffects of essential oils can vary according to their chemical composition, the present study sought to investigate the biological activities of essential oils extracted from three food condiments: *Thymus vulgaris* L., *Salvia officinalis* L., and *Ocimum basilicum* L., popularly known as thyme, sage, and basil, respectively, purchased in bulk. Our questions were the following: (i) Do essential oils have bactericidal and bacteriostatic effects against pathogenic bacteria such as *Salmonella choleraesuis* and *L. monocytogenes*? (ii) Do essential oils have antioxidant activities observable through different methods? (iii) Do essential oils have insecticidal activities against *Drosophila suzukii*? (iv) Are these natural products as effective as some of the commercially available synthetic antibiotics, antioxidants, and insecticides? The answers to these questions are extremely important for understanding the effectiveness of essential oils, comparing them with commercial products, and contributing to the use of more sustainable, ecologically correct, and safe products.

2 | MATERIALS AND METHODS

2.1 | Extraction of essential oils

Dried leaves of *T. vulgaris*, *S. officinalis*, and *O. basilicum* were obtained from the Central Market of Belo Horizonte, MG, Brazil. The essential oils were extracted by the hydrodistillation method over a period of 1 h using the modified Clevenger apparatus (Brasil, 2010). After extraction, the essential oils and hydrolates were separated by centrifugation for 15 min (Fanem Baby I model 206 BL, São Paulo, Brazil) at 965.36*g*. The essential oils were separated in amber glass containers and stored under refrigeration at 4°C and the hydrolates were discarded. The yield of essential oils was determined according to the method described by Pimentel et al. (2006), and is expressed as the percentage of dry weight relative to the weight of the plant material (% w/w DWB).

2.2 | Identification and quantification of constituents present in essential oils

To identify the constituents present in essential oils, a gas chromatograph (Shimadzu Corporation, model QP2010 Plus, Kyoto, Japan) coupled to a mass spectrometer (GC–MS) and equipped with a capillary column of fused silica (30 m × 0.25 mm) with a DB5 bonded phase (0.25 μm thick) was used (Adams, 2017). Helium was the carrier gas (White Martins, Rio de Janeiro, Brazil) at a flow rate of 1.0 mL min^{-1}, and the injector and detector temperatures were 220 and 240°C, respectively. The initial column temperature was 60°C. The temperature increased at a rate of 3°C min^{-1} to 240°C, followed by an increase at 10°C min^{-1} to 300°C, where it remained constant for 7 min. A 0.5-μL aliquot of the sample dissolved in hexane (1:100) (Sigma-Aldrich®, St. Louis, MO, USA) was injected. The MS was operated using the following parameters: 70 eV ionization potential, ion source temperature of 200°C, scan speed of 1000 Da s^{-1}, and scan interval of 0.50 fragments s^{-1}. Analyses were performed in full scan mode, ranging from 45 to 500 Da. Data on chemical constituents of the essential oils were obtained from the LabSolutions LC/GC Workstation 2.72. For the identification of components, the retention indices were calculated using the equation of Van den Dool and Kratz (1963) and the standards of the homologous series of n-alkanes (nC8–nC18). The retention indices were compared with those existing in the literature (Adams, 2017). The FFNSC 1.2, NIST 107, and NIST 21 mass spectral libraries were also employed to compare the spectral data of the constituents of the essential oils with similarities greater than 95%. The quantification of essential oil constituents was performed by gas chromatography using a flame ionization detector (Shimadzu GC-2010, Kyoto, Japan). The operating conditions were the same as those used in the qualitative analysis, except for the detector temperature (300°C). The percentages of essential oil constituents were calculated using the area normalization method.

2.3 | Evaluation of the antibacterial activity of essential oils

2.3.1 | Bacterial strains

Standard strains of *L. monocytogenes* (ATCC 19117) and *S. choleraesuis* (ATCC6539) were obtained from the Microorganism Culture Collection of the Mycotoxins and Food Mycology Laboratory of the Federal University of Lavras, Lavras, MG, Brazil. *Salmonella choleraesuis* strains were reactivated in Brain Heart Infusion Broth (BHI, HiMedia Laboratories Pvt. Ltd., Mumbai, India), and *L. monocytogenes* strains (ATCC 19117) were reactivated in Brain Heart Infusion Broth (BHI, HiMedia Laboratories Pvt. Ltd., Mumbai, India) enriched with 0.6% yeast

extract (Merck, Germany) at 37°C for 24 h. *Salmonella choleraesuis* strains were seeded on the surface of Petri dishes containing Mueller Hinton Agar (KASVI, Pinhals, PR, Brazil), whereas *L. monocytogenes* strains were seeded on Brain Heart Infusion Agar (BHI, HiMedia Laboratories Pvt. Ltd., Mumbai, India). After the incubation period of 24 h at 37°C, the bacterial cells were suspended in saline solution (0.9% NaCl w/v). Bacterial inocula were standardized to turbidity equal to that of a 0.5 McFarland standard solution, resulting in a culture solution of 10^8 CFU mL^{-1}. The turbidity was adjusted to a range of 0.08–0.1 Å by measuring the absorbance at 625 nm in a UV/Vis spectrophotometer (Shimadzu UV-160 1 PC) (CLSI, 2015).

2.3.2 | Agar disk diffusion method

The antimicrobial activities of the essential oils from *T. vulgaris*, *S. officinalis*, and *O. basilicum* were evaluated by the disk diffusion method (CLSI, 2019). Initially, 100 µL of standardized inoculum (10^8 CFU mL^{-1}) was distributed on Petri dishes containing 20 mL of solidified Mueller Hinton Agar (KASVI, Pinhals, PR, Brazil). Sterile filter paper disks with a diameter of 6 mm were soaked with the essential oils or with the antibiotic chloramphenicol (1 mg mL^{-1}) and then placed on the solidified plates. Plates were incubated at 37°C for 24 h. The diameters of the inhibition zones were measured in millimeters with a caliper. Each assay was performed in triplicate.

2.3.3 | Determination of the minimum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) tests were performed by the microdilution method, with some modifications (CLSI, 2019), using 96-well polystyrene microplates. Ten microliter aliquots of standardized bacterial inocula (10^8 CFU mL^{-1}) were added to 300 µL of Mueller Hinton Broth solution containing 1% (v/v) surfactant (Tween 80, Synth) and essential oils at concentrations of 283.32, 141.66, 70.83, 35.42, 17.71, 8.85, 4.43, 2.21, 1.11, 0.55, 0.28, and 0.14 mg mL^{-1} for *T. vulgaris*; 279.94, 139.97, 69.99, 34.99, 17.50, 8.75, 4.37, 2.19, 1.09, 0.55, 0.27, and 0.14 mg mL^{-1} for *S. officinalis*; and 291.06, 145.53, 72.77, 36.38, 18.19, 9.10, 4.55, 2.27, 1.14, 0.57, 0.28, and 0.14 mg mL^{-1} for *O. basilicum*. The microplates were incubated at 37°C for 24 h. The positive control consisted of a liquid medium, Mueller Hinton broth, surfactant, and bacterial suspensions, and the negative control consisted of the liquid medium. The same procedure was performed for the antibiotic chloramphenicol (Sigma Aldrich) using concentrations ranging from 0.00048 to 1.0 mg mL^{-1}.

The minimum bactericidal concentration (MBC) was determined according to the CLSI (2015), with some modifications. After microplate incubation, 10 µL of each well was seeded onto plates containing Mueller Hinton Agar (KASVI) (20 mL) and incubated at 37°C for 24 h. The lowest concentration that did not grow in the medium was considered to be the MBC. After incubation for 24 h, 20 µL of rezasurin solution (0.01% w/v) (Sigma-Aldrich®) was added to each well as an indicator of cellular activity and mixed. The microplates were incubated for 2 h at a temperature of 25°C, and the change in color from blue to pink was observed. The MIC was considered to be the lowest concentration of essential oil in which there was no bacterial growth.

2.3.4 | Scanning electron microscopy

The effect of the essential oils and the antibiotic on the morphology of the bacteria was evaluated using electron micrographs obtained using scanning electron microscopes (SEM) types LEO EVO 40 and TESCAN CLARA. Samples in MIC and positive and negative controls were preserved in Karnovsky fixative solution (2.5% glutaraldehyde, 2% formaldehyde, 0.05 mol L^{-1} cacodylate buffer at pH 7.2 [Sigma-Aldrich, São Paulo, Brazil] and 0.001 mol L^{-1} $CaCl_2$) and stored at 4°C for 24 h. Then, 30 µL aliquots of the samples were added to coverslips containing 0.5% poly-L-lysine, and the samples were air-dried for 10 min. The samples were washed three times for 10 min with sodium cacodylate buffer (0.05 mol L^{-1}, pH 7.2) and sequentially dehydrated with 25%, 50%, 75%, and 90% acetone for 10 min and three times with 100% acetone for 10 min. The samples were dried in the critical point apparatus (Bal-Tec CPD 030 Balzers, Liechtenstein) to complete the drying process with carbon dioxide as the transition fluid. The samples were mounted on stubs, coated with gold sputter (Baltec model SCD 050 Balzers, Liechtenstein), and kept in the desiccator with silica gel until observation by SEM (Oliveira et al., 2017).

2.4 | Antioxidant activity of essential oils

2.4.1 | Stabilization of the 2,2-diphenyl-1-picrylhydrazyl radical

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical stability assay was determined according to the method described by Teixeira et al. (2022). To test tubes, 300 µL of ethanolic solutions of the essential oils at concentrations of 15, 25, 50, 100, 150, 250, and 500 µg mL^{-1} and 2700 µL of ethanolic DPPH solution (40 mg L^{-1}) were added. A negative control containing 2700 µL of DPPH stock solution and 300 µL of ethanol was prepared, and the positive control contained the antioxidant 2,6-di-tert-butyl-4-hydroxytoluene (BHT) at the same concentrations as that of the essential oils. Analyses were performed in triplicate and readings were performed at 515 nm on a UV/Vis spectrophotometer (Shimadzu UV-160 1PC). The percentage of antioxidant activity (% AA_{DPPH}) was determined using Equation (1), and the antioxidant activity of the essential oils was expressed in IC_{50} (µg mL^{-1}), calculated from the equation for the graph of concentration versus $\%AA_{DPPH}$:

$$\%AA_{DPPH} = 1 - \left[\left(\frac{A_{sample}}{A_{control}}\right) \times 100\right] \quad (1)$$

where A_{sample} is the absorbance of the analyzed sample and $A_{control}$ is the absorbance of the negative control.

2.4.2 | β-Carotene bleaching assay

The assessment of antioxidant activity by the β-carotene bleaching assay was performed according to the method of Ferreira et al. (2019), with some modifications. An emulsion was prepared in a round bottom flask by adding 0.003 g of β-carotene, 0.2 g of linoleic acid, 1 g of Tween-20, and 15 mL of chloroform. After homogenization, the chloroform was evaporated on a rotary evaporator (Büchi Rotavapor R114) for 1 h at 50°C. The residue was dissolved in 500 mL of oxygen-saturated distilled water solution (emulsion A). A total of 300 µL of each of the essential oils was dissolved in ethanol at concentrations of 15, 25, 50, 100, 150, 250, and 500 µg mL^{-1}, and 2700 µL of emulsion A was added to the test tubes. The absorbance was read at 470 nm using a spectrophotometer (Shimadzu UV-1601PC) at time zero. The tubes were incubated at 50°C, protected from light, for 1 h, and the absorbance was read again at 470 nm. The negative control was prepared by adding 300 µL of ethanol and 2700 µL of emulsion. BHT was used as a positive control at the same concentrations used for the essential oils. Assays were performed in four replications, and the percentage of antioxidant activity by the β-carotene bleaching assay (%AA β-carotene) was calculated using Equation (2). The antioxidant activities of the essential oils were expressed as the IC_{50} (µg mL^{-1}), determined from the equation of the curve of concentration versus percentage of $AA_{β\text{-carotene}}$.

$$\%AA_{\beta-\text{carotene}} = 100 \times \left\{1 - \left[\frac{(A_0 - A_t)}{(A_{00} - A_{0t})}\right]\right\} \quad (2)$$

where A_0 and A_{00} represent the sample and negative control absorbances at time zero, and A_t and A_{0t} correspond to the sample and negative control absorbances after 1 h of incubation at 50°C.

2.5 | Evaluation of insecticidal activity

2.5.1 | Rearing of *D. suzukii*

The adult *D. suzukii* flies used in the bioassays were obtained from a stock colony created in the Molecular Entomology and Ecotoxicology Laboratory of the Entomology Department of the Federal University of Lavras. Laboratory rearing was conducted under controlled conditions at 25 ± 2°C; relative humidity of 50 ± 10% and a photoperiod of 12L:12D. The flies were fed a diet prepared with water, brewer's yeast, corn flour, sugar, agar, propionic acid, and methyl 4-hydroxybenzoate (Nipagin®) (Andreazza et al., 2016; Mendonça et al., 2019).

2.5.2 | Evaluation of the toxicity of essential oils on *D. suzukii*

Mortality bioassays of *D. suzukii* flies were conducted according to Insecticide Resistance Action Committee contact/ingestion exposure protocol number 026 (IRAC, 2011), with modifications (De Souza et al., 2022; Pineda et al., 2023). Two-cm dental cottons were inoculated with 2.2 mL of DMSO, aqueous sugar solution (20% w/v) and *T. vulgaris* essential oil (concentrations ranging from 1 to 5 µL mL^{-1}, which corresponded to 0.94 and 4.72 mg mL^{-1}); *S. officinalis* (concentrations between 3 and 7 µL mL^{-1}, which corresponded to 2.79 and 6.53 mg mL^{-1}) and *O. basilicum* (concentrations between 0.1 and 2.75 µL mL^{-1}, which corresponded to 0.09 and 2.67 mg mL^{-1}) and transferred to 200-mL glass vials. Twenty-five unsexed insects of the same age (48–72 h after emergence) were introduced into the glass vials. These flasks were closed with foam caps and kept in the laboratory at a temperature of 25 ± 2°C, relative humidity of 50 ± 10% and photoperiod of 12L:12D. The tests were performed in quadruplicate, and the synthetic insecticide Spinetoram (Delegate®) (concentrations ranging from 0.000128 to 0.0016 mg mL^{-1}) was used as a positive control. The negative control was an aqueous sugar solution (20% w/v) containing 2.2 mL of DMSO. After 24 h, the mortality of the flies was visually evaluated. Flies that did not move after brush stimulation were considered dead.

2.6 | Statistical analyses

The treatments were applied through 4 × 2 (essential oils/positive control × bacteria) and 4 × 7 (essential oils/positive control × concentrations) factorial designs, respectively, for disk diffusion and antioxidant activities. Analysis of variance and Tukey test with a significance level of 5% ($p \leq 0.05$) were performed using the Sisvar software. The IC_{50} data were submitted to a completely randomized design, and the means were compared by the Tukey test with a 5% significance level ($p \leq 0.05$) using the Sisvar software (Ferreira, 2011).

Dose-mortality data were submitted to probit analysis (SAS Institute, Cary, NC, USA) to estimate lethal concentrations (LC_{50} and LC_{95}). Confidence intervals for toxicity rates (TR) were estimated according to the method of Robertson et al. (2017), and their values were considered significantly different if the range did not include the value 1.

3 | RESULTS AND DISCUSSION

3.1 | Chemical composition of essential oils

Aromatic and medicinal plants produce a wide range of secondary metabolites, like essential oils, to cope with different stress situations. The diversity of stress conditions resulted in diversity in chemical compositions of the secondary metabolites and offered opportunities for their application in different areas of interest (Ed-Dra et al., 2020).

Our findings showed that the yields of essential oils, based on dry weight, differed between plant species, being 0.44%, 0.1%, and 0.25%, respectively, for essential oils from *T. vulgaris*, *S. officinalis*, and *O. basilicum*. A total of 13, 10, and 13 constituents were identified in the essential oils from *T. vulgaris*, *S. officinalis*, and *O. basilicum*, respectively (Table 1).

TABLE 1 Chemical composition of the essential oils from *Thymus vulgaris*, *Salvia officinalis*, and *Ocimum basilicum*.

Peak	Compound	RT (min)	RI_{ref}	RI_{cal}	Area (%)		
					Thymus vulgaris	*Salvia officinalis*	*Ocimum basilicum*
1	α-Pinene	6.465	932	933	0.61	0.22	–
2	Camphene	6.958	946	950	0.33	5.16	–
3	**β-Pinene**	7.806	974	979	–	**11.73**	–
4	Myrcene	8.064	988	987	–	0.08	0.83
5	α-Terpinene	9.007	1014	1017	0.39	–	–
6	***p*-Cymene**	9.354	1020	1025	**22.83**	3.25	–
7	Limonene	9.533	1024	1029	0.28	0.62	–
8	**1,8-Cineole**	9.664	1026	1032	0.82	**10.21**	**12.69**
9	γ-Terpinene	10.613	1054	1057	1.19	–	–
10	**Linalool**	12.211	1095	1099	2.39	-	**27.70**
11	**Camphor**	14.287	1141	1147	–	**63.37**	–
12	Borneol	15.351	1165	1172	1.50	4.61	–
13	Terpinen-4-ol	15.704	1174	1180	1.02	–	1.12
14	α-Terpinenol	16.331	1186	1195	–	–	1.41
15	Bornyl acetate	20.200	1284	1283	–	0.75	–
16	**(E)-Anethole**	20.211	1282	1287	1.33	–	**33.06**
17	**Thymol**	20.464	**1289**	**1293**	**62.67**	–	–
18	**Carvacrol**	**20.781**	**1298**	**1300**	**4.64**	–	–
19	Eugenol	23.127	1351	1356	–	–	0.84
20	(E)-Methyl cinnamate	24.488	1383	1.376	–	–	4.59
21	Methyl-eugenol	25.146	1398	1.403	–	–	6.34
22	α-*trans*-Bergamotene	26.550	1432	1432	–	–	2.73
23	Germacrene D	28.533	1480	1484	–	–	0.89
24	γ-Cadinene	29.834	1512	1513	–	–	0.10
25	α-epi-Muurolol	34.839	1640	1640	–	–	7.70
Composition							
	Monoterpene hydrocarbons				25.63	21.06	0.83
	Sesquiterpene hydrocarbons				0.00	0	3.72
	Oxygenated monoterpenes				74.37	78.19	42.92
	Oxygenated sysquiterpenes				0.00	0.00	7.70
	Other				0.00	0.75	44.83
	Total identified (%)				100.00	100.00	100.00

Note: The bold values are majority compounds.
Abbreviations: RI_{cal}, calculated retention index; RI_{ref}, reference retention index (Adams, 2017); RT, retention time.

The principal constituents in the essential oil from *T. vulgaris* were thymol, *p*-cymene, and carvacrol (Table 1); those in the essential oil from *S. officinalis* were camphor, β-pinene, and 1,8-cineole; and those in the essential oil from *O. basilicum* were (*E*)-anethole, linalool, and 1,8-cineole. Similar to the results obtained in the present study, Zanotto et al. (2023) found thymol (45.95%) as the principal constituent in the essential oil from *T. vulgaris*, followed by *p*-cymene (25.11%). Liu et al. (2021) observed that thymol (26.18%) was the principal constituent found in the essential oil from *T. vulgaris*, followed by 1,3,8-*p*-menthatriene (25.16%), limonene (12.37%), α-pinene (8.47%) and α-terpineol (4.92%). Studies carried out by Orhan-Yanıkan et al. (2022) evaluated the chemical composition of the essential oil from *T. vulgaris* and obtained d-carvone (44.88%), ϒ-terpinene (15.69%) and *p*-cymene (10.76%) as the main constituents.

The results obtained by Temerdashev et al. (2020) partially corroborate those found in this study, in which camphor (37.29%) was the most abundant compound in the essential oil from *S. officinalis*, followed by constituents with a lower abundance, such as α-thujone (21.87%) and 1,8-cineole (9.76%). Some constituents detected in this study were also reported by Assaggaf et al. (2022). They evaluated the chemical composition of the essential oil from *S. officinalis* and observed that the amounts of constituents are influenced by

phenological stages such as the vegetative phase, the beginning of the flowering phase, and the full flowering phase. The main compounds identified were naphthalenone (20.81%–22.9%), camphor (14.35%–16.29%), eremophilene (7.25%), *trans*-caryophyllene (8.91%) and 1,8-cineole (10.75%).

Brandão et al. (2022) studied the essential oil from *O. basilicum* and observed that the principal constituents were linalool (26.89%), 1,8-cineole (23.62%), and camphor (15.69%). At the same time, Alimi et al. (2022) reported that trazole (80.87%) and linalool (16.12%) were the main constituents of the essential oil from *O. basilicum*, with a yield of 0.24%.

Oxygenated monoterpene was the largest chemical class that predominated in the three essential oils. Factors such as genetic composition, species origin, growing regions and portion of the plant, availability of minerals in the soil, soil type, environmental conditions such as temperature, amount of daylight, frequency of irrigation, fertilizers, and extraction methods influence plant metabolism and, consequently, the synthesis of secondary metabolites. Jointly with extraction methods, such factors can result in different yields and chemical composition profiles of essential oils of the same species (Da Cunha Honorato et al., 2023; Hosseini et al., 2021; Rioba et al., 2015).

3.2 | Antibacterial activity

3.2.1 | Disk diffusion methods

The largest inhibition zones were observed with the essential oil obtained from *T. vulgaris*. Substantial inhibitory effects on both bacterial strains were observed, resulting in inhibition zone diameters of 26.38 ± 0.33 and 46.16 ± 0.16 mm for *L. monocytogenes* and *S. choleraesuis*, respectively. In comparison, the essential oils extracted from *O. basilicum* and *S. officinais* displayed relatively weak activity against these bacterial strains. The inhibition diameters measured 8.57 ± 0.21 mm for *L. monocytogenes* and 13.30 ± 0.61 mm for *S. choleraesuis* ($p \leq 0.05$) when the essential oil from *O. basilicum* was tested. These halos measured 9.27 ± 0.15 and 12.05 ± 0.13 mm when the essential oil from *S. officinalis* was tested against *L. monocytogenes* and *S. choleraesuis* ($p < 0.05$), respectively. Concerning the treatment with the commercial antibiotic, the inhibition halo recorded was 18.10 ± 1 and 22.49 ± 0.39 mm for *L. monocytogenesus* and *S. choleraesuis*, respectively ($p \leq 0.05$) (Figure 1).

Ed-Dra et al. (2020) reported that the antibacterial activity can be classified according to the inhibition diameter. It is considered weak when the inhibition zone is less than or equal to 12.0 mm, intermediate when this zone is between 12.1 to 20.0 mm, and strong when it is greater than or equal to 20.1 mm. This activity depends on factors such as bacterial strain and the chemical composition of the essential oils.

The greater antibacterial activity of *T. vulgaris* essential oil on bacteria is probably related to the presence of thymol, which has effective antibacterial effects on many pathogens (Jafri & Ahmad, 2020;

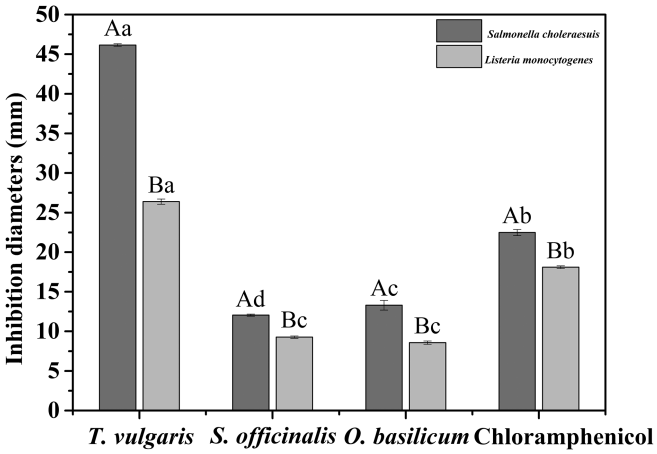

FIGURE 1 Inhibition diameters (mm) of essential oils tested on strains of *Salmonella choleraesuis* and *Listeria monocytogenes*. Means followed by the same lowercase letter compare each essential oil/positive control within each bacterium, and those followed by the same capital letter compare the bacteria within each essential oil/positive control by Tukey's test at 5% probability.

Liu et al., 2021). The strain that was most sensitive to the essential oils was *S. choleraesuis*, a Gram-negative bacterium. Typically, Gram-negative bacteria are known to be resistant to different essential oils. This resistance is because Gram-negative bacteria have a more complex membrane, limiting the penetration of hydrophobic components through the lipopolysaccharide layer (Lakhdari et al., 2020; Oualdi et al., 2023). The antibiotic chloramphenicol exhibited antibacterial activity against both strains.

The antibacterial activity of essential oils is influenced by the essential oil concentration and the bacterial strain tested. The antibacterial activities of the essential oils can be due to their ability to interfere with the selective permeability barrier of cell membrane structures and the consequent loss of control of chemiosmosis. Furthermore, essential oils can cause cytoplasmic coagulation and damage to lipids and proteins (Firdous et al., 2023).

3.2.2 | Determination of MIC and MBC

The essential oil from *T. vulgaris* and the antibiotic chloramphenicol exhibited bacteriostatic and bactericidal activities against both bacteria. *S. officinalis* and *O. basilicum* essential oils acted only as bacteriostatic agents (Table 2). According to Firdous et al. (2023), the biological activities of essential oils are typically difficult to correlate with a specific compound. However, there is likely a relationship between the most abundant components of the essential oil and its antibacterial activity.

The greatest activity against the bacteria was observed for the essential oil from *T. vulgaris*. The lowest MIC was observed with this essential oil, probably because of the presence of the principal constituent thymol, a phenolic monoterpene. The second highest activity was observed for the essential oil from *S. officinalis*, in which the

TABLE 2 Minimum inhibitory and bactericidal concentrations of essential oils and the positive control against *Salmonella choleraesuis* and *Listeria monocytogenes*.

	Salmonella choleraesuis		*Listeria monocytogenes*	
Essential oil/positive control	**MIC (mg mL^{-1})**	**MBC (mg mL^{-1})**	**MIC (mg mL^{-1})**	**MBC (mg mL^{-1})**
Thymus vulgaris	8.85	283.32	17.71	70.83
Salvia officinalis	17.50	NI	139.97	NI
Ocimum basilicum	18.19	NI	145.53	NI
Chloramphenicol	0.0020	0.063	0.004	0.063

Abbreviation: MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; NI, no inhibition at the concentration utilized.

principal constituent was camphor, a ketone. The lowest activity was found for the essential oil from *O. basilicum*, in which (*E*)-anethole, an ether, predominated.

Essential oils with a high phenolic monoterpene proportion, especially thymol, have the strongest antibacterial characteristics when compared to others that do not have phenolic monoterpenes in their composition (Goodarzi et al., 2023). For Liu et al. (2021), Radünz et al. (2020), and Sharma et al. (2023) the thymol acts by destroying the lipid structure of the bacterial cell wall, resulting in the disruption of the integrity of the bacterial cell membrane, the loss of cell content such as ions, ATP, and nucleic acids and, finally, cell death.

Auezova et al. (2020) and Gharib et al. (2017) observed that the main site of action of anethole in bacterial cells occurs on the phospholipid membrane because this constituent is capable of interacting with polar choline groups and acylhydrophobic chains of phospholipids, incorporating itself into the lipid bilayer and fluidizing it. Aydemir et al. (2018) state that *trans*-anethole also has the capacity of inhibition by quorum sensing as a mechanism of action that is used by pathogenic bacteria to control the expression of virulence genes. Disruption of quorum sensing systems leads to bacterial death.

Nogueira et al. (2021) evaluated the antibacterial activity of different standards against Gram-positive and Gram-negative bacteria. They determined that oxygenated aromatic compounds containing aldehyde, ketone, phenolic, or acidic groups possessed antibacterial properties superior to those derived from oxygenated aliphatic terpenes. This study's findings are consistent with those of Kalemba and Kunicka (2003) and Nogueira et al. (2021), who assert that the biological activity of essential oil constituents decreases in the following order: phenols, aldehydes, ketones, alcohols, ethers, and hydrocarbons. Lipophilic compounds composed only of carbon have an affinity for plasma membrane lipid layers. However, polar groups with hydrophilic characteristics' can interact with polar cell structures such as proteins and carbohydrates, thereby ensuring that the essential oils possess high antimicrobial activity (Nogueira et al., 2021).

Salmonella choleraesuis is a Gram-negative bacterium that is less resistant to essential oils than *L. monocytogenes*, as observed through the comparison of the MIC values with those of antibiotics. These results align with those of Silva et al. (2021), who evaluated the MIC and MBC of eugenol and linalool on *Escherichia coli*, *L. monocytogenes Salmonella*, and *S. aureus*, where Gram-positive bacteria were more resistant than Gram-negative bacteria. Hence, the intrinsic properties of each bacterium's cell wall structure likely play a role in facilitating or preventing penetration by essential oil constituents (Silva et al., 2021).

Paudel et al. (2019) studied the influence of nanoemulsions of cinnamon essential oil on different strains of *L. monocytogenes* and *Salmonella* spp. They observed lower MIC values for *Salmonella* spp strains than for *L. monocytogenes*, (0.039% and 0.78%, respectively), and the same MBC value for *L. monocytogenes* and *Salmonella* spp strains (0.78%). This result shows that the antibacterial activity of essential oils is related to the chemical components present, the bacterial strains tested, and the methods used (Ed-Dra et al., 2020; Silva et al., 2021).

Studies carried out by Ed-Dra et al. (2020), Gomes-Carneiro et al. (1998), and Kotan et al. (2007) showed that camphor, the principal compound in the essential oil from *S. officinalis*, exhibits low activity or is inactive against some bacteria, which agrees with the moderate activity of this essential oil. Almeida et al. (2023) stated that the minor components and the synergism of the components of essential oils are important to the microbial activity of essential oils.

The lower sensitivity of *L. monocytogenes* to essential oils and antibiotics is probably due to the high resistance of this bacterium to antibiotics already consolidated in the market, such as ampicillin, tetracycline, chloramphenicol, and streptomycin, as well as to adverse environmental stresses such as low pH and food preservation technologies such as pasteurization, high hydrostatic pressure, sonication, microwaves, and irradiation. This resistance of *L. monocytogenes* makes it a worrisome problem because it is responsible for many foodborne outbreaks that can be lethal (Bahrami et al., 2020; Ulusoy & Chirkena, 2019; Wu et al., 2022).

Studies performed by Ulusoy and Chirkena (2019) show that *L. monocytogenes* is resistant to antibiotics of human and veterinary importance such as ampicillin, tetracycline and chloramphenicol, streptomycin, erythromycin, and penicillin G, among others. Resistance to the antibiotic tetracycline is related to the presence of ribosomal protection genes; resistance to the antibiotic trimethoprim is due to the acquisition of genes that encode trimethoprim-resistant dihydrofolate reductases; and resistance to the antibiotic gentamicin is due to the presence of an efflux pump conferring resistance to benzalkonium chloride (Baquero et al., 2020). Therefore, the substances that inhibit the growth and development of this pathogenic bacteria in food, such as essential oils, are being increasingly studied.

3.2.3 | Scanning electron microscopy

Morphological changes in bacterial cells exposed to treatments are observed using electromicrographs. These images show the differences between untreated *S. choleraesuis* and *L. monocytogenes* (control) bacteria and those subjected to sublethal stress through treatments (MIC) of essential oils from *T. vulgaris*, *S. officinalis*, *O. basilicum*, and the antibiotic chloramphenicol (Figure 2).

Untreated bacterial cells (Figure 2A,F) had a smooth surface and an intact cell membrane. Treatment with the essential oils and the antibiotic resulted in significant changes in the structures of the bacteria, leaving damaged, deformed cells and ruptured membranes. Morphological changes in bacterial cells after exposure to the MIC included wrinkled and deformed surfaces (Figure 2D) and collapsed surfaces (Figure 2H,I). The presence of holes in the bacterial wall was also observed (Figure 2C).

In some treatments, morphological deformations were observed on cell surfaces, such as circular and wavy elevations (Figure 2B,E,G,J). According to Pagnossa et al. (2021), this deformation might be the result of overexpression of fatty acid derivatives and their precursors, such as amides and esters. Fatty acids are essential for the formation of the cell membrane, and after recovery from sublethal stress, they can accumulate disorderly on the surface of the cell wall of bacteria.

According to Turgis et al. (2009), the type of damage observed by SEM can be different for different bacteria, even those treated with the same essential oil, as was observed in the present study. The antibacterial mechanisms of essential oils do not occur in a single mode of action, but rather through the multiple action of constituents that can act on different areas of bacterial cells (Ed-Dra et al., 2020).

The mechanisms of action of essential oils include degradation of the cell wall, damage to the cytoplasmic membrane, coagulation of the cytoplasm, damage to membrane proteins, increased permeability that causes the leakage of cell contents, reduction in the proton motive force, electron flow, active transport, coagulation of cell contents, lower intracellular ATP synthesis, destruction of the DNA structure and reduction of membrane potential (Firdous et al., 2023). In general, the biological activities of essential oils are the result of synergistic, antagonistic, or both effects that result from their complex mixtures of terpenes and phenylpropanoids (Jaouadi et al., 2023).

3.3 | Antioxidant potential of the essential oils

The antioxidant activity of the essential oils from *T. vulgaris*, *S. officinalis*, and *O. basilicum* and the positive control BHT were determined by spectrophotometric techniques that were based on the elimination of DPPH radicals and bleaching of β-carotene. Two different methods were used to assess the antioxidant capacity of the components present in the essential oils because a single method cannot identify all the possible mechanisms that characterize an antioxidant (Jaouadi et al., 2023). A correlation was observed between antioxidant activities and concentration, with a dose-dependent relationship in the two methods used. The variation in the results observed between the samples is related to the difference in the composition of the essential oils.

The IC_{50} represents the concentration required to stabilize and safeguard 50% of the free radicals and lipid substrates in the DPPH and β-carotene tests, respectively. Thus, a lower IC_{50} denotes greater antioxidant activity for the essential oil being studied. In determining antioxidant activity through DPPH free radical scavenging, discoloration from purple to yellow occurs when antioxidants are present (Rezende et al., 2022). The highest levels of antioxidant activities were observed for the BHT control (IC_{50} 9.89 ± 0.08 μg mL^{-1}), followed by the essential oil from *T. vulgaris* (IC_{50} 231.13 ± 0.53 μg mL^{-1}), as is presented in Table 3. The essential oil from *T. vulgaris* is well known

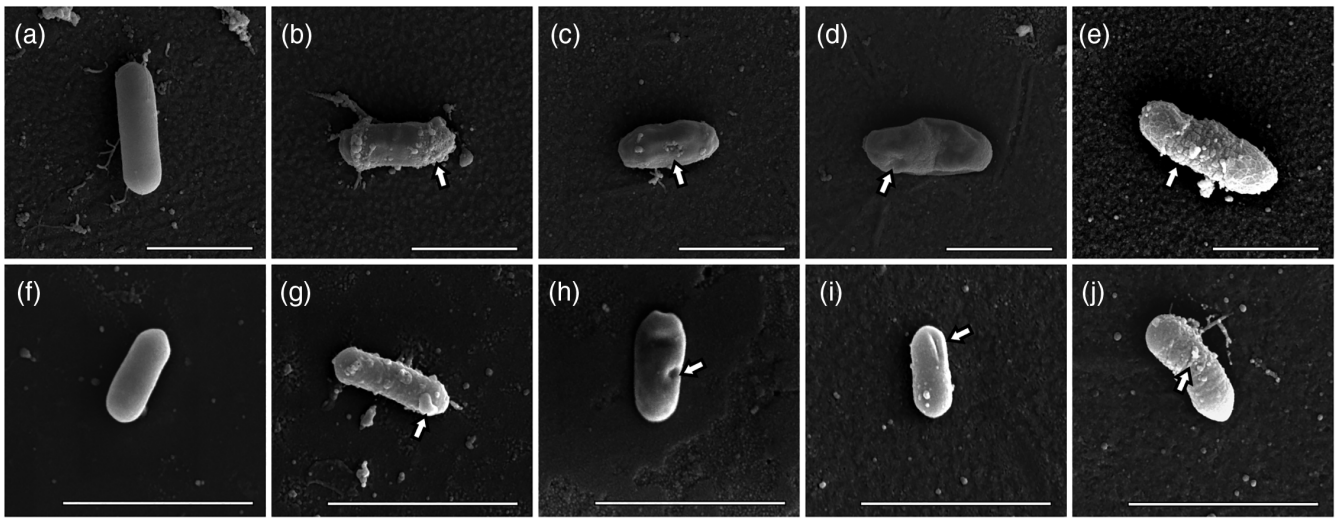

FIGURE 2 Scanning electron micrographs showing *Salmonella choleraesuis* ([A] untreated; [B] [C–E] treated) and *Listeria monocytogenes* ([F] untreated; [G–J] treated) with the essential oils from *Thymus vulgaris*, *Salvia officinalis*, *Ocimum basilicum*, and chloramphenicol, respectively. The scale bars in A, B, C, D, E, F, G, H, I, and J are 2 μm.

for its high in vitro antioxidant activity that is often linked to constituents having phenolic characteristics such as thymol, which has been proven to have an effective antioxidative effect (Sharma et al., 2023). Thymol's antioxidative property stems from its oxidation–reduction nature, where it functions as a reducing agent by donating hydrogen atoms to free radicals to scavenge them and stabilize them by resonance (Orlo et al., 2023).

A lower antioxidant activity was observed for the essential oil from *S. officinalis* (IC_{50} > 500 μg mL^{-1}). The chemical composition of this essential oil does not favor the elimination of free radicals, such as DPPH, through the donation of electrons or hydrogen atoms because this essential oil contains camphor (63.37%), a ketone. This constituent, when donating an electron for the stabilization of the DPPH radical, does not generate a resonance hybrid. It forms a chemically unstable species, unlike phenolic compounds (Dewick, 2009).

Research by Ferreira et al. (2019) reports that constituents with phenolic characteristics confer the ability to stabilize free radicals by transferring protons or electrons. The antioxidant activity of essential oils tends to be lower or even absent when there is an absence of phenolic constituents. The greatest capacity of the constituents of essential oils to donate hydrogens or electrons to stabilize free radicals are in this order: phenylpropanoids, phenolic terpenoids, alcohols, and terpenes (mono and sesquiterpenes).

TABLE 3 Antioxidant activity (IC_{50}) values obtained from testing essential oils extracted from *Thymus vulgaris*, *Salvia officinalis*, and *Ocimum basilicum*, using 2,6-di-tert-butyl-4-hydroxytoluene (BHT) as a positive control.

	Antioxidant method	
	DPPH	β-Carotene
Essential oil/positive control	IC_{50} (μg mL^{-1})	
Thymus vulgaris	231.13 ± 0.53[b]	15.25 ± 0.38[c]
Salvia officinalis	>500	136.00 ± 1.84[b]
Ocimum basilicum	434.67 ± 1.21[a]	167.41 ± 1.73[a]
BHT	9.89 ± 0.08[c]	7.44 ± 0.27[d]

Note: Means followed by the same lowercase letters in the columns do not differ by Tukey's test at the 5% level of probability.
Abbreviation: DPPH, 2,2-diphenyl-1-picrylhydrazyl.

To evaluate the inhibition of lipid peroxidation, the β-carotene method was used, in which the ability of essential oils to protect lipid substrates from oxidation can be verified. During the test, the orange color of β-carotene fades in the absence of an antioxidant. This discoloration is caused by the formation of free radicals after the loss of a hydrogen atom from a methylene diallyl group of linoleic acid, which reacts with the double bonds of the β-carotene molecule, promoting its degradation (Teixeira et al., 2022).

A lower IC_{50} for the β-carotene bleaching test was observed for the samples, that is, better results than for the DPPH free radical reduction test. BHT (IC_{50}: 7.44 ± 0.27 μg mL^{-1}) had the greatest protective activity, followed by the essential oils from *T. vulgaris* (IC_{50}: 15.25 ± 0.38), *S. officinalis* (IC_{50}: 136.00 ± 1.84 μg mL^{-1}), and *O. basilicum* (IC_{50}: 167.41 ± 1.73 μg mL^{-1}).

3.4 | Evaluation of the toxicity of essential oils for *D. suzukii*

The toxicities of the essential oils for *D. suzukii* flies after 24 h of application are presented in Table 4. Mortality levels were obtained using dose-mortality assays described by the probit model (adequacy of fit tests with low χ^2 values [<12] and high *p*-values [>0.05]). The LC_{50} of the synthetic insecticide (Delegate) was 0.0009 mg mL^{-1}. The toxicities of essential oils from *T. vulgaris*, *S. officinalis*, and *O. basilicum* were LC_{50} = 1.24 mg mL^{-1}, LC_{50} = 3.51 mg mL^{-1}, and LC_{50} = 1.19 mg mL^{-1}, respectively.

A lower LC_{50} (1.19 mg mL^{-1}) was observed for the essential oil from *O. basilicum*, that is, a higher toxicological activity. Thus, the toxicity ratios (TR) were calculated relative to this essential oil. The essential oil from *T. vulgaris* was equally lethal, and that from *S. officinalis* was one-third as lethal to *D. suzukii* as the essential oil from *O. basilicum*.

A higher concentration of essential oils was necessary to obtain the same LC_{50} as was obtained with the positive control, Delegate. Corroborating the results of this study, Dam et al. (2019), Jang et al. (2017), and Park et al. (2017) state that synthetic insecticides generally exhibit insecticidal activity approximately a 1000 times greater than that of the essential oils tested. Although a lower concentration of synthetic insecticides was required when compared to essential

TABLE 4 Estimate of the LC_{50} and LC_{95} (in mg mL^{-1}) of essential oils from *Thymus vulgaris*, *Salvia officinalis*, and *Ocimum basilicum* and the synthetic insecticide (Delegate) against *Drosophila suzukii*.

Essential oil/positive control	Number of insects	LC_{50} (95% IF) (mg mL^{-1})	LC_{95} (95% IF) (mg mL^{-1})	χ^2	*p*	TR LC_{50} (95% IF)
Thymus vulgaris	600	1.24 (1.14–1.33)	2.93 (2.55–3.55)	4.8124	0.1861	1.07 (0.92–1.08)
Salvia officinalis	500	3.51 (3.18–3.78)	8.63 (7.42–10.88)	2.7617	0.2514	3.06 (3.07–3.08)
Ocimum basilicum	800	1.19 (1.08–1.28)	2.29 (2.1–2.5638)	4.1497	0.5281	a
Delegate	900	0.0009 (0.0008–0.0016)	0.00158 (0.0013–0.0021)	6.8971	0.0753	—

Note: Where LC_{50} is the lethal concentration for 50% of the individuals and LC_{95} is the lethal concentration for 95% of the individuals; (95% IF) represents the 95% fiducial range; χ^2 is the chi-square for lack of fit to the probit model; *p* is the probability associated with the chi-square statistic; TR: toxicity ratio = LC_{50} of the oil over the LC_{50} of the oil with lowest LC_{50} value, with a 95% confidence limit.
[a]Oil used as a reference for calculating the TR LC_{50}.

oils, they could induce much higher hazards to nontarget organisms than essential oils and their components (Jang et al., 2017).

De Souza et al. (2022) evaluated the effects of different essential oils on *D. suzukii* and observed greater toxicological results for the essential oil from *Illicium verum*, obtaining LC_{50} values of 1.9 μL mL^{-1}. Their results corroborate those obtained in this study. This high insecticidal activity of the essential oil from *I. verum* was related to the action of its principal constituent, the phenylpropanoid (*E*)-anethole (99.61%). The principal constituent present in the essential oil of *O. basilicum* presented an LC_{50} of 1.23 μL mL^{-1}, showing that the E–Z isomerism of the anethole is probably not crucial for the toxic activity against *D. suzukii*. The essential oil of *I. verum*, whose principal constituent was (E)-anethole, caused changes to the epithelial thickness and carbohydrate distribution in the midgut of *D. suzukii* (De Souza et al., 2022).

Thymol has toxic neurological effects and causes neuronal degeneration through direct binding to GABA receptors in *Drosophila melanogaster*, in addition to showing a highly significant level of repellency towards males and females of *D. suzukii* (Finetti et al., 2020; Nesterkina et al., 2023).

Wang et al. (2021) evaluated the effect of the essential oil from peppermint and its constituents menthone, (−)-menthol, menthyl acetate, (R)-(+)-limonene, nerol, (+)-fenchone, (−)-α-thujone, camphor, and norcamphor on the *D. melanogaster* and *D. suzukii* flies. They observed that camphor is a strong repellent for *D. melanogaster*. However, camphor had little repellent activity against *D. suzukii*, which corroborates the results of the present study. There are differences in the olfactory systems of these insects, possibly reflecting their distinct ecological and behavioral adaptations. In addition to *D. melanogaster*, camphor has a strong repellent activity against many species of mosquitoes, including the red flour beetle (*Tribolium castaneum*) and the tobacco beetle (*Lasioderma serricorne*) (Wang et al., 2021).

According to Finetti et al. (2020), the biological activity of essential oil constituents is related to functional groups and different modes of action in insects. Minority constituents of essential oils can interact and act synergistically, resulting in important activities (Pan et al., 2022).

For De Souza et al. (2022), direct contact with essential oils through fumigation, and, principally, ingestion can cause a reduction in the thickness of muscle fibers and the absence of glycogen in the thorax, deformities in the abdomen, wings, legs and pronotum. The small difference in the toxicity ratios between the essential oils studied shows that the essential oils from *T. vulgaris*, *S. officinalis*, and *O. basilicum* have the potential to be used in the development of new insecticides for the management of the fly *D. suzukii*. However, further studies are needed to assess the mode of action of the essential oils on the fly and their toxicity to nontarget organisms.

Being formulated with only a single active ingredient, synthetic products, such as antioxidant (BHT), antibacterial (chloramphenicol) and insecticide (delegate) are consequently more efficient, as the active ingredient of the formulations effectively reaches the specific target. However, synthetic insecticides have many side effects, such as environmental problems, human health, and insect resistance. Essential oils, which are a complex mixture of terpenes and phenylpropanoids (Ferreira et al., 2019; Majeed et al., 2023; Rezende et al., 2022; Wang et al., 2023), proved to be a good alternative to synthetic products because their constituents can act synergistically, in addition to having different modes of action that could delay the development of resistance.

4 | CONCLUSION

Our results show, firstly, that the three essential oils have bacteriostatic activities against the bacteria *S. choleraesuis* and *L. monocytogenes*; however, only the essential oil from *T. vulgaris* showed bactericidal activity. Secondly, the essential oils have antioxidant activity through the two methods evaluated, whereas the greatest activity was observed for the essential oil from *T. vulgaris* because of its chemical composition. Thirdly, essential oils have insecticidal activity against the *D. suzukii* fly, with the essential oil from *O. basilicum* being the most effective. These results confirm that essential oils have varying biological activities that are intimately linked to their chemical compositions. Knowledge about biological activities is fundamental to determining the implementation and sustainability implications of essential oils. Yet, more studies are still needed to overcome some of the challenges facing the use of essential oils in practice. The volatility is one of the major barriers to the practical uses of essential oils in agriculture, health, and food-based applications that will require specifically designed delivery systems allowing gradual release. Nanotechnology and coating-based essential oils delivery strategies are some of the promising approaches to help improve controlled release. Moreover, from an ecotoxicology perspective, the risk assessment of the potential side effects of essential oils based products on nontarget organisms needs to be further investigated before they can be advocated as alternatives to synthetic compounds.

AUTHOR CONTRIBUTIONS

Cassia Duarte Oliveira: conceptualization; investigation; writing – original draft; methodology; writing– review & editing; formal analysis. Maria das Graças Cardoso: conceptualization; investigation; funding acquisition; writing – original draft; methodology; writing – review & editing; formal analysis; project administration; resources; supervision. Luis Roberto Batista: conceptualization; writing – original draft; methodology; writing – review & editing. Eduardo Alves: conceptualization; writing – original draft; methodology. Maria Beatriz Pereira Rosa, Vanuzia Rodrigues Fernandes Ferreira, Luciano de Souza, Maria Pineda, and Antonia Isadora Fernandes: investigation; methodology. David Lee Nelson: writing – review & editing; investigation; methodology. Khalid Haddi: conceptualization; writing – original draft; methodology; writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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